Ecological Approaches to Dental Caries Prevention: Alteration of Biofilms by Natural Products and Casein-Phosphopeptide-Amorphous Calcium Phosphate

Nebu Ivan Philip

BDS, MDS

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ABSTRACT

Background:

Dental caries is today widely recognised to be a biofilm-mediated disease triggered by dysbiosis in the resident oral microbiome. Despite the evolution of caries aetiological theories, disease preventive strategies are still largely dependent on the physicochemical effects of fluoride on the demineralization-remineralization caries equilibrium. The inability of fluoride to significantly influence the cariogenic bacterial challenge and reverse the microbial dysbiosis is one of the reasons the disease persists in many high caries-risk individuals and population groups. Countering the microbiome dysbiosis with conventional oral microbiocides is not recommended as their broad-spectrum antimicrobial effects will eliminate even health-associated plaque microflora. Comprehensive caries management protocols need to encompass agents that not only influence the de-/remineralization balance, but also include measures that can have a beneficial modulating effect on the ecology of the dental plaque microbiome.

Objectives:

The overall aims of this PhD project were to examine whether selected natural products and novel remineralizing agents like casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) can disrupt key virulence properties of cariogenic bacteria without broadly affecting microbial viability. The study also aimed to explore whether any virulence inhibitory effects of cariostatic natural products and CPP-ACP could translate into beneficial ecological effects both in *in vitro* biofilm models and in a clinical environment.

Methods:

A literature review was performed to identify natural products with potential cariostatic effects for investigation in this study. Agar diffusion and broth microdilution assays were used to assess whether any of the selected natural products could inhibit the growth of planktonic cultures of a caries-associated bacteria species (*Streptococcus mutans*) without affecting the viability of a health-associated oral commensal bacterial species (*Streptococcus sanguinis*). Based on the results of the planktonic bacterial culture study, specific natural products were further selected to explore their effects on cariogenic biofilm virulence properties. The biofilm studies were conducted in an active attachment *in vitro* model using monospecies *S. mutans* biofilms, dual-species *S. mutans-Candida albicans* biofilms, and saliva-derived polymicrobial biofilms growing in a cariogenic environment. The natural products were assessed for their effects on biofilm metabolic activity, acidogenicity,

microbial and exopolysaccharide (EPS) biovolumes, structural architecture, microbial counts and ecological microbial changes. Besides the natural products, the effect of CPP-ACP on acidogenicity, microbial counts and ecology of saliva-derived polymicrobial biofilms was also examined in a separate study. Finally, a randomised controlled trial (RCT) was conducted in a high caries-risk population group to evaluate the plaque ecological effects of a CPP-ACP dentifrice and a combined CPP-ACP – natural product dentifrice with regards to effecting species level changes of caries- and health-associated bacteria.

Results:

1. The planktonic culture study identified several natural products with significant antimicrobial effects. However, none of the selected natural products were able to selectively inhibit the growth of *S. mutans* without also affecting the viability of *S. sanguinis*.
2. Fruit berry extracts, though they lacked significant growth inhibitory effects, were selected for the biofilm virulence studies. This was based on literature reports that suggested polyphenols present in dark-coloured berries could exert biological activity against cariogenic virulence factors.
3. A standardized polyphenol-rich cranberry extract was found to be the most effective berry extract in disrupting virulence properties of *S. mutans* biofilms.
4. The virulence inhibitory effects of the cranberry extract extended to dual-species *S. mutans-C. albicans* biofilms, although higher concentrations of the extract were required for significant effects.
5. Twice-daily treatment of saliva-derived polymicrobial biofilms growing in a cariogenic environment with the cranberry extract inhibited biofilm virulence factors and drove a beneficial ecological change in the biofilm microbial community.
6. CPP-ACP demonstrated cariogenic virulence-attenuating attributes that influenced beneficial microbial ecological changes in saliva-derived polymicrobial biofilms that were growing in a cariogenic environment.
7. The RCT demonstrated that dentifrices containing CPP-ACP and polyphenol-rich cranberry extracts were able to effect a species level shift in the ecology of the dental plaque microbiome, resulting in a microbial community that had lower levels of bacteria associated with dental caries, and elevated levels of bacterial species associated with health.

Conclusions:

The results of this research project confirm the virulence inhibitory and microbial ecological effects of a sugar-free polyphenol-rich cranberry extract. This suggests the potential of incorporating the commercially available cranberry product into daily use oral care products as a useful strategy for better long-term control over dental caries. This study also provides evidence that CPP-ACP has beneficial virulence attenuating and ecological effects on saliva-derived polymicrobial biofilms growing in a cariogenic environment. Furthermore, a CPP-ACP dentifrice was shown to beneficially modulate dental plaque microbial composition in a high caries-risk population group. Thus, the effects of CPP-ACP are not limited to its well-established remineralizing effects on tooth surfaces, but also extend to having beneficial prebiotic actions on dental plaque biofilms. Although the cranberry extract did not appear to significantly enhance the ecological effects of CPP-ACP, optimisation of the cranberry extract concentration added to the CPP-ACP dentifrice could result in a synergistic ecological effect. Future studies with the cranberry extract incorporated into a fluoride dentifrice without CPP-ACP will be needed to resolve whether the cranberry extracts can have clinically useful ecological effects of their own.

DECLARATION BY AUTHOR

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Nebu Ivan Philip

PUBLICATIONS DURING CANDIDATURE

Peer-reviewed papers

1. **Philip N**, Suneja B, Walsh LJ. Ecological Approaches to Dental Caries Prevention: Paradigm Shift or Shibboleth? **Caries Research 2018**; 52(1):153-165.
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4. **Philip N**, Walsh LJ. Cranberry Polyphenols: Natural Weapons against Dental Caries. **Dentistry Journal 2019**; 7(1):20-24.
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Contributor	Statement of Contribution
Philip, N. (Candidate)	Conception (100%) Writing - original draft manuscript (100%) Writing - critical review and editing (10%)
Suneja, B.	Writing - critical review and editing (10%)
Walsh, L.J.	Writing - critical review and editing (80%) Supervision (100%) Final approval for submission

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Philip, N. (Candidate)	Conception (100%) Writing - original draft manuscript (100%) Writing - critical review and editing (10%)
Suneja, B.	Writing - critical review and editing (10%)
Walsh, L.J.	Writing - critical review and editing (80%) Supervision (100%) Final approval for submission

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Philip, N. (Candidate)	Conception (100%) Writing - original draft manuscript (90%) Writing - critical review and editing (10%)
Leishman, S.J	Writing - critical review and editing (10%)
Walsh, L.J.	Writing - original draft manuscript (10%) Writing - critical review and editing (80%) Supervision (100%) Final approval for submission

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Philip, N. (Candidate)	Conception (100%) Writing - original draft manuscript (100%) Writing - critical review and editing (10%)
Walsh, L.J.	Writing - critical review and editing (90%) Supervision (100%) Final approval for submission

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Philip, N. (Candidate)	Conception (100%) Writing - original draft manuscript (100%) Writing - critical review and editing (100%)

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Philip, N. (Candidate)	Conception (100%) Writing - original draft manuscript (100%) Writing - critical review and editing (10%)
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Contributor	Statement of Contribution
Philip, N. (Candidate)	Data Collection (100%) Analysis (100%) Interpretation (80%) Writing - original draft manuscript (90%)
Bandara, H.M.H.N.	Designed experiment methodology (50%) Interpretation (10%) Writing - critical review and editing (30%) Supervision (40%)
Leishman, S.J.	Designed experiment methodology (50%) Interpretation (10%) Writing - critical review and editing (30%) Supervision (40%)
Walsh, L.J.	Conception (100%) Writing - original draft manuscript (10%) Writing - critical review and editing (40%) Supervision (20%) Funding (100%) Final approval for submission

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Contributor	Statement of Contribution
Philip, N. (Candidate)	Conception (10%) Designed experiment methodology (20%) Data Collection (100%) Analysis (100%) Interpretation (80%) Writing - original draft manuscript (90%)
Bandara, H.M.H.N.	Conception (70%) Designed experiment methodology (80%) Interpretation (20%) Writing - critical review and editing (60%) Supervision (80%)
Leishman, S.J.	Writing - critical review and editing (10%) Supervision (10%)
Walsh, L.J.	Conception (20%) Writing - original draft manuscript (10%) Writing - critical review and editing (30%) Supervision (10%) Funding (100%) Final approval for submission

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Contributor	Statement of Contribution
Philip, N. (Candidate)	Conception (20%) Designed experiment methodology (20%) Data Collection (100%) Analysis (100%) Interpretation (100%) Writing - original draft manuscript (90%)
Bandara, H.M.H.N.	Conception (40%) Designed experiment methodology (80%) Writing - critical review and editing (50%) Supervision (80%)
Leishman, S.J.	Writing - critical review and editing (10%) Supervision (10%)
Walsh, L.J.	Conception (40%) Writing - original draft manuscript (10%) Writing - critical review and editing (40%) Supervision (10%) Funding (100%) Final approval for submission

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Philip, N. (Candidate)	Conception (80%) Designed experiment methodology (60%) Data Collection (100%) Analysis (80%) Interpretation (80%) Writing - original draft manuscript (90%)
Bandara, H.M.H.N.	Writing - critical review and editing (20%) Supervision (40%)
Leishman, S.J.	Designed experiment methodology (40%) Analysis (20%) Interpretation (20%) Writing - critical review and editing (40%) Supervision (50%)
Walsh, L.J.	Conception (20%) Writing - original draft manuscript (10%) Writing - critical review and editing (40%) Supervision (10%) Funding (100%) Final approval for submission

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Contributor	Statement of Contribution
Philip, N. (Candidate)	Conception (80%) Designed experiment methodology (60%) Data Collection (100%) Analysis (80%) Interpretation (80%) Writing - original draft manuscript (90%)
Bandara, H.M.H.N.	Writing - critical review and editing (20%) Supervision (40%)
Leishman, S.J.	Designed experiment methodology (40%) Analysis (20%) Interpretation (20%) Writing - critical review and editing (40%) Supervision (50%)
Walsh, L.J.	Conception (20%) Writing - original draft manuscript (10%) Writing - critical review and editing (40%) Supervision (10%) Funding (100%) Final approval for submission

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Philip, N. (Candidate)	Conception (20%) Designed experiment methodology (20%) Data Collection (100%) Analysis (60%) Interpretation (80%) Writing - original draft manuscript (90%)
Bandara, H.M.H.N.	Writing - critical review and editing (10%) Analysis (10%)
Leishman, S.J.	Designed experiment methodology (70%) Analysis (30%) Interpretation (20%) Writing - critical review and editing (40%) Supervision (70%)
Healy, D.L.	Designed experiment methodology (10%) Writing - critical review and editing (10%) Supervision (20%)
Walsh, L.J.	Conception (70%) Writing - original draft manuscript (10%) Writing - critical review and editing (40%) Supervision (10%) Funding (100%) Final approval for submission

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Research Involving Human or Animal Subjects

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"No man goes before his time - unless the boss leaves early" - Groucho Marx

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DEDICATION

To my wife and son,

Their enormous sacrifices have made this possible.

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LIST OF ABBREVIATIONS

AAA	Amsterdam Active Attachment
AAPD	American Academy of Pediatric Dentistry
ACP	amorphous calcium phosphate
ACY	anthocyanin
ADA	American Dental Association
AMP	antimicrobial peptide
ANOVA	analysis of variance
ANZCTR	Australian New Zealand Clinical Trial Registry
ACY	anthocyanin
A-PAC	A-linked proanthocyanidin
ATCC	American type culture collection
BHI	brain heart infusion
CAMBRA	caries management by risk assessment
CARS	caries associated with restorations and sealants
CAT	caries-risk assessment tool
CFU	colony forming unit
CHX	chlorhexidine
CGMP	caseinoglycomacropeptide
CPP	casein phosphopeptide
CPP-ACFP	casein phosphopeptide-amorphous calcium fluoride phosphate
CPP-ACP	casein phosphopeptide-amorphous calcium phosphate
CSPS	Calcium sodium phosphosilicate
Ct	Cycle threshold

CV	crystal violet
DCRAM	Dundee caries risk assessment model (DCRAM)
DP	degree of polymerisation
DPP	dentin phosphoprotein
DNA	deoxyribonucleic acid
EAER	electrically accelerated and enhanced remineralization
ECC	early childhood caries
EPH	ecological plaque hypothesis
EPS	exopolysaccharide
*f*TCP	Functionalized β-Tricalcium Phosphate
Gtf	glucosyltransferase
GRAS	generally recognised as safe
HA	hydroxyapatite
ICCMS	International Caries Classification and Management System
ICDAS	International Caries Detection and Assessment System
ICNARA	International Conference on Novel Anticaries and Remineralizing Agents
LB	lactobacilli
MIC	minimum inhibitory concentrations
MID	minimum intervention dentistry
mL	millilitre
MS	mutans streptococci
NDM	nondialyzable material
NHANES	National Health and Nutrition Evaluation Survey
nHA	nanohydroxyapatite
NSPH	non-specific plaque hypothesis

OD	optical density
QS	quorum sensing
P	levels of statistical significance (*P*-value)
PAC	proanthocyanidin
PBS	phosphate buffered saline
ppm	parts per million
qPCR	real-time quantitative polymerase chain reaction
RCT	randomised controlled trial
rpm	rounds per minute
RNA	ribonucleic acid
SD	standard deviation
sHA	saliva-coated hydroxyapatite
STMP	sodium trimetaphosphate
SPH	specific plaque hypothesis
STAMP	specifically targeted antimicrobial peptide
STEM	system for total environmental management
TGA	Therapeutic Goods Administration
TLM	traffic light matrix system
WHO	World Health Organisation
WSL	white spot lesion

1. INTRODUCTION TO THE THESIS

This chapter provides a brief background to the thesis and outlines the flow of the thesis chapters.

1.1 BACKGROUND TO THE THESIS

Ecological approaches to dental caries prevention is an area of research that is attracting increasing attention from investigators around the world. The recent years have seen an explosion of exciting developments in this field from *Streptococcus mutans*-antagonistic probiotic bacteria that are natural inhabitants of the oral niche [Huang *et al.,* 2016; Lopez-Lopez *et al.,* 2017], prebiotics like arginine incorporated into fluoride dentifrices [Li *et al.,* 2015; Nascimento, 2018], antimicrobial peptides that specifically target *S. mutans* [Guo and Edlund, 2017; Liu *et al.,* 2016], to exopolysaccharide matrix degrading free radicals released from pH-responsive catalytic nanoparticles [Gao *et al.,* 2016]. The use of cariostatic natural products to modulate the dental plaque microbiome and prevent caries lesions is another potential ecological strategy that requires further research. Natural products with cariogenic virulence inhibiting properties could supplement the remineralizing effects of fluoride and significantly enhance preventive capabilities of daily use oral care products. Their natural origin suggest they could find easier acceptance among the general public compared to commonly used synthetic microbiocides. This should allow them to be used for long-term control over the disease, making them particularly beneficial for high caries-risk groups. The research challenge is to identify a natural plant extract or phytochemical that can specifically inhibit cariogenic virulence factors without broadly affecting microbial viability.

Ideally, the remineralizing agent itself should be able to reverse any dysbiosis and maintain a symbiotic oral microbiome. However, fluoride, regarded as the gold standard among remineralizing agents, has minimal to no effects on microbial composition of dental plaque [Adams *et al.,* 2017; Koopman *et al.,* 2015; Reilly *et al.,* 2016; Reilly *et al.,* 2014]. The milk protein-based remineralizing technology called casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) may hold greater promise in effecting a beneficial shift in the dental plaque microbial ecology. Several reports in literature suggest that regular use of CPP-ACP products lowered salivary *S. mutans* counts [Emamieh *et al.,* 2015; Plonka *et al.,* 2013; Pukallus *et al.,* 2013; Subramanian and Naidu, 2009; Yetkiner *et al.,* 2014]. Moreover, recent *in vitro* studies have shown that treating multi-species biofilms growing in a cariogenic environment with CPP-ACP not only reduced the abundance of cariogenic bacterial species, but also effected a concomitant increase in the abundance of health-associated bacterial species [Philip *et al.,* 2019; Dashper *et al.,* 2018]. However, the large majority of the CPP-ACP studies have focussed on its remineralizing effects and there is a need for further research on its plaque ecological effects.

Recognising the need to develop newer approaches to dental caries prevention, this research project aimed to address some of the aforementioned challenges with specific regards to virulence attenuating and ecological effects of natural products and CPP-ACP. The long-term aim of this research would be to develop an oral care product combining polyphenol-rich natural plant or fruit extracts with the milk-derived CPP-ACP for dental caries prevention.

1.2 OUTLINE OF THE THESIS

The chapters in this thesis are divided into: (i) a broad review of the literature to identify the research gaps and define the aims and objectives of the research; and (ii) the experimental studies conducted to address the aims of the research.

The study flow diagram below illustrates the logical flow of thesis chapters relating to the literature review performed.

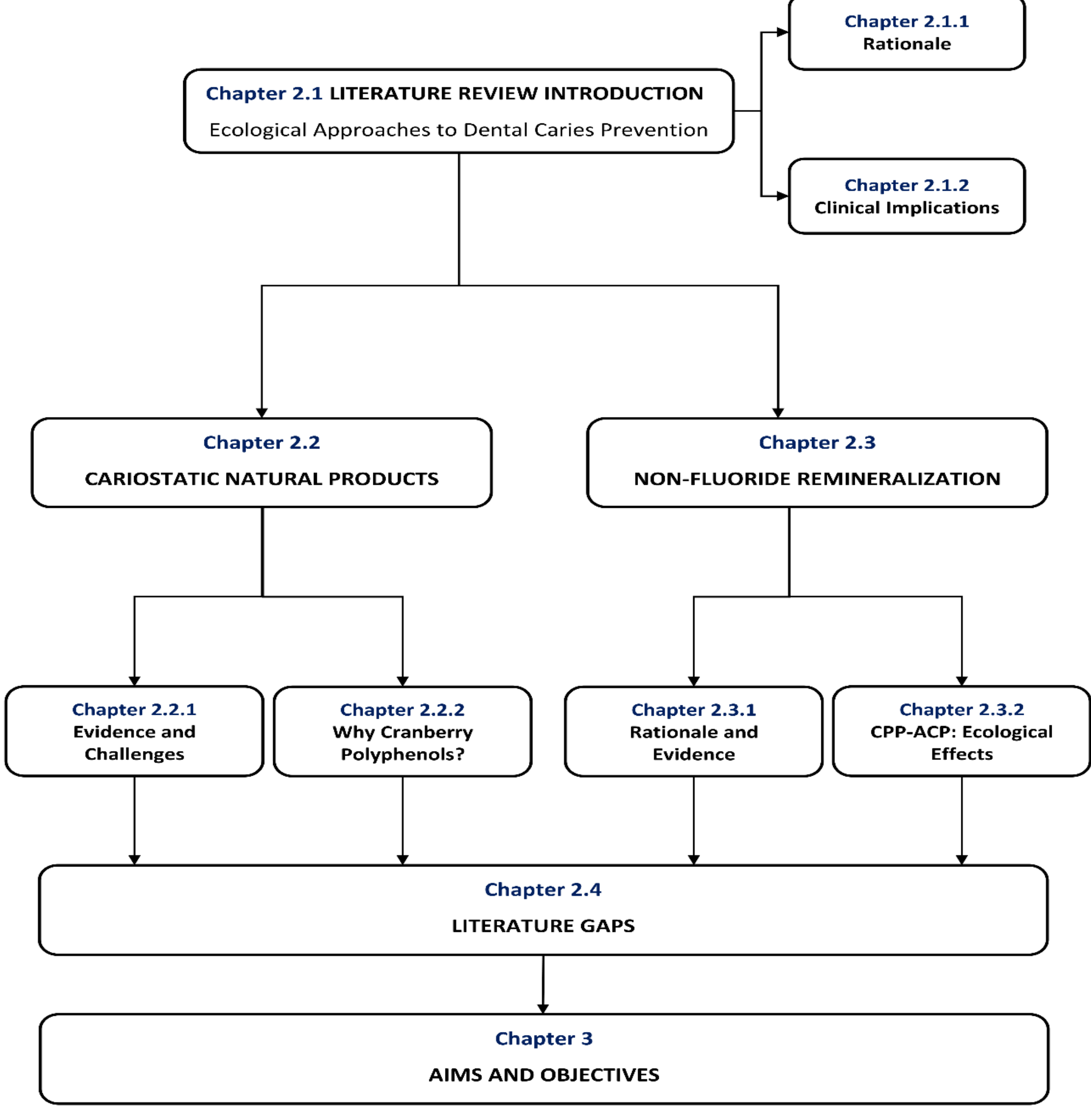

Figure 1. Flow of literature review with corresponding chapters

The methodology followed for the experimental studies was based on recommendations by the International Conference on Novel Anticaries and Remineralizing Agents (ICNARA) [Maltz and Beighton, 2012]. ICNARA suggests the screening process for new agents must involve a variety of assay systems of increasing complexity starting from simple tests with planktonic cells to be followed by a series of biofilm studies (from mono-species to polymicrobial biofilms) using models like the Amsterdam Active Attachment model [Exterkate *et al.,* 2010]. Lastly, a randomised controlled trial in a high caries-risk group is recommended to substantiate any *in vitro* results.

The study flow diagram below illustrates the logical flow of thesis chapters relating to the experimental studies conducted for this research project.

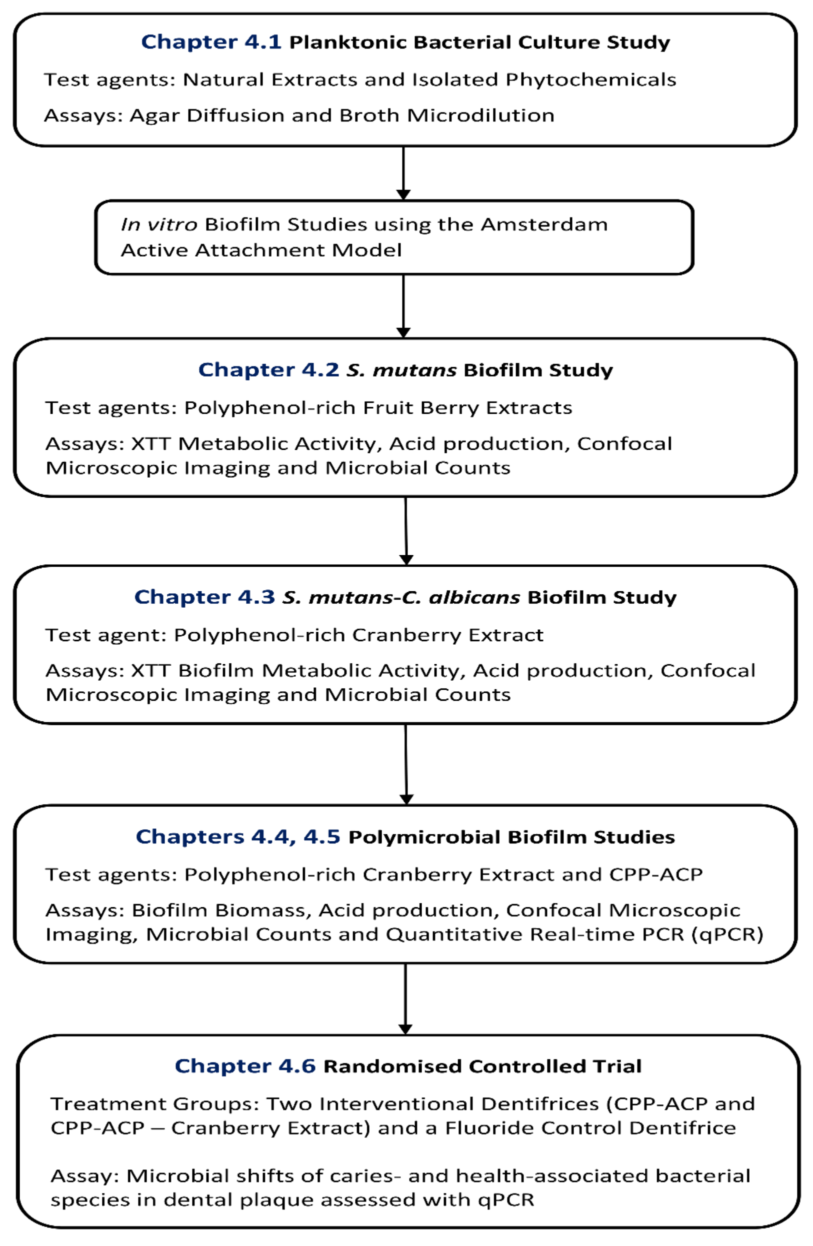

Figure 2. Flow of experimental studies and corresponding chapters

2. REVIEW OF THE LITERATURE

2.1 LITERATURE REVIEW: INTRODUCTION

2.1.1 REVIEW PAPER 1

Ecological Approaches to Dental Caries Prevention: Paradigm Shift or Shibboleth?

This paper discusses the rationale for developing alternate methods for dental caries control that go beyond the traditional caries preventive measures. The paper further reviews the different ecological approaches to dental caries prevention and the current status of these ecological caries preventive strategies.

The paper was published on 11th January 2018 in *Caries Research*, the official publication of the European Organisation for Caries Research (ORCA).

Caries Res 2018 52(1): 153-165.

This chapter is comprised of the publication as it stands in print.

ABSTRACT

Contemporary paradigms of dental caries aetiology focus on the ecology of the dental plaque biofilm and how local environmental factors can modulate this to cause disease. The crucial role that a healthy oral microbiome plays in preventing caries and promoting oral health is also being increasingly recognized. Based on these concepts, several ecological preventive approaches have been developed that could potentially broaden the arsenal of currently available caries preventive measures. Many of these ecological approaches aim for long-term caries control by either disrupting cariogenic virulence factors without affecting bacterial viability or include measures that can enhance the growth of diverse health-associated microbial communities in the oral microbiome. This paper argues for the need to develop ecological preventive measures that go beyond conventional caries preventive methods and discusses whether these ecological approaches can be effective in reducing the severity of caries by promoting stable, health-associated oral biofilm communities.

Oral health is integral to general well-being, with profound individual and societal implications that extend well beyond the functions of the craniofacial complex. Although largely preventable, diseases such as dental caries are major public health concerns, imposing a costly burden on health services. Traditional caries epidemiological measures do not adequately reflect the social impacts, economic costs, and health care system effects of the disease [Casamassimo *et al.,* 2009].

At the individual level, control of dental caries remains largely dependent on twice-daily mechanical oral hygiene in the form of tooth brushing with fluoride dentifrices, a preventive approach that has been in place for over 50 years. Based on the current understanding of the dental caries process, several ecological preventive strategies have been developed or are currently under investigation, suggesting a future where caries prevention will not be narrowly focussed on fluoride therapies. This review will explore the rationale supporting the need for alternate methods of dental caries control, and will discuss the current status of some ecological approaches to biofilm modification and caries prevention.

DENTAL CARIES: A BIOFILM-MEDIATED MULTIFACTORIAL DISEASE

Dental caries belongs to a group of diseases that are considered "complex" or "multifactorial," with no single causation pathway, and therefore, are not amenable to simplistic preventive solutions such as the elimination of "one type of organism" or merely enhancing "tooth resistance" [Fejerskov, 2004]. Dental caries is now widely recognized to be an endogenous, biofilm-mediated disease that occurs when acidogenic/aciduric members of resident oral flora obtain a selective ecological advantage over other species, disrupting the homeostatic balance of the plaque biofilm and initiating the disease process [Marsh and Martin, 1999]. Modern molecular analyses and microbial culture techniques have demonstrated that an entire range of bacteria, not just mutans streptococci (MS) or lactobacilli (LB), can contribute to the caries process at different stages [Tanner *et al.,* 2016], and that even fungi such as *Candida albicans* can significantly enhance cariogenic virulence of plaque biofilms [Koo and Bowen, 2014]. A MS or LB dominated microbiome may be found only at the advanced stages of the disease, where the increased severity and frequency of biofilm acidification results in the oral microbiome becoming less microbially diverse [Takahashi and Nyvad, 2008]. Indeed, it is not the bacterial genotype per se but their shared phenotypic characteristics (being acidogenic and aciduric) that are more important for driving the microbial ecological shift that leads to dental caries [Takahashi and Nyvad, 2011].

While the ionic aspects of the dental caries process have been the focus of research for decades, new insights into the aetiology and microbial aspects of this biofilm-mediated disease have engendered novel concepts and approaches for its prevention and control [ten Cate and Cummins, 2013]. A consensus is now emerging that caries preventive measures should aim to not only correct the environmental pressures responsible for the plaque biofilm dysbiosis, but also help to maintain a healthy, microbially diverse, resident microbiome [Marsh *et al.,* 2015]. These new approaches acknowledge the possibility that bacteria yet to be identified and cultured may participate in the caries process, and aligns with engaging in a more comprehensive approach towards preventing dental caries as a disease at the patient level [Walsh, 2011].

RATIONALE FOR NEWER METHODS OF DENTAL CARIES CONTROL

Ecological Strategies

The historical focus in preventing and managing oral diseases was on eliminating dental plaque from teeth. However, contemporary evolving evidence is increasingly highlighting the beneficial aspects of a healthy oral microbiome [Kilian *et al.,* 2016]. Commensal plaque microflora have a symbiotic relationship with the host, not only acting as a barrier to opportunistic pathogens, but also carrying out metabolic processes that benefit the host [He *et al.,* 2011; Schlafer *et al.,* 2017]. Acute infections of the oral mucosa are rare because of the interplay between the host immune system and microbial symbionts [Zaura *et al.,* 2014]. Likewise, the pro-and anti-inflammatory activities of resident bacteria help maintain homeostasis in the oral cavity [Devine *et al.,* 2015]. A moderate amount of healthy plaque has also been shown to prevent erosive enamel lesions and hypersensitivity [Honorio *et al.,* 2010].

Based on this changed understanding of the importance of a healthy oral microbiome, caries preventive strategies should ideally take an ecological approach to the holobiont. Indiscriminate or "shotgun" suppression of almost the entire oral biota, without understanding the overall effects on plaque ecology, is unlikely to have long-term success in controlling the disease [Caufield *et al.,* 2001]. Instead, preventive and curative products should either specifically target cariogenic bacteria without affecting other resident microflora, or they should inhibit virulence factors (e.g., glucan synthesis or acid production) rather than bacterial viability [ten Cate and Zaura, 2012]. Measures that enhance colonization of health-promoting microbial communities can also help in correcting the ecological imbalance in cariogenic biofilms. The advantage of such an approach is that the impacts of low pH environments generated by acidogenic organisms can be counterbalanced by

ammonia production from other bacteria. Following an ecological approach to caries prevention can potentially preserve the favourable effects that the host derives from the resident oral microbiome, while reducing cariogenic virulence factors that are responsible for plaque biofilm dysbiosis.

Aetiological Factors

The control and prevention of any disease should preferably focus on the aetiological factors involved. For dental caries, this would be the periodic disorganisation of the oral plaque biofilm by mechanical oral hygiene, along with dietary modification to reduce exposure to fermentable carbohydrates [Cury and Tenuta, 2008]. Unfortunately, individual oral hygiene measures have only limited impact in caries prevention [Bellini *et al.,* 1981; Hujoel *et al.,* 2006; Nyvad B, 2003]. It has also been suggested that tooth brushing has been effective in preventing caries mainly because it brings fluoride into the oral cavity at regular intervals, rather than any particular efficiency in disrupting cariogenic plaque biofilms [ten Cate and Zaura, 2012]. Dietary modification, which requires individuals to restrict their exposure to sucrose and other fermentable substrates, is particularly difficult to achieve in present-day society where cariogenic foods are easily available [Duggal and van Loveren, 2001]. Furthermore, restriction of sucrose intake alone is unlikely to completely prevent dental caries if frequent intake of other starches persisted [Bradshaw and Lynch, 2013]. Thus, while minimising the aetiological factors contributing to the disease is critical, additional preventive measures commensurate with individual risk status may still be required in many segments of the population.

Caries Epidemiology

For a long time, dental caries was a pandemic that affected children and adults almost universally. The landmark discovery of fluoride as an agent that could prevent dental caries, and the widespread use of fluoride-based caries preventive programmes, have been responsible for the significant reductions seen in caries prevalence of developed countries in the latter half of the 20th century [Fejerskov, 2004]. However, the latest Global Burden of Disease report revealed that untreated caries in permanent teeth still remains the most common human disease condition worldwide [Kassebaum *et al.,* 2015]. Doubts have also been expressed on whether the earlier decline in caries prevalence has continued into this new century [Gimenez *et al.,* 2016]. Recent caries prevalence studies in United States and Australia indicate tooth decay may in fact be increasing again [Chrisopoulos *et al.,* 2016; Dye *et al.,* 2007]. A similar trend has been seen in countries such as Norway and Iceland, which had initially shown the biggest improvements, but are

now registering increases in caries experience [Agustsdottir *et al.,* 2010; Haugejorden and Birkeland, 2005]. The flattening of prevalence rates that has occurred even in dentally aware populations where individuals commonly brush their teeth with fluoridated dentifrices is concerning and underscores the need to develop additional caries preventive measures that are synergistic with or complementary to fluoride.

Fluoride Alone may not be Sufficient

While fluoride is a highly effective and economical agent for dental caries prevention and will remain the mainstay of any caries preventive program, it must be recognised that in many situations fluoride alone may not be sufficient. Even with regular fluoride use, carious lesions can still develop when there are more than six dietary sugar exposures per day [Ccahuana-Vasquez *et al.,* 2007; Duggal *et al.,* 2001]. The ready availability of cariogenic snack foods and drinks in the modern consumer culture can overwhelm the benefits of community water fluoridation and daily use of a fluoride dentifrice. The limit to the repair potential of fluoride could partly explain the reversal of the caries decline being observed in contemporary epidemiological reports from developed countries [Agustsdottir *et al.,* 2010; Dye *et al.,* 2017].

The cariostatic actions of fluoride are largely attributed to its physiochemical ability to inhibit enamel demineralisation and enhance remineralisation [Cury and Tenuta, 2008]. Recent laboratory studies have confirmed the notion that under appropriate conditions fluoride ions can also influence critical MS virulence factors, significantly reducing acidogenicity, aciduricity, and glucan formation [Domon-Tawaraya *et al.,* 2013; Pandit *et al.,* 2015; Pandit *et al.,* 2013]. Metabolome analysis of plaque biofilms has demonstrated that fluoride can repress acid production *in vivo* too [Takahashi and Washio, 2011]. However, questions still remain on the extent to which these antibacterial mechanisms contribute towards caries preventive effects. A recent study showed that the brief fluoride exposure from toothpastes or mouthwashes could not sustain anti-acid production activity, with the biofilms recovering acidogenicity over time regardless of the fluoride concentration used [Dang *et al.,* 2016]. Concerns have also arisen regarding the emergence of microorganisms that are more resistant to the effects of fluoride on microbial metabolism [Liao *et al.,* 2017; Mitsuhata *et al.,* 2014].

Preventive approaches that combine fluoride with other protective agents have been advocated to enhance the ability of fluoride to modify biofilms and diminish the cariogenic bacterial challenge [Li *et al.,* 2015]. Fluoride-antimicrobial combinations have been recommended based on

the 2-part rationale that fluoride can reduce the critical pH at which dissolution starts, while effective antimicrobial agents can decrease the depth of the Stephan curve pH drop following consumption of fermentable carbohydrates [Øgaard, 2000]. Randomised controlled trials (RCTs) have shown that fluoride-chlorhexidine interventions significantly reduced bacterial load and pH drop from glucose metabolism [Giertsen and Scheie, 1995], and most importantly, lowered caries increment in high-risk groups [Featherstone *et al.,* 2012]. Sytematic reviews have also found high-quality evidence that fluoride-triclosan toothpastes give a small improvement in reducing coronal caries compared to conventional fluoride dentifrices [Blinkhorn *et al.,* 2009; Riley and Lamont, 2013]. However, the use of such broad-spectrum antimicrobial agents may not be ideal, as the aim of antimicrobial treatment in caries prevention should preferably be to modify the plaque biofilm ecology by weakening cariogenic virulence factors rather than eliminating microflora.

An alternative to the fluoride-antimicrobial approach is combining fluoride with agents that promote an overall community-wide microbial shift by encouraging the growth of health-associated bacteria, thereby beneficially rebalancing the biofilm ecology and potentially resulting in better long-term dental caries control. Among such biofilm-modifying oral products, there is now sufficient evidence supporting fluoride-arginine combinations as a new standard of care for caries prevention [ten Cate and Cummins, 2013; Zheng *et al.,* 2015; Zheng *et al.,* 2017]. Likewise, a dentifrice containing enzymes and proteins was shown to significantly shift the ecology of the oral microbiome resulting in a community with a stronger association to health [Adams *et al.,* 2017].

Caries Risk

Over time, the distribution of caries in the community has changed from a disease that was pandemic in society, to now being endemic in specific risk groups. A cross-sectional study concluded that 75% of the caries-risk burden tends to reside in 25–40% of the population [Macek *et al.,* 2004]. Likewise, there is evidence for genetic differences that exist with respect to caries susceptibility [Bretz *et al.,* 2005; Opal *et al.,* 2015], with not everyone in a population group benefiting to the same extent from traditional caries preventive programmes [ten Cate, 2013]. The pattern of dental caries has also changed over the years, from a rapidly progressing disease of childhood, to a slowly progressing disease that can occur or persist throughout adulthood and old age [Lagerweij and van Loveren, 2015]. Data from contemporary population studies have revealed that carious lesions are also increasingly localized to specific tooth sites [Anderson, 2002]. In children and adolescents, occlusal surfaces are the sites most likely to have experienced caries [Carvalho, 2014; Carvalho *et*

al., 2016]. The increasing life expectancy of dentition also means that older adults with exposed root surfaces are at greater risk of experiencing root caries during their lifetime [Gati and Vieira, 2011]. With fluoride known to exert its cariostatic actions primarily on smooth enamel surfaces, susceptible occlusal/root surfaces remain relatively predisposed to acid dissolution [ten Cate, 2009]. Caries preventive strategies must therefore go beyond conventional methods (personal oral hygiene, fluoride dentifrices, and limiting sugar exposures) to better protect at-risk surfaces in at-risk patients.

Fluoride Safety

The U.S Centers for Disease Control and Prevention ranked community water fluoridation as one of the 10 great public health achievements of the 20th century. Despite continued broad support within the dental profession worldwide, the polarised debate on the safety of fluorides used for caries prevention still continues in sections of the popular and scientific press. Recent reports can only add to the alarmist picture for sections of the general public who are not informed consumers of the scientific literature on this topic. For example, a 2012 systematic review and meta-analysis concluded that high fluoride exposure may lower IQ levels in children [Choi *et al.,* 2012], a finding which attracted media attention and resulted in the inclusion of fluoride amongst chemicals classified as developmental neurotoxicants [Grandjean and Landrigan, 2014]. There are however a number of concerns with how the 2012 review was conducted (all selected studies were from China, fluoride exposure came from multiple sources not just drinking water, definition of "high" fluoride levels varied widely, and the probability of confounding as covariates were not controlled), and the authors themselves were extremely conservative, only concluding that "our results support the possibility of adverse effects" advocating future research to evaluate dose-response relations based on individual-level measures of exposure over time. However, such caveats are universally overlooked when studies are reported in the media, and this fans extremist views on the safety of fluoride used for caries prevention.

Additionally, dental fluorosis from excessive intake of fluoride during the period of tooth formation is a persisting concern that is raised by anti-fluoride lobbyists. While emphasising that fluoride is safe when used at the recommended levels for community water fluoridation or dentifrice, behaviours such as swallowing large amounts of dentifrice are not. In modern times there are also now opportunities for "halo" exposure from various other sources of fluoride, which has

triggered downward revision of recommended levels for community water fluoridation (from 0.7-1.2 mg/L to 0.7 mg/L), to ensure that the risk for dental fluorosis does not increase [E.P.A., 2011].

Ecological caries preventive approaches synergistic with fluoride can potentially allow dental products to be designed with lower concentrations of fluoride and these could be useful for caries prevention in infants and young children, as well as for patients reluctant to use oral care products with high fluoride concentrations.

ECOLOGICAL APPROACHES TO CARIES PREVENTION

Antimicrobial Peptides (AMPs)

AMPs are a heterogeneous group of molecules with unique antimicrobial characteristics that have great potential for controlling bacterial infections and modifying biofilms. AMPs have a broad range of antibacterial, antiviral, and antifungal activity mediated by selectively interacting electrostatically with negatively charged components of cell membrane phospholipids, resulting in membrane permeabilization and disruption, leading to cell death [Koczulla and Bals, 2003]. To escape the actions of bilayer-disruptive AMPs would entail changing membrane composition and organisation, a ‘costly’ process in evolutionary terms, meaning that AMPs have very low resistance rates compared to common antibiotics [Zasloff, 2002]. Besides naturally secreted salivary AMPs (lactoferrin, cathelicidins, histatins, defensins), a number of AMPs have been synthesized in the laboratory, and these include specific anti-caries peptides that have shown the potential to modify plaque biofilms and inhibit dental caries.

The specifically targeted antimicrobial peptide (STAMP) is a synthetic fusion peptide with two independent functional domains, consisting of a *S. mutans*-selective ‘targeting domain’ designated as C16, and a ‘killing domain’ designated as G2. C16 is derived from a fragment of the *S. mutans* competence stimulating peptide (CSP), while G2 is derived from a broad-spectrum antimicrobial peptide [Eckert *et al.,* 2012]. C16G2 had antimicrobial mechanisms similar to traditional AMPs, and critically, its membrane disrupting activity specifically targets *S. mutans* from multispecies biofilms without affecting closely related non-cariogenic oral streptococci [Eckert *et al.,* 2006; Kaplan *et al.,* 2011]. More recently, an *in vitro* study on human saliva-derived polymicrobial biofilms was able to demonstrate that treatment with C16G2 not only eliminated *S. mutans*, but also resulted in a more benign oral microbial community with increased populations of health associated bacteria and fewer harmful Gram-negative bacteria [Guo *et al.,* 2015]. C16G2 is

recognized by the U.S Food and Drug Administration as an investigational new drug for dental caries prevention and has successfully completed phase II clinical trials, where it was delivered to patients in the form of a dental gel loaded in trays.

Another promising anti-caries AMP is a synthetic α-helical antimicrobial decapeptide designated KSL-W, which can selectively destabilize the cell membranes of cariogenic bacteria including *S. mutans*, *S. sobrinus*, and *L. acidophilus* [Leung *et al.,* 2015; Na *et al.,* 2007]. This peptide resits enzymatic degradation in human saliva for 1 hr, and the potential use of KSL-W as an anti-biofilm agent in a chewing gum formulation has been suggested [Faraj *et al.,* 2007; Na *et al.,* 2007]. More recently, a hydroxyapatite-binding antimicrobial was designed, based on the fusion of specific hydroxyapatite-binding heptapeptide with KSL-W, and this bioconjugate was shown to have improved oral retention and antibacterial efficacy [Huang *et al.,* 2016b]. Other AMPs that have shown *in vitro* antimicrobial activity against cariogenic bacteria and impaired biofilm formation include a synthetic peptide called L-K6 (derived from the naturally occurring peptide temporin-1CEb), a short synthetic amphiphilic peptide known as 1018, low-cost plant-derived AMPs like retrocyclin or protegrin, and a amphipathic α-helical peptide containing only 12 amino acids named GH12 [Liu *et al.,* 2016; Shang *et al.,* 2014; Tu *et al.,* 2016; Wang *et al.,* 2015].

Limitations of AMPs include their potential toxicity, susceptibility to proteases, high cost of peptide production, and reduced cationic activity of most AMPs in physiological fluids like saliva. With regards to their use for dental caries prevention, questions still remain on whether anti-caries AMPs will be able to function against a background of excessive acid production often seen in high caries-risk individuals [Maltz and Beighton, 2012]. Before any clinical recommendations can be made it will be essential to test such agents in a caries-conducive oral environment, and to evaluate whether the desired outcome of reduced caries increment in at-risk population groups can be achieved.

Probiotics

The term probiotics refers to “live micro-organisms, which, when administered in adequate amounts, confer a health benefit on the host” [Teughels *et al.,* 2008], and is based on the Noble-prize winning pioneering work of Metchnikoff [1907] for maintaining a healthy gut flora. This concept of implanting a harmless effector strain into the host’s microflora to maintain or restore a natural microbiome by inhibition of pathogenic microorganisms is attractive and has been also used to support health-associated microbes or restore diversity in the oral plaque biofilm. The

mechanisms by which probiotics re-establish ecological balance in oral biofilms are not fully understood, but probiotic bacteria are believed to have both local and systemic effects [Meurman, 2005]. The local anti-caries effects may include competitive inhibition with cariogenic bacteria for nutrition or adhesive surfaces [Terai *et al.,* 2015], selective co-aggregation of MS without disturbing other oral flora [Lang *et al.,* 2010; Twetman *et al.,* 2009], and bacteriocin-producing probiotics targeting MS [Burton *et al.,* 2013]. The most commonly used and studied probiotics belong to the *Lactobacillus* and *Bifidobacterium* bacterial genera, although not all their strains have the same efficacy in the inhibition of *S. mutans* growth or biofilm formation [Schwendicke *et al.,* 2017].

Evidence supporting the application of probiotics for preventing dental caries is controversial, with recent reports suggesting potential harmful effects for some probiotic bacterial strains [Gruner *et al.,* 2016]. One of the problems identified in using probiotics for caries prevention is that the commonly available *Lactobacillus* and *Bifidobacterium* probiotic bacteria are themselves acidogenic and aciduric, and could contribute to the caries process if such bacteria are allowed to colonise the oral cavity [Maltz and Beighton, 2012]. Recent *in vitro* biofilm studies have confirmed this apprehension with different strains such as *Lactobacillus salivarius* W24 [Pham *et al.,* 2009], *Lactobacillus rhamnosus* GG [Schwendicke *et al.,* 2014a], *Bifidobacteria animalis lactis* BB12 [Schwendicke *et al.,* 2014b], *Lactobacillus rhamnosus* LB21 [Fernandez *et al.,* 2015], and *Lactobacillus acidophilus* LA-5 [Schwendicke *et al.,* 2017] have all been shown to lower biofilm pH. In fact, some *Lactobacilli* and *Bifidobacteria* strains have greater cariogenic attributes than even MS [Beighton *et al.,* 2010]. Thus, while displacing *S. mutans* from plaque biofilms may in principle be a desirable, substituting them for even more cariogenic bacteria will not be useful [Schwendicke *et al.,* 2014a]. Some of the non-acidogenic alternatives to *Lactobacillus* and *Bifidobacterium* that have shown promising early results include Streptococcus salivarius M18 [Burton *et al.,* 2013], heat-inactivated BB12 [Schwendicke *et al.,* 2014b], and *Weissella cibaria* CMU [Jang *et al.,* 2016].

Another limitation with the traditional use of gut-associated *Lactobacillus* and *Bifidobacterium* probiotic species to promote oral health, is that these non-oral bacterial strains may not efficiently colonise the oral niche, which is vital for the long-term success of probiotics [Lopez-Lopez *et al.,* 2017]. Even the use of a bacteriocin-producing strain of *S. salivarius* may not succeed, as *S. Salivarius*, while a typical member of the oral soft tissue flora, has limited ability to colonise tooth surfaces. However, very recently two natural oral commensal species, *Streptococcus dentisani and Streptococcus* A12 that were isolated from the supragingival plaque of caries-free

individuals, have demonstrated promising probiotic effects against dental caries. Both these 'active colonisers' have a double probiotic action, as they can not only inhibit the growth of MS, but also moderate plaque pH through their arginolytic actions [Huang *et al.,* 2016a; Lopez-Lopez *et al.,* 2017].

The current decade has seen several systematic reviews and meta-analyses evaluating the effectiveness of using traditional gut-associated probiotics for caries prevention. A meta-analysis of studies with surrogate caries markers (MS and/or LB counts) concluded that these probiotics significantly decreased MS counts, but there was insufficient data on whether caries increment was reduced as well [Laleman *et al.,* 2014]. A similar inference was also reached by a qualitative systematic review of probiotic caries studies, concluding that clinical recommendations would be premature without more comprehensive RCTs showing actual reduction in individual caries experience [Twetman and Keller, 2012]. Relatively few RCTs of oral probiotics have used clinical dental caries indicators to prove the efficacy of probiotics in preventing or treating dental caries [Burton *et al.,* 2013; Nase *et al.,* 2001; Stecksen-Blicks *et al.,* 2009; Taipale *et al.,* 2013]. A comprehensive systematic review utilizing evidence from these RCTs, concluded that current evidence is insufficient for recommending probiotics in controlling dental caries [Gruner *et al.,* 2016].

Taken together, currently available data indicate that while traditional probiotic bacteria may have a beneficial effect on the gut flora and systemic health, a beneficial and clinically significant effect on the oral flora is yet to be demonstrated with sufficient rigor. However, observations from two major clinical trials in children [Näse *et al.,* 2001; Stecksén-Blicks *et al.,* 2009] support the intriguing concept of a 'metabolic domino effect', with reductions in caries risk seen to be accompanied by improvements in general health. Particularly promising for caries prevention is the move away from gut-associated probiotic bacteria to resident oral probiotic strains, such as the two *S. mutans*-antagonistic bacterial species *S. dentisani and A12.* Further evidence on the ability of these oral probiotics to inhibit dental caries is awaited with interest.

Prebiotics

The prebiotic approach involves feeding resident microbiota with specific nutrients to create conditions that favour the growth and dominance of healthy bacteria in the biofilm. The nutritional stimulation of endogenous beneficial oral flora to restore microbial balance and promote oral health has been validated in mixed species models [Slomka *et al.,* 2017]. Oral prebiotic substrates that are especially valuable to prevent caries include arginine, arginine-rich peptides, and urea, as these

foods when metabolized create alkalising effects that counteract the acidogenic environment created by cariogenic bacteria. Many commensal bacteria are able to use arginine or urea to generate ammonia by the arginine deaminase system or urease enzymes respectively [Bradshaw and Marsh, 1998; Nascimento *et al.,* 2009]. Multiple studies have shown that bacterial production of alkaline metabolites such as ammonia can play a major role in biofilm pH homeostasis and beneficially alter the de-/remineralization equilibrium [Burne and Marquis, 2000; Burne *et al.,* 2012; Nascimento *et al.,* 2014]. A substantial body of evidence from microbiological, genetic, biochemical analyses, and clinical studies has now accumulated confirming that the modulation of the alkalinogenic potential of dental biofilms is a promising strategy for caries control [Liu *et al.,* 2012].

The preventive potential of oral alkali production has resulted in the development of commercial oral care products that utilize arginine to promote a healthy resident oral microbiome. *In vitro* biofilm experiments found that fluoride-arginine combinations synergistically inhibited *S. mutans* but enriched *S. sanguinis* growth within multispecies biofilms, while maintaining a “streptococcal pressure” against the potential growth of oral anaerobe *P. gingivalis* in the alkalised biofilm [Zheng *et al.,* 2015]. Fluoride-arginine combinations can also supress exopolysaccharide (EPS) production, thus targeting another critical virulence factor for cariogenic biofilms [Zheng *et al.,* 2015]. Human *in situ* studies and several double-blinded RCTs using a fluoride dentifrice containing 1.5% arginine and an insoluble calcium compound have shown significantly greater protection against carious lesions than a fluoride dentifrice alone [Cantore *et al.,* 2013; Kraivaphan *et al.,* 2013; Petersen *et al.,* 2015; Srisilapanan *et al.,* 2013; Yin *et al.,* 2013]. A systematic review and meta-analysis of the anti-caries effects of arginine-containing dentifrice formulations concluded that arginine products provided a superior preventive effect over matched formulations containing fluoride alone [Li *et al.,* 2015]. Other authors have been more conservative in their conclusions, either citing insufficient evidence in support of a caries preventive effect for arginine, or expressing concerns over the higher cost of arginine-fluoride dentifrices versus any additional caries preventive effect these may provide [Astvaldsdottir *et al.,* 2016]. However, the preponderance of evidence does seem to suggest the arginolysis is an effective approach to improve oral health and balance the microbial ecology.

Sugar Polyols

Sucrose has been designated as the ‘arch criminal’ in the caries process for a long time [Newbrun, 1969], and the search for alternative non-fermentable sweeteners has attracted much

attention. Data collected from *in vitro* and *in vivo* studies indicate that such sugar substitutes can exhibit potential anti-caries effects through a number of different mechanisms [Matsukubo and Takazoe, 2006]. Xylitol, a naturally occurring five-carbon sugar polyol, is the non-nutritive sweetener that has been most extensively researched over the past four decades for its potential cariostatic effects. Xylitol inhibits MS growth by disrupting their energy production processes, leading to a futile energy cycle and cell death [Marttinen *et al.,* 2012]. Although not all MS strains were inhibited by xylitol in this manner, even xylitol-resistant bacteria were found to be less virulent after xylitol treatment [Trahan, 1995]. The predominant delivery vehicle for xylitol has been chewing gums, although xylitol dentifrices, candies, lozenges, and mouthrinses have also been used with varying degrees of success. A substantial body of evidence suggests that 5-6 g of xylitol per day delivered over three exposures are needed for worthwhile anti-caries effects [Milgrom *et al.,* 2009]. Among other sugar polyols, erythritol, has been attracting increasing attention as it is has been shown to be more effective than xylitol and sorbitol, and importantly, its anti-caries effects were shown to persist for up to three years [de Cock *et al.,* 2016; Falony *et al.,* 2016; Honkala *et al.,* 2014].

Despite an immense body of literature, the caries preventive effects of xylitol products remain inconclusive because of inconsistent study outcomes. While numerous studies have indicated xylitol has beneficial effects on surrogate endpoints (MS levels, plaque pH, acid production), evidence for worthwhile reductions in caries experience remains equivocal, and there is a need for more double-blind placebo-controlled RCTs, focussing on optimal dosage, delivery vehicle, and possible synergism with other preventive agents [Milgrom *et al.,* 2012; Twetman, 2009]. In fact, the more recent data is concluding that there is limited evidence to show xylitol is effective in the fight against dental caries. A double-blind cluster-RCT using xylitol gummy bears found that polyol consumption did not provide any additional benefit over other caries preventive measures [Lee *et al.,* 2015]. Recent Cochrane systematic reviews also found only very low to low quality evidence on xylitol effectiveness which was not sufficient to determine whether xylitol-containing products can prevent caries in infants, children or adults [Duane, 2015; Riley *et al.,* 2015]. Other issues that limit the usefulness of sugar polyols include their high costs and low compliance in high-risk patients because of the need for daily long-term use [Gold, 2016].

Quorum-sensing Targets

Another approach that may maintain and support a healthy oral plaque ecology is to interfere with the fundamental cell-cell communications system between biofilm bacteria. This

process of quorum sensing (QS) is mediated through small diffusible hormone-like molecules (pheromones) and their specific receptors. For MS, the stress-dependent QS system is primarily comprised of the competence stimulating peptide and its ComD/ComE two-component signal transduction system for communication between biofilm cells of the same species, while interspecies signalling is mediated via the autoinducer-2 (AI-2) molecule produced by LuxS [Senadheera D and Cvitkovitch DG, 2008]. The CSP-mediated QS system in *S. mutans* affects biofilm formation, acidogenicity, aciduricity, genetic transformation, bacteriocin production, stress response, and ability to produce persister phenotypes [Leung *et al.,* 2015]. Targeting QS signalling pathways could provide a promising avenue in the development of novels therapeutics to alter cariogenic biofilms. As QS is not directly involved in processes essential for bacterial growth, targeting QS will allow less virulent bacteria to remain in the biofilm, and also will not impose selective pressures that can lead to development of antibiotic resistance [Rasmussen and Givskov, 2006].

Interfering with CSP signalling systems has been shown to inactivate a wide range of bacteriocins and mutacins that play an important role in the sustained existence of *S. mutans* in the dental plaque [Cvitkovitch *et al.,* 2003; Qi *et al.,* 2004]. The addition of an exogenous CSP can disrupt signalling events of *S. mutans* and induce cell death [Qi *et al.,* 2005]. Another novel QS-modifying compound 3-Oxo-N, was seen to significantly minimise lactic acid accumulation without affecting biofilm growth even in the presence of fermentable sugars, representing a promising agent for maintaining a healthy, non-cariogenic microbial ecology in dental plaque [Janus *et al.,* 2016].

Natural Products

Natural products include secondary metabolites or phytochemicals derived from plants, fruits, herbs or spices. They offer a rich source of structurally diverse molecules with a wide range of biological activities and could prove useful as alternative or adjunctive anti-caries agents [Jeon *et al.,* 2011]. Potential cariostatic mechanisms identified include inhibition of bacterial growth, adhesion, acidogenicity, aciduricity, and glucan synthesis [Ferrazzano *et al.,* 2011; Jeon *et al.,* 2011].

Polyphenols from propolis (apigenin and *tt*-farnesol) and cranberry proanthocyanidins have been shown to exert useful ecological effects on the plaque biofilm. Apigenin is a potent inhibitor of water-insoluble glucan synthesis, while *tt*-farnesol disrupts *S. mutans* membrane permeability and acid production [Koo and Jeon, 2009]. An animal study found a combination of these two phytochemicals with fluoride suppressed dental caries without affecting the viability of oral flora,

being as potent as a fluoride-chlorhexidine control in caries inhibition, but without the broad antibacterial action of the control [Koo *et al.,* 2005]. Similarly, cranberry proanthocyanidins, which lack significant biocidal activity, can modify plaque biofilms by reducing acidogenicity, aciduricity, and glucan synthesis [Duarte *et al.,* 2006; Gregoire *et al.,* 2007], and this translated into cariostatic effects *in vivo* [Koo *et al.,* 2010]. A number of other polyphenol compounds have been found to be effective in killing *S. mutans,* with the minimal inhibitory concentrations of some bioactive molecules like xanthorrrhizol (from Javanese turmeric, *Curcuma xanthorrhiza*) or macelignan (from nutmeg, *Myristica fragrans*) almost comparable to chlorhexidine [Chung *et al.,* 2006; Hwang *et al.,* 2000]. While most of the tested anti-caries phytochemicals showed growth inhibitory or anti-adhesive effects, a potentially interesting natural agent in caries prevention is *Galla Chinensis*, which was uniquely able to beneficially regulate the de-/remineralization balance of dental hard tissues [Cheng *et al.,* 2015].

An analysis of how phytochemical research has impacted oral care in the period from 2000-2015 found that despite many *in vitro*, *in vivo*, and clinical studies testing natural products derived from plants, only 11% of studies were in phase IV clinical trials [Freires and Rosalen, 2016]. Similar conclusions were reached in a systematic review of the anti-caries effects of essential oils and their isolated constituents, which found that most studies were conducted in the laboratory and did not provide botanical characterization or compositional data on the natural product being tested [Freires *et al.,* 2015]. The emphasis in caries research using natural products has mostly focussed on microbial aspects of the disease process, but effects on de-/remineralization processes also need to be explored [Jeon *et al.,* 2011]. Natural products remain a largely unexplored source of effective and non-toxic anti-biofilm molecules that could potentially be used in combination with fluoride as useful alternatives to broad-spectrum biocides like chlorhexidine or triclosan. However, future research needs to focus on translational approaches to advance the development of effective anti-caries products containing phytochemicals or essential oils.

Replacement Therapy with "Designer" Bacteria

Modifying dental plaque by replacing *S. Mutans*, a member of the normal microbiota, with a less virulent effector strain has been an established concept for many years [Hillman, 1978]. The rationale for bacterial replacement therapy against dental caries is that relatively avirulent strains of MS are most likely to occupy the same ecological niche in plaque as their more cariogenic counterparts thereby reducing the overall cariogenicity of the plaque biofilm [Sun *et al.,* 2009].

A number of "designer" bacteria have been studied for bacteriotherapy against cariogenic biofilms including a glucan synthesis-defective mutant of *S. mutans*, variants of *S. salivarius* (TOVE-R), and a recombinant alkali-generating ureolytic *S. mutans* strain [Clancy *et al.,* 2000; Tanzer *et al.,* 1974; Tanzer *et al.,* 1985].The most extensive research in using genetically modified bacteria for preventing dental caries used a wild-type *S. mutans* strain that naturally produces an antibiotic called mutacin 1140 capable of killing all other strains of *S. mutans* [Hillman, 2002]. This strain was genetically modified by deleting the open reading frame for lactate dehydrogenase, to yield a viable strain called BCS3-L1, that still produced wild-type levels of mutacin 1140, but notably produced no lactic acid [Hillman *et al.,* 2000]. In laboratory and animal models, the BCS3-L1 strain proved to have significantly reduced cariogenic potential. It persistently and pre-emptively colonized tooth sites normally occupied by wild-type *S. mutans* strains, with no reported adverse effects [Hillman *et al.,* 2000]. To overcome safety issues and to enable the altered strain to be implanted into human oral biofilms for clinical trials, additional genetic modifications of BCS3-L1 was done to facilitate its rapid elimination should an adverse event manifest itself. This strain was designated as A2JM and was extensively tested to assure its safe use in Phase I clinical trials [Hillman *et al.,* 2007].

It has also been suggested that hypocariogenic strains exhibiting only defects in acid production are unlikely to compete successfully with wild-type strains for initial plaque locations, and on this principle, a *S. mutans* strain that was deficient in the *gcrR* gene was genetically engineered for bacterial replacement therapy [Pan *et al.,* 2013; Sun *et al.,* 2009]. The *gcrR* gene functions as a negative transcriptional regulator of the *gbpC* gene, which encodes the *Glucan-binding lectin (GBL), an adhesin that is ubiquitous on S. mutans surfaces, and plays an important role in initial bacterial aggregation and adhesion to tooth surfaces. The* MS-*gcr*R-def mutant bacteria showed reduced acid production, out-competed wild *S. mutans* strains with its strong early colonization ability, and lowered caries incidence *in vivo* [Pan *et al.,* 2013].

Modulation of oral plaque biofilms with genetically engineered "designer" bacteria has great potential through fostering a healthy oral environment, which prevents the dominance of cariogenic bacteria. A single treatment regimen could lead to persistent colonization by the effector strain affording lifelong protection, with minimal need for patient compliance. Whilst there have been encouraging results with genetically modified strains, the concept of replacement therapy needs to be tested for effectiveness in highly cariogenic environments. Even if successful, the widespread

acceptance of genetically-engineered "designer" bacteria may prove to be difficult for emotional, ethical, and legal reasons.

Other Approaches

In addition to the strategies discussed above, several other approaches for modifying plaque biofilm ecology are currently under investigation including using compounds that specifically affect bacterial virulence proteins [Horst *et al.,* 2012], free radicals released from pH-responsive catalytic nanoparticles that can degrade the EPS matrix [Gao *et al.,* 2016], calcium phosphate-osteopontin particles that can inhibit biofilm formation and reduce the fall in pH without affecting bacterial viability [Schlafer *et al.,* 2017], graphene oxide [He *et al.,* 2015] and ceramic water [Nomura *et al.,* 2017].

CONCLUSIONS

There is no doubt that fluoride will continue to be the mainstay of any caries prevention protocol as it still remains the most effective and economical protective agent against dental caries. However, fluoride alone may not offer complete protection against the disease, and it is generally recognized that the effectiveness of fluoride could be enhanced when combined with additional cariostatic agents [NIH, 2001]. Moreover, current paradigms emphasise the importance of maintaining a healthy and stable oral plaque biofilm for long-term disease control. One way to do this is to limit or exclude refined sugars from the diet. However, within the constraints of present-day consumer culture behavioural dietary changes are difficult to achieve and sustain. Adopting ecological preventive measures can help in correcting the disturbed plaque ecology and drive the advent and persistence of a symbiotic oral microbiome. These could be valuable tools in achieving long-term dental caries control, allowing the clinician to shift to a biological model for the management of the disease.

It is imperative that the effectiveness of ecological preventive approaches be evaluated for success in individuals who consume a conventional diet containing a fairly high level of sugars before any clinical recommendations are made [Beighton, 2009]. Furthermore, rather than surrogate endpoints like lower MS levels or reduced acid production, the critically important outcome for all new caries preventive measures will be whether they can ensure a significant reduction in individual caries experience.

2.1.2 REVIEW PAPER 2

Beyond *Streptococcus mutans*: Clinical Implications of the Evolving Dental Caries Aetiological Paradigms and its Associated Microbiome

This paper reviews the evolving dental caries aetiological concepts and highlights the clinical evidence for adopting an ecological approach to caries prevention, risk-assessment and treatment.

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ABSTRACT

Aetiological concepts of dental caries have evolved over the years from being considered as a disease initiated by nonspecific microorganisms, to being regarded as an “infectious” disease caused by specific bacteria, to the current paradigms that emphasise a “mixed bacterial-ecological approach” as being responsible for lesion initiation and pathogenesis. These aetiological paradigms are not just intellectual concepts but have important implications on how clinicians manage this age-old disease in the twenty-first century. Despite evidence-backed recommendations for adopting more biological measures to counter the disease, a significant proportion of dentists continue following traditional caries management guidelines in their daily clinical practice. This paper will review the evolving dental caries aetiological concepts and highlight the current evidence for adopting a more ecological approach to caries prevention, risk assessment, and treatment.

Dental caries remains the most common chronic disease in children (being five time as common as asthma), and is a major contributor to tooth loss in adults [Benjamin, 2010]. In fact, the most recent Global Burden of Disease report revealed that untreated caries in permanent teeth was the most prevalent human disease condition worldwide, with untreated caries in primary teeth being the tenth most prevalent disease [Kassebaum *et al.,* 2015]. Dental caries is now recognised to belong to a group of diseases like cancer or diabetes that are considered "complex" or "multifactorial" with no single causation pathway [Fejerskov, 2004]. There have been important paradigm shifts in the aetiological concepts of the disease and it is now widely recognized that the earlier singular focus on *Streptococcus mutans* to assess caries risk status or success of preventive measures and treatment interventions is no longer a viable strategy. This article highlights the evolution of dental caries aetiological theories over the years and how this continues to have important implications for caries prevention, risk assessment, and treatment.

EVOLVING PARADIGMS OF DENTAL CARIES AETIOLOGY

The role of microbes in the development of caries lesions was suggested as early as 1890 with the chemoparasitic theory of Miller [1973], and by the end of the nineteenth century it was commonly accepted that dental diseases were caused by the non-specific overgrowth of bacteria in dental plaque [Black, 1899]. According to this non-specific plaque hypothesis (NSPH) it was the quantity of plaque that determined levels of pathogenicity without discriminating between different levels of bacterial virulence. Applying the NSPH, it was recommended that the best way for preventing caries would be the mechanical removal of as much plaque as possible by tooth brushing or flossing [Rosier *et al.,* 2014]. By the mid-twentieth century, Keyes and Fitzgerald's famous animal model experiments revolutionised caries aetiological concepts by demonstrating that dental caries was an "infectious and transmittable" disease [Keyes, 1960; Tanzer, 1995]. In 1976, Loesche announced the specific plaque hypothesis (SPH), postulating that dental caries was an "infection" caused by specific bacteria within dental plaque [Loesche, 1976]. The specific bacteria that have for long been the *cause célèbre* for caries initiation and progression belonged to a group of Gram-positive acidogenic and aciduric bacterial species, now designated as mutans streptococci (MS), of which *Streptococcus mutans* and *Streptococcus sobrinus* are the most common in humans. For decades most diagnostic, preventive and therapeutic interventions were directed against these microorganisms. For example, the SPH proposed the use of antibiotics against specific bacterial species as a method to prevent and treat dental caries [Loesche *et al.,* 1977].

However, it is now acknowledged that Robert Koch's postulates on infectious diseases that focussed on specific pathogens as causative agents of disease are not applicable for microbial community-based diseases like dental caries or periodontitis. The bulk of the data that supports a relation between MS and dental caries can be considered associative rather than causative [Beighton, 2005]. There is also evidence showing that individuals with high MS levels do not necessarily develop caries lesions, while lesions have been detected even in the absence of MS [Galaviz *et al.,* 2005; Kleinberg, 2002]. Furthermore, the specific bacteria suggested to be responsible for caries are actually part of the indigenous microflora and unlike foreign pathogens cannot be eliminated for long from the oral cavity by use of antibiotics. Thus, while dental caries is of course microbially induced, the important point is that it is caused by endogenous bacteria that are normal part of the resident microbiome, and not by specific exogenous bacteria acquired from outside the host, as in other infectious diseases [Fejerskov, 2004].

The Bacterial-Ecological Approach

Contemporary concepts of dental caries aetiology and pathogenesis emphasise a "mixed bacterial-ecological approach" as being responsible for lesion initiation and progression [Kleinberg, 2002]. Rather than being considered as an "infectious disease" caused by a specific organism, dental caries is now understood to be a biofilm-mediated polymicrobial disease [Marsh, 1994]. Caries lesions develop due to a catastrophic ecological shift in the plaque biofilm microbial flora, instigating an imbalance in the physiologic equilibrium between tooth mineral and biofilm fluid, and ultimately tipping the caries balance towards demineralization and lesion formation [Marsh, 2003]. According to this ecological plaque hypothesis (EPH), the critical factors that trigger an upsurge in the acidogenic/aciduric component of the oral microbiome are local environmental conditions like frequent dietary sugar exposures or salivary dysfunction. Dental caries can thus be considered to be an endogenous infection which may occur when members of resident flora obtain a selective ecological advantage over other species, disrupting the homeostatic balance of the biofilm, and thereby initiating the disease process [Marsh and Martin, 1999].

Takahashi and Nyvad proposed an extension of the caries EPH to explain the relation between the dynamic changes in the phenotypic/genotypic properties of plaque bacteria and the de-/remineralization equilibrium of the caries process [Takahashi and Nyvad, 2008, 2011]. They suggested that the oral plaque biofilm is a dynamic microbial ecosystem with different microbial communities associated with the three reversible stages of the caries process (dynamic stability

stage, acidogenic stage, and aciduric stage). Mutable bacterial ecological succession takes place in the plaque biofilm at each stage of the caries process, depending on the severity and frequency of biofilm acidification [Takahashi and Nyvad, 2011]. It is only when the acidogenic environment is prolonged that highly aciduric bacteria like MS or lactobacilli, begin to dominate and replace the "low pH" non-MS, *Actinomyces* spp., or *Veillonella* spp. that are the main species populating the plaque microflora in the early stages of incipient (non-cavitated) carious lesions. Even at the highly aciduric stage, microbial composition and mineral loss can be reversed provided the acidogenic/aciduric properties of the biofilm are modified by adopting effective preventive measures such as restricting dietary sugar exposures [Takahashi and Nyvad, 2008]. The significance of the extended EPH model is that it is not the bacterial genotype *per se*, but the phenotypic characteristics (acidogenic and aciduric properties) and their regulatory parameters that are more relevant for causing a microbial ecological shift leading to caries [Takahashi and Nyvad, 2011]. There is now a consensus that any bacterial species can participate in the caries process as long as they are aciduric and dominant [Bowden, 1984].

These caries ecological concepts have been confirmed by recent DNA- and RNA-based molecular studies that have uncovered an extraordinarily diverse microbial ecosystem, where *S. mutans* accounts for a very small fraction (0.1% -1.6%) of the bacterial community implicated in the caries process [Simon-Soro and Mira, 2015]. Oral microbiologists have now expanded the principal bacterial species in caries-associated microbiomes from the traditional culture-isolated cariogenic bacteria like *S. mutans*, *S. sobrinus*, *Lactobacillus,* and *Actinomyces* to include bacterial species like non-MS*, Bifidobacterium*, *Scardovia wiggsiae*, *Prevotella* spp., *Selenomonas*, *Olsenella* spp., *Atopobium* spp., *Capnocytophaga* and many more [Aas *et al.,* 2008; Gross *et al.,* 2012; Tanner *et al.,* 2016]. In addition, bacterial-fungal associations can also synergistically enhance cariogenic biofilm virulence, with *Candida albicans* frequently detected in high numbers in plaque biofilms of children with early childhood caries (ECC) [de Carvalho *et al.,* 2006; Raja *et al.,* 2010]. This was an intriguing observation as *Candida* does not normally associate well with *S. mutans*, nor colonise teeth or metabolise sucrose effectively on its own [Koo and Bowen, 2014]. However, recent studies have demonstrated a symbiotic relationship exists between *S. mutans* and *C. albicans,* mediated through the influence of bacterially-derived glucosyltransferases, contributing to the increased severity of ECC [Falsetta *et al.,* 2014; Gregoire *et al.,* 2011; Kim *et al.,* 2017]. These findings prompt the possibility of incorporating anti-*Candida* therapy also in the treatment of virulent ECC [Koo and Bowen, 2014].

The polymicrobial nature of carious lesions implies that consortia formed by multiple microorganisms act collectively, probably synergistically, to initiate and advance the disease [Simon-Soro and Mira, 2015]. The 16S RNA-based estimates of microbial diversity over carious lesions also show different microbial consortia are formed in the dental plaque of different individuals. These microbial combinations have essentially the same functional profile, indicating that focussing on metabolic output of microbial communities would be more useful in controlling the disease regardless of the specific microbial compositions involved in the process [Simon-Soro and Mira, 2015; Takahashi and Nyvad, 2011].

Health-associated Microbial Communities

Another evolving paradigm in our understanding of the caries-associated microbiome is the role that health-promoting oral microbial communities can play in mitigating acid produced by cariogenic bacteria [Dewhirst, 2016]. Many oral commensal bacteria can counter the low pH produced by acidogenic organisms by using arginine or urea to generate ammonia [Nascimento *et al.,* 2009]. Such alkaline metabolites produced by "healthy" bacteria can play a major role in maintaining biofilm homeostasis to beneficially alter the de-/remineralisation equilibrium. Research has also shown microbial biodiversity to be crucial to health, with plaque and saliva of healthy individuals revealed to be much more diverse than originally hypothesized [Filoche *et al.,* 2010]. Results from clinical studies have shown that oral microbiomes of children with ECC were significantly less diverse than those of children without ECC, confirming that diversity in plaque microbial communities is essential for maintaining oral health [Kanasi *et al.,* 2010]. From the applied viewpoint, the use of broad-spectrum biocides like chlorhexidine for long-term caries control may not be desirable as they would cause an indiscriminate suppression of the entire oral microbiota [Caufield *et al.,* 2001].

Genetic Factors

Genetic factors can also be responsible for microbial ecological shifts that lead to disease. An individual's genotype can potentially prevent the existence of certain beneficial bacteria or allow pathogenic species to reside and contribute to its unique microbiome [Turnbaugh *et al.,* 2007]. The criticism of the caries EPH is that it does not consider host genetic susceptibility to caries [Rosier *et al.,* 2014]. A salient observation from the unethical Vipeholm study was that about 20% of the individuals who received high frequency cariogenic snacks for two years did not develop dental caries, and this genetic resistance to caries was further supported by the observation that parents

and siblings of these individuals also showed lower caries prevalence than the rest of the population [Böök and Grahnén, 1953; Gustafsson *et al.,* 1954]. There is now additional recent evidence that certain at-risk individuals and population groups are genetically more susceptible to dental caries [Bretz *et al.,* 2005; Opal *et al.,* 2015; Wang *et al.,* 2013], implying that hereditary factors should also be considered while planning individual caries management protocols.

In summary, the current ecological model of dental caries presents a complex picture of a multifactorial, pH-driven disease whose onset and progression is influenced by multiple pathogens, systemic effects, and hereditary components layered on interactions of diet, behavioural, environmental, socioeconomic, and physiological risk factors. The caries process can be considered as a model system of dynamic amphibiosis, where under normal environmental conditions the biofilm microorganisms live in a symbiotic relationship with the host, characterised by commensalism and mutualism.[Ruby and Goldner, 2007] The nature of this symbiosis may shift under changing local environmental conditions with mutualism becoming parasitism, and this dynamic adaptation is the basic principle that underlines our current understanding of endogenous disease processes like dental caries.

ORAL CARE IMPLICATIONS

The current aetiological paradigms of the dental caries have important consequences for oral care. Complex diseases like dental caries that arise from the concerted action of biofilm-embedded polymicrobes, risk-conferring behaviours, environmental influences, and genes present clinical challenges on how these multiple contributing factors can be assessed in a way that can be translated into effective approaches for caries prevention, risk assessment, and therapy. Contemporary evidence-backed recommendations for management of dental caries, based on the ecological-biofilm concepts of the disease, have revolutionised many conventional caries management philosophies, and clinicians need to be aware of the emerging evidence with regards to prevention, risk-assessment and treatment of dental caries.

Dental Caries Prevention

With the mechanism of dental caries now well established, new strategies are being sought for better targeted caries prevention based on a scientific understanding of the processes involved. The earlier focus on determining the specific causative agents is giving way to ecology-based propositions where the disease is seen as the output of a skewed microbial community caused by

environmental changes. While fluoride will remain the cornerstone of any dental caries preventive protocol, a consensus is emerging that preventive measures should also focus on remediation of environmental pressures responsible for the plaque biofilm dysbiosis, while maintaining the resident microbiota at levels compatible with health [Marsh *et al.,* 2015].

Advising patients to reduce dietary exposure to fermentable carbohydrates is clearly the most effective approach to prevent acidification and the detrimental ecological shift of the plaque biofilm. However, requiring individuals to modify their dietary behaviour as part of caries prevention, is usually the most difficult advice for patients to adhere to [Duggal and van Loveren, 2001]. Additional preventive measures that are synergistic with fluoride have thus been advocated for high caries-risk groups [NIH, 2001]. Ecological caries preventive approaches are probably the next frontier in the long-standing attempts to obtain control over this ubiquitous disease. These preventive measures are based on the current caries aetiological paradigms, where an ecologically balanced and diverse microbiome is seen as the key to long-term control over the disease. Ecological preventive approaches can broadly be divided into measures that either enhance the growth of health-promoting microbial communities within the plaque biofilm, or antimicrobial strategies that can weaken cariogenic biofilm virulence properties without eliminating the entire microbiome. Examples include prebiotics, probiotics, antimicrobial peptides (AMP), sugar polyols, phytochemicals, quorum-sensing (QS) targets, and genetically-modified designer bacteria.

The ecological preventive approach that is probably the most evidence-backed is the use of prebiotics like arginine or urea. Prebiotics are nutritional substrates that commensal bacteria can breakdown to alkalise the biofilm, thus preventing the overgrowth of acidogenic/aciduric bacteria. The nutritional stimulation of endogenous oral flora to restore microbial balance and promote oral health has been validated in mixed species models [Slomka *et al.,* 2017]. A substantial body of evidence from microbiological, genetic, and biochemical analyses suggests that alkali generation in dental biofilms contributes to inhibition of dental caries [Liu *et al.,* 2012]. Additionally, human *in situ* studies, double-blinded RCTs, and systematic reviews have demonstrated that a 1.5% arginine-containing fluoride toothpaste (Colgate Maximum Cavity Protection plus Sugar Acid Neutraliser™) provided significantly greater protection against caries than a fluoride dentifrice alone [Cantore *et al.,* 2013; Kraivaphan *et al.,* 2013; Li *et al.,* 2015; Petersen *et al.,* 2015; Srisilapanan *et al.,* 2013]. Although other reviews have questioned the dentifrice's preventive efficacy and its comparative higher costs [Astvaldsdottir *et al.,* 2016; Shaw *et al.,* 2015], the preponderance of evidence does

seem to suggest that arginine-fluoride dentifrices offer a new standard of care for caries prevention in high-risk patients [ten Cate and Cummins, 2013]. An alternative to arginine dentifrices may be a toothpaste containing enzymes and proteins (Zendium™), that was recently shown to boost oral defences by promoting a beneficial shift in the oral microbial ecology [Adams *et al.,* 2017]. However, more comprehensive randomised controlled trials (RCTs) regarding its caries preventive effects are required before firm clinical recommendations can be made.

The use of probiotic bacteria (lactobacilli and bifidobacteria) to support health-associated microbes or restore diversity in the oral plaque biofilm is enjoying growing popularity as an ecological method to control dental caries. However, while probiotics may indeed improve surrogate caries markers (e.g. reduced MS counts) [Laleman *et al.,* 2014], there are doubts on whether this actually results in any reduction in individual caries experience [Twetman and Keller, 2012]. Another drawback of using traditional probiotics for caries prevention is the potentially harmful acidogenic effects of many commonly used oral probiotic bacterial strains. [Gruner *et al.,* 2016] Furthermore, the traditional use of gut-associated lactobacilli and bifidobacteria probiotic species to promote oral health may not succeed as these non-oral bacterial strains cannot efficiently colonise the oral niche, which is vital for the long-term success of probiotics. A more promising breakthrough in the use of oral probiotics for caries prevention is the recent identification of two *S. mutans*-antagonistic oral probiotic strains, *Streptococcus dentisani and Streptococcus* A12. Both these bacteria are natural oral commensal species having double probiotic action, as they not only inhibit the growth of major oral pathogens but can also moderate plaque pH through their arginolytic actions [Huang *et al.,* 2016; Lopez-Lopez *et al.,* 2017]. However, clinical recommendations would be premature at this stage without evidence from RCTs showing significant reductions in caries increment on using these oral probiotics.

Another long-standing approach to promote healthy biofilms is the use of non-fermentative sugar polyols (e.g. xylitol, sorbitol, erythritol) that are believed to inhibit virulent bacteria allowing healthy bacteria to dominate in the biofilm. Despite an immense body of literature, the caries preventive effects of xylitol oral products remain inconclusive, with more recent data concluding that there is limited evidence to show that xylitol provided any additional benefit beyond other preventive measures [Duane, 2015; Lee *et al.,* 2015; Riley *et al.,* 2015]. Among the sugar polyols, erythritol may be the one that hold most promise, as new studies indicate that it may be more effective than xylitol and sorbitol, and critically, its anti-caries effects were shown to persist for up

to three years after the intervention ended [de Cock *et al.,* 2016; Falony *et al.,* 2016; Honkala *et al.,* 2014].

The other side of the ecological preventive approach is to use antimicrobial agents to modify the plaque biofilm. It is logical to assume that caries prevention can potentially be improved by combining fluoride, which predominantly has physiochemical effects on the hard dental tissues, with biological agents that can reduce the severity of the bacterial attack on teeth. However, many of the commonly available toothpastes and mouthwashes use broad-spectrum antimicrobials which can cause an undesirable suppression of even the healthy plaque microflora. There is now a consensus that antimicrobials used for dental caries prevention should be more subtle in their anti-plaque actions, aiming to undermine bacterial virulence properties (e.g. glucan synthesis or acid production) rather than bacterial viability [ten Cate and Zaura, 2012]. Promising antimicrobial measures with biofilm-modifying cariostatic action include specifically targeted antimicrobial peptides (STAMP) [Kaplan *et al.,* 2011], natural phytochemicals [Koo *et al.,* 2010; Koo *et al.,* 2005], genetically-engineered *S. mutans* strains [Hillman *et al.,* 2007; Pan *et al.,* 2013], and QS-targeting compounds [Janus *et al.,* 2016; Qi *et al.,* 2005]. Although promising, the ultimate success of these antimicrobial ecological approaches will depend on their ability to effectively act in the highly cariogenic oral environments, often found in high-risk individuals, where there is frequent biofilm acidification due to multiple sugar exposures.

The changed paradigms of dental caries has implications for dental public health programmes too. A consequence of dental caries being a multifactorial disease is that a particular preventive program that is successful in one population group, need not be so in another population group with different cultural and behavioural habits. The complexity of the disease also implies that dental caries can never be 100% preventable at the individual or societal level [Fejerskov, 2004]. What this implies is that a well-trained dentist should regularly monitor patients for any change in their oral environmental conditions to avoid new lesions.

Caries-risk Assessment

Caries-risk assessment (CRA) is fundamental to modern caries management protocols, assisting the clinician in making decisions regarding the type of diagnostic procedures, preventive/treatment interventions, and recall intervals required for each patient. When high caries-risk patients are effectively identified, more resources and intensive preventive interventions can be directed towards them, while avoiding redundant diagnostic procedures and therapy in

patients at low-risk of developing caries lesions. Past caries experience has been demonstrated to be the best risk predictor of the disease in every age group [Mejare *et al.,* 2014; Powell, 1998]. However, this metric does not provide an explanation for the heightened risk, and thus may not suggest the best avenue for prevention.

Microbiological tests on the levels of MS or lactobacilli in plaque or saliva have traditionally been used to predict an individual's risk of developing caries lesions. However, with an entire consortium of acidogenic/aciduric bacterial species now known to be involved in the caries process such microbial tests have limited applicability in assessment of caries activity and in risk prediction [Bowden, 1997]. For instance, eating habits and socio-economic status of children and their caregivers have been shown to be better predictors of ECC than MS titre levels [Nunn *et al.,* 2009]. The high MS levels detected in patients with severe ECC can be considered an incidental consequence of the frequent acidification of plaque by poor eating habits, rather than an early risk predictor [Takahashi and Nyvad, 2011]. The fact that *S. wiggsiae* has been shown to have the same prevalence as *S. mutans* in ECC lesions, or that *Bifidobacterium dentium* was found in cavitated lesions but never in healthy plaque, suggests that assessing combined levels of several indicator aciduric bacterial taxa may be more useful for risk prediction [Henne *et al.,* 2015]. Microbiological tests should only be considered as one component of the patient's caries risk history, establishing a norm for the individual patient, with any deviations from this indicating a change in the oral environment [Bowden, 1997].

The recognition of the multifactorial aetiology of dental caries has led experts and professional bodies to develop multivariate CRA systems accounting for each of the disease-associated factors, assessing them not in isolation but with regards to their unique and dynamic interactions. Examples of some of the currently recommended CRA protocols include the cariogram model [Petersson *et al.,* 2002], the caries management by risk assessment (CAMBRA) system [Featherstone *et al.,* 2007; Ramos-Gomez *et al.,* 2007], the American Academy of Paediatric Dentistry's caries-risk assessment tool (CAT) [AAPD, 2013], systems of the American Dental Association [ADA, 2009, 2011], the Traffic Light Matrix system (TLM) [Ngo and Gaffney, 2005], and the Dundee caries risk assessment model (DCRAM) [MacRitchie *et al.,* 2012].

The clinical utility of these protocols to guide the design of precise personalised care has been questioned with a systematic review finding that the validity of some the commonly used CRA protocols (cariogram, CAMBRA, CAT, and ADA) to accurately predict caries risk is limited [Tellez *et*

al., 2013]. This has been confirmed with the cariogram model shown to be not "particularly useful in identifying high caries risk patients in a low-caries community" [Holgerson *et al.,* 2009], while the CAMBRA protocol was not able to significantly distinguish between the low and moderate caries-risk groups [Tellez *et al.,* 2015]. A more recent critical review suggested that it was fallacious to transfer and apply population risk estimates to assess individual caries risk [Divaris, 2016]. For example, ECC risk factors shown to be consistently correlated with caries prevalence in large population studies are poor predictors of individual lesion occurrence.

However, many of these CRA tools still have excellent pedagogical value, facilitate communication with patients, and have the potential to enhance oral care by identifying the specific causes for the caries activity, allowing the clinician to customise the treatment plan according to individualised needs of patients [Divaris, 2016; Domejean *et al.,* 2017]. For a more comprehensive risk assessment and personalised caries care plan, clinicians may be advised to adopt the international caries classification and management system (ICCMS) [Pitts and Ekstrand, 2013], or the system for total environmental management (STEM) protocol [Walsh, 2008], as these include explicit patient-level assessments as part of the overall disease management.

Dental Caries Treatment

Caries treatment approaches have progressed over an extended timeframe from the early extractive phase to the commonly practiced restorative phase through to the currently recommended preventive/preservative phase [Pitts, 2004]. The evidence for the evolution of caries management philosophies from a restorative-only approach to a preventive/preservative approach has now been available for at least a couple of decades [Elderton, 1993; Mertz-Fairhurst *et al.,* 1998]. Unfortunately, caries management continues to be skewed to "drilling" and "filling", with a recent systematic review and meta-analysis finding that a significant proportion of dentists continue to intervene invasively (restoratively) on carious lesions where clinical recommendations indicate less invasive therapies should be used [Innes and Schwendicke, 2017]. The largely technical and mechanistic approaches to tooth restoration ignores the emerging evidence strongly recommending more biological approaches to controlling disease causative factors and the need for clinicians to adopt the principles of minimum intervention dentistry (MID) for treating non-cavitated and cavitated lesions [Pitts and Zero, 2016]. The overarching approach to caries management in the 21st century should be to "preserve the tooth structure and restore only when necessary" [Ismail *et al.,* 2013].

The rationale underpinning many of the current recommendations for managing caries lesions are based on the aetiological paradigms of EPH. Fundamental to the EPH is that unless there is an attempt to interfere with the environmental factors driving the biofilm dysbiosis, the patient is likely to suffer from repeated episodes of the disease and the clinician will encounter frequent failure of any restorative or preventive treatment rendered. Most restoration failure is due to secondary caries associated with restorations and sealants (currently referred to as CARS), highlighting that patient risk factors should be regularly monitored and actively managed [Domejean *et al.,* 2017]. While repairing cavitations (the end result of the disease process) is important, these are merely symptoms of the disease, and alleviating symptoms by restorations alone cannot proffer a "final solution" to the disease [Walsh, 2008]. It is wrong to assume that drilling out a caries lesion and placing a restoration eliminates bacteria and stops the disease process [Featherstone, 2000]. With the extended EPH establishing a firm association between plaque microbiome diversity and the de-/remineralization caries equilibrium, a biological understanding of the disease process and its associated microbiome is vital for successful clinical management of dental caries.

There are now excellent papers detailing the contemporary guidelines for operative management of caries lesions in permanent and primary teeth based on the recommendations of the International Caries Consensus Collaboration (ICCC) [Banerjee *et al.,* 2017; Schwendicke *et al.,* 2016]. These consensus recommendations underscore the pathological basis of dental caries as a biofilm disease and emphasise that both the prevention of new lesions and management of existing lesions should primarily focus on biofilm management rather than tissue removal. Although some of these recommendations may seem counterintuitive to decades of clinical practice and dental training, they are in keeping with our current understanding of the dental caries disease process. For instance, in teeth with deep cavitated lesions, it is recommended that carious tissue be removed only to create conditions for long-lasting, tightly sealed restorations, and all the bacterially contaminated (soft/infected dentine) or demineralised tissue close to the pulp need not be removed [Schwendicke *et al.,* 2016]. This makes sense once it is accepted that dental caries is tissue destruction caused by bacterial metabolism in the biofilm – if the disease process can be arrested by modifying the biofilm, the symptoms of the disease (demineralised dentine) is removed purely to create a sufficiently large surface to bond to and optimise the longevity of a restoration. Once bacteria are sealed into the tooth, the biofilm is physically prevented from accessing nutrition and an actively carious lesion becomes an arrested lesion.

A number of studies have clearly shown that the microbiological load in infected dentine is progressively reduced when it is sealed off from the oral environment [Bjorndal *et al.,* 1997; Mertz-Fairhurst *et al.,* 1998; Paddick *et al.,* 2005; Weerheijm *et al.,* 1999]. The gradual reduction in lesion activity allows time for the pulp-dentine complex to lay down tertiary and peri-tubular dentine providing further protection to the pulp complex and reducing risk of pulp exposure [Ricketts *et al.,* 2013]. Multiple RCTs and systematic reviews have not found any detrimental effects to the pulp by sealing in bacteria [Bjorndal *et al.,* 2010; Hesse *et al.,* 2014; Innes *et al.,* 2007, 2011; Mertz-Fairhurst *et al.,* 1998; Orhan *et al.,* 2010; Ricketts *et al.,* 2013]. These include the classical studies of the Mertz-Fairhurst group which showed that bonded and sealed composite restorations placed over frank cavitations arrested the clinical progress of these lesions even at 10 years, profoundly changing our concepts of how much demineralised dentine may be left during cavity preparation [Kidd, 2016]. A similar rationale supports the use of the Hall technique, where preformed stainless steel crowns are placed directly over asymptomatic carious primary molar teeth without removing any carious tissue (and hence not requiring any local anaesthesia or tooth preparation). This radical treatment option gave rise to a great deal of controversy and questions in the past [Croll *et al.,* 2015; Nainar, 2012], but as authors of a recent seminal paper on the Hall technique assert "emotion, misinformation, and outdated ideas have been used in arguments against Hall crowns rather than logic, understanding or evidence" [Innes *et al.,* 2017]. There is now robust evidence showing that the Hall technique, when appropriately used in indicated teeth (clear band of dentine between carious lesion and pulp), has success rates superior to comparator treatment [Boyd *et al.,* 2017; Innes *et al.,* 2007, 2011; Innes *et al.,* 2015; Santamaria *et al.,* 2014]. Given the high success rate and patient acceptability of the Hall technique, clinicians still reluctant to offer this treatment option, need to examine why they are treating a child more invasively when a less invasive option is available [Innes and Manton, 2017].

Many of the core principles of MID elucidated by Walsh and Brostek [2013] are also in keeping with the current aetiological and preventive/preservative paradigms of dental caries. These MID principles include: recognition (of disease contributory factors); re-orientation (of the contributory lifestyle factors); remineralisation (of both cavitated and non-cavitated lesions); repair (only when other solutions are not possible); and review (to ensure healthy oral and life environment is maintained) [Innes and Manton, 2017; Walsh and Brostek, 2013]. The chance for MID to be successful is increased if dental caries is not considered as an infectious disease but rather a behavioural disease with a bacterial component.

By approaching the clinical situation of caries from a biological standpoint, the vicious cycle of treatment and retreatment of CARS can be terminated. This is done most effectively by evaluating and tackling the risk factors that fuel the disease process and adopting one of the several biological management options available. These contemporary caries management philosophies are consistent with the ICCMS or STEM, whose intergral aims are to preserve tooth structure with non-operative biological care at the initial stages, and conservative tooth-preserving operative care at at more extensive stages of the carious lesion.

CONCLUSIONS

About 700-800 bacterial species have been identified from the human oral microbiome making the mouth the most microbiologically diverse environment in the body. Both traditional as well as newly identified bacterial species have an important role in the caries process, and from an ecological point of view it may be more important to describe what the bacteria are doing in the biofilm community rather than which bacteria are present [Takahashi, 2015]. Given the polymicrobial nature of dental caries, it is predicted that diagnostic, preventive, and treatment strategies directed toward specific bacterial species will not be universally effective [Simon-Soro and Mira, 2015]. Another important change in perspective is the realisation that there are beneficial members of the oral microbiome and an understanding of health and disease requires knowledge of all microorganisms, not just a select few pathogens.

Dental caries affects all age groups, and can have particularly devastating, life-long consequences in young children, while in the elderly it can compromise an individual's ability to eat leading to malnutrition and expediting mortality. It is thus critical that dentists recognize the evolving aetiological paradigms of the dental caries process and its clinical implications. Oral health care professionals need to incorporate contemporary evidence-based guidelines into their routine practice for the benefit of their patients. Further education and training for dental practitioners, coupled with fairer remunerative schemes, would encourage a change in favour of the provision of more biological approaches towards prevention and treatment of dental caries [Suga *et al.,* 2014].

2.2 LITERATURE REVIEW: NATURAL PRODUCTS

2.2.1 REVIEW PAPER 3

Potential Role for Natural Products in Dental Caries Control

This paper presents the emerging evidence on the anti-cariogenic properties of natural products and the rationale for their use in controlling dental caries. The paper also discusses the challenges and future research directions needed to translate the cariostatic potential of natural products into clinically relevant oral care products.

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This chapter is comprised of the publication as it stands in print.

ABSTRACT

Dental caries is today recognised as a polymicrobial biofilm-mediated behavioural disease resulting from an unfavourable ecological change in the oral microbiome. In addition to fluoride-based preventive measures, the importance of adopting ecological approaches to dental caries prevention is being increasingly highlighted. This has resulted in renewed interest in the anti-cariogenic properties of a number of natural products, especially those that have the ability to inhibit cariogenic virulence properties or bacterial adhesion without disrupting the key health benefits of the resident oral microbiome. Other cariostatic mechanisms identified for natural products include phytochemicals that can inhibit demineralization and enhance remineralization thereby beneficially rebalancing the caries equilibrium, and natural non-fermentable dietary sweeteners that can reduce the acidogenic challenge posed by sugar-laden foods or beverages. There are however a number of significant challenges to which future research needs to be directed in order to translate the potential of cariostatic natural products into clinically relevant oral care products for dental caries control.

The term “natural products” is generally used to refer to secondary metabolites or phytochemicals derived from plants, herbs, spices, and fruits. While not essential nutrients, these bioactive chemicals are believed to be responsible for much of the disease protection conferred by diets rich in plant-based products [Arts and Hollman, 2005]. The influence of diet on oral health is indisputable, as can be clearly observed in populations consuming high quantities of polyphenol-rich foods [Signoretto *et al.,* 2010; Taylor *et al.,* 2005]. Both Eastern and Western civilisations have used natural products to prevent or treat oral maladies, from recipes of herbal mouthwashes and dentifrices mentioned in the Ebers Papyrus, to the use of plant-based products to cure bad breath by Greek and Roman physicians. The advent of the “Antibiotic Era” in the mid-20th century did shift the emphasis away from natural product-based formulations. However, the emergence of antibiotic-resistant bacterial strains and other adverse effects of synthetic chemotherapeutics have rekindled interest in the search for biocompatible natural antimicrobial agents, including those with activity against cariogenic biofilms.

Dental caries results from a deleterious shift in the microbial ecology of the resident oral microbiome, rather than from any exogenous “infection” acquired from outside the host [Fejerskov, 2004]. Under normal oral environmental conditions, the dental plaque biofilm has a symbiotic relationship with the host characterised by commensalism and mutualism [Ruby and Goldner, 2007]. However, deleterious lifestyle factors (e.g. frequent exposure to fermentable carbohydrates) can alter local environmental conditions, causing biofilm dysbiosis, resulting in the acidogenic/aciduric members of resident oral flora gaining a selective ecological advantage over other commensal species. This disrupts the homeostatic balance of the biofilm and initiates the dental caries disease process [Marsh, 2003]. While dental caries is undoubtedly a microbial disease, it is imperative not to perturb or lose the beneficial functions delivered by the resident oral microbiome by non-specific mechanical plaque removal or application of broad-spectrum antimicrobials [Philip *et al.,* 2018; Yoo *et al.,* 2011]. Consumer oral care products should ideally focus on maintaining the composition and activity of healthy plaque microflora rather than eliminating them completely [Marsh *et al.,* 2015]. Natural products have exhibited the potential to maintain the delicate balance needed to control oral microflora at levels compatible with health and could be valuable adjuncts to control biofilm-mediated oral diseases like dental caries.

Various antimicrobial agents have been recommended for caries control in high-risk individuals, from biocides like chlorhexidine [Ribeiro *et al.,* 2007], triclosan [Chen *et al.,* 2010], and

cetylpyridinium chloride [Holbeche *et al.,* 1976] to antibiotics like vancomycin [De Paola *et al.,* 1974], based on their ability to reduce levels of mutans streptococci (MS). However, most of these chemotherapeutic agents exert a broad-spectrum of antimicrobial action, suppressing even the health-associated commensal microflora. Randomised controlled trials (RCTs) and systematic reviews have indicated that their long-term effectiveness in preventing new lesions in highly caries-active patients is limited [Plonka *et al.,* 2013; Twetman, 2004]. Natural products that can specifically target bacterial adherence, glucan synthesis, or biofilm matrix acidification without killing beneficial microbes are an attractive alternative to traditional broad-spectrum microbicides [Jeon *et al.,* 2009]. Furthermore, oral products containing synthetic antimicrobials can cause a number of adverse effects making their long term use impractical [Eley, 1999; Marsh, 1991]. Natural antimicrobials, on the other hand, generally have excellent biocompatibility allowing for the improved compliance and public acceptance of oral care products containing them.

Cariostatic natural agents acting in concert with the anti-caries effects of fluoride could be a promising strategy for long-term dental caries prevention [Jeon *et al.,* 2011]. Animal model caries studies have shown that fluoride-phytochemical combinations were as potent as fluoride-chlorhexidine controls in caries inhibition, with the added benefit that the resident oral microbiota were not affected [Koo *et al.,* 2010; Koo *et al.,* 2005]. Evidently, the emerging field of phytodentistry can be particularly promising for the prevention of biofilm-mediated oral diseases like dental caries [Chenicheri *et al.,* 2017]. This paper discusses the cariostatic mechanisms of some of the most promising natural products and the research directions that are needed to translate the potential of natural products into commercial oral care products for dental caries control. The search strategy for this narrative review involved performing a comprehensive literature search to capture all published studies (up to December 2017) specifically related to natural products with anti-caries effects. The databases searched were MEDLINE (via PubMed), EMBASE, and Web of Science using a combination of controlled vocabulary and text words. The reference list of all selected studies were also hand searched for additional relevant studies.

NATURAL PRODUCTS: CLASSIFICATION AND CARIOSTATIC MECHANISMS

Naturally occurring bioactive compounds possess structures that have high chemical diversity and biochemical specificity that make them attractive candidates for drug discovery and development [Koehn and Carter, 2005]. Phytochemicals from natural sources have been classified into three broad groups based on their chemical structure [Chinou, 2008]: (i) polyphenols, which

are the most widely distributed phytochemicals in the plant kingdom, (e.g. flavonoids, Fig. 3); (ii) terpenoids, which include the essential oils used in some anti-plaque mouthwashes (e.g. thymol and eugenol); and (iii) alkaloids, which are nitrogen-containing compounds (e.g. sanguinarine).

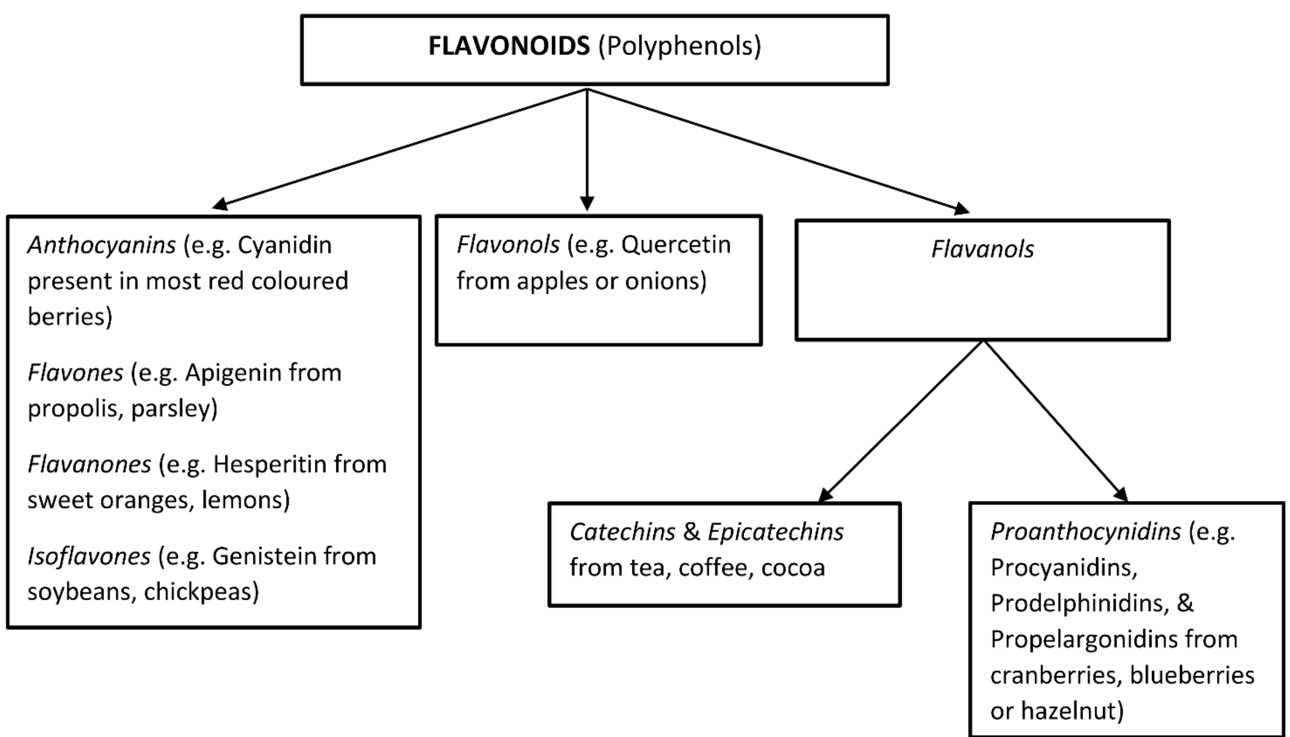

Figure 3. Different classes of flavonoids and their food sources

Three potential antimicrobial mechanisms have been identified for natural products to inhibit dental caries: (i) antibacterial activity through the inhibition of bacterial growth and metabolic activity; (ii) inhibition of glucan synthesis through suppressing glucosyltransferase (Gtf) activity; and (iii) inhibition of bacterial adherence [Jeon *et al.,* 2009]. In addition to these antimicrobial properties, other proposed modes of cariostatic action include modulation of the demineralization-remineralization caries equilibrium, and natural products that provide a source of non-fermentable sugar substitutes. A summary of selected key natural products, their reported anti-caries mechanisms, and the bioactive phytochemicals believed to be responsible for these cariostatic actions is presented in Table 1 below.

Table 1. Natural products with anti-caries activity

Natural Product Source	Studies [authors, year]	Bioactive phytochemicals	Cariostatic Effects
Cacao bean husk	Matsumoto *et al.,* 2004; Ooshima. *et al.,* 2000; Osawa *et al.,* 2001; Venkatesh *et al.,* 2011	Oleic acid, linoleic acid and epicatechins	Oleic & linoleic acids have bactericidal actions at concentrations of 30 µg/mL. Epicatechins displayed anti-Gtf activity.
Cinnamon (*Cinnamomum camphora*)	Chaudhari *et al.,* 2012; Gupta *et al.,* 2011	Cinnamaldehyde and eugenol	Anti-bacterial action against MS.
Cranberry (*Vaccinium macrocarpon*)	Duarte *et al.,* 2006; Gregoire *et al.,* 2007; Koo *et al.,* 2010	Polyphenols like PACs, flavonols, phenolic acids and anthocyanins	Inhibits acidogenicity and aciduricity. Disrupts glucan synthesis with anti-Gtf activity. Reduces caries lesion development *in vivo*
Crustacean shells	Busscher *et al.,* 2008; Hayashi *et al.,* 2007; Tarsi *et al.,* 1997	Low molecular-weight chitosans	Inhibitory effects on *S. mutans* adhesion to tooth surfaces.
Galla chinensis	Cheng *et al.,* 2011; Cheng *et al.,* 2008, 2010; Xie *et al.,* 2008	Gallic acid and other polyphenols	Inhibits demineralization, enhances remineralization and fluoride effects. Inhibits polymicrobial biofilm development.
Ginger (*Zingiber officinale*)	Hasan *et al.,* 2015	Flavonoids and gingerols	Inhibits multiple *S. mutans* virulence factors including glucan synthesis, adherence, F_1F_0-ATPase activity and acidogenicity.
Guava (*Psidium guajava*)	Jain *et al.,* 2014; Prabu *et al.,* 2006	Guaijaverin	Bacterial growth inhibitory activity with MICs against *S. mutans* between 2-4 mg/mL.
Hop bracts (*Humulus lupulus*)	Shinada *et al.,* 2007; Tagashira *et al.,* 1997	Not known	Inhibitory effects against cellular adherence, Gtf activity and plaque accumulation.
Javanese Turmeric (*Curcuma xanthorrhiza*)	Hwang *et al.,* 2000; Hwang *et al.,* 2000a	Xanthorrrhizol	Growth inhibition of *S. mutans* with MIC of 2 µg/mL

Table 1. Continued

Natural Product Source	Studies [authors, year]	Bioactive phytochemicals	Cariostatic Effects
Liquorice (*Glycyrrhiza uralensis*)	Ahn *et al.,* 2012; Ahn *et al.,* 2015; Sela *et al.,* 1987	Glycyrrhizin, licorisoflavan A, methoxyficifolinol and diprenylgenistein	Dose-dependent inhibition of bacterial adherence, Gtf activity and biofilm development.
Mulberry (*Morus alba*)	Islam *et al.,* 2008	Deoxynojirimycin	Anti-adhesion effects against *S. mutans.*
Neem (*Azadirachta indica)*	Pai *et al.,* 2004; Wolinsky *et al.,* 1996	Gallotannins	Inhibited glucan synthesis and reduced dental plaque formation/MS counts.
Nutmeg (*Myristica fragrans*)	Chung *et al.,* 2006; Yanti *et al.,* 2008	Macelignan	MIC against *S. mutans* and *Lactobacilli* of 3.9 µg/mL and 2 µg/mL respectively.
Peppermint (*Mentha piperita*)	Rasooli *et al.,* 2008	Menthol and menthone	Anti-MS and anti-biofilm effects
Propolis from *Apis mellifera*	Koo *et al.,* 2005; Koo *et al.,* 2006	Apigenin and *tt*-farnesol	Apigenin displays potent anti-Gtf activity. *tt*-farnesol disrupts *S. mutans* membrane permeability.
Rosemary (*Rosmarinus officinalis*)	Bernardes *et al.,* 2010; Tsai *et al.,* 2007	Oxygenated monoterpenes	Antibacterial effects with MICs against *S. sobrinus* of 16 mg/mL. Anti-Gtf activity.
Sanguinaria canadensis (Bloodroot)	Dzink and Socransky, 1985]	Sanguinarine	Antimicrobial activity against various oral pathogens including *S. mutans*
Stevia rebaudiana (Sweetleaf)	Brambilla *et al.,* 2014; Das *et al.,* 1992; Gamboa and Chaves, 2012]	Stevioside and rebaudioside A	Non-fermentable natural sweetener. Impairs plaque biofilm metabolism.
Sweet orange (*Citrus sinensis*) **Lemon** (*Citrus limon*)	Hiraishi *et al.,* 2011; Islam *et al.,* 2012]	Hesperidin	Beneficially shifts the de-/remineralisation caries equilibrium.
Tea (*Camellia sinensis*) Green tea Oolong tea Black tea	Hirasawa *et al.,* 2006; Nakahara *et al.,* 1993; Ooshima *et al.,* 1994; Ooshima *et al.,* 1998; Otake *et al.,* 1991; Touyz and Amsel, 2001	Catechins, theaflavins and tannins	Inhibits bacterial growth, aggregation, acidogenicity and Gtf activity

PAC: proanthocyanidin; MIC: minimum inhibitory concentration; MS: mutans streptococci; Gtf: glucosyltransferase

Several naturally derived bioactive compounds have demonstrated strong growth inhibitory effects against MS. It has been reported that the minimum inhibitory concentrations of xanthorrrhizol from Javanese turmeric, [Hwang *et al.,* 2000] or macelignan from nutmeg [Chung *et al.,* 2006] against planktonic *Streptococcus mutans* are almost comparable to that for chlorhexidine. However, it is not known whether the bacterial growth inhibitory effects of these antimicrobial phytochemicals would be selective for cariogenic bacterial species without affecting growth of health-associated bacterial species. Natural antimicrobials with broad-spectrum of action may still have a role to play in oral conditions where broad antimicrobial action is required and could be incorporated into oral care products instead of synthetic chemical microbiocides.

More relevant for caries prevention are polyphenols that can inhibit lesion development by targeting the unique virulence properties of cariogenic bacteria without affecting the viability of the oral commensal microbial communities. Such an ecological approach is more likely to succeed compared to bactericidal methods as it inhibits disease pathogenesis without stimulating the evolution of resistant bacteria or disrupting the resident microbiome [Cegelski *et al.,* 2008]. Tea (*Camellia sinensis*), one of the most widely consumed beverages, is a rich source of polymeric polyphenols with well-documented strong evidence of anti-Gtf activity [Hirasawa *et al.,* 2006; Nakahara *et al.,* 1993; Ooshima *et al.,* 1998; Otake *et al.,* 1991]., This could be the reason why Japanese tea drinkers have been observed to have reduced incidence of dental caries compared to other population groups [Taylor *et al.,* 2005]. The slow release of catechins, theaflavins and tannins from tea leaves is likely to contribute to the caries preventive effects of the non-fluoride component of tea that has been demonstrated in several human clinical trials and animal studies [Elvin-Lewis and Steelman, 1986; Onisi *et al.,* 1981; Ooshima *et al.,* 1994]. Besides tea phenolics, flavonoids found in cranberry, beehive propolis, parsley, and red wine grapes (proanthocyanidins, apigenin, and myricetin), gallotannins present in Neem (*Azadirachta indica)*, and epicatechins derived from cacao bean husk also possess potent anti-Gtf activity [Jeon *et al.,* 2009; Koo *et al.,* 2010; Koo *et al.,* 2006; Matsumoto *et al.,* 2004; Osawa *et al.,* 2001; Wolinsky *et al.,* 1996]. Inhibition of bacterial Gtf enzymes will reduce the synthesis of insoluble glucans, which are key virulence factors for cariogenic biofilms as they provide specific binding sites for bacterial adhesion and co-aggregation, support biofilm bulk and stability, and serve as a reserve carbohydrate source for enhanced acid production [Bowen and Koo, 2011].

Acidogenicity, aciduricity, and adhesion are other key cariogenic virulence factors that have been found to be affected by specific phytochemicals. Cranberry phenolics (especially the A-type proanthocyanidins) have been shown to reduce the acid production induced glycolytic pH drop in biofilms, besides disrupting the F_1F_0-ATPase proton-translocating pumps responsible for maintaining the aciduric properties of *S. mutans* [Duarte *et al.,* 2006b; Gregoire *et al.,* 2007]. Natural products have also demonstrated the ability to inhibit sucrose-dependent and sucrose-independent bacterial adhesion to tooth surfaces [Badet and Quero, 2011; Islam *et al.,* 2008; Steinberg *et al.,* 2004; Tarsi *et al.,* 1997]. The anti-adhesion effects of natural products are believed to be mediated through the reduction of cell surface hydrophobicity, which is an important factor in the initial attachment of bacteria to the pellicle-coated tooth surface [Yamanaka *et al.,* 2004]. Anti-adhesion natural agents can thereby reduce the total mass of biofilm without significantly affecting bacterial viability. A good example of natural anti-adhesion agents are the low molecular-weight chitosans (obtained from marine crustacean shells), that have been found to reduce initial bacterial adhesion to salivary pellicle coated surfaces [Busscher *et al.,* 2008; Tarsi *et al.,* 1997]. The excellent biocompatibility of chitosans has already seen their inclusion in some dentifrices, mouth rinses, and chewing gums [Busscher *et al.,* 2008; Hayashi *et al.,* 2007].

Besides antimicrobial cariostatic effects, some natural extracts also appear to be adept at shifting the demineralization-remineralization caries balance. *Galla chinensis*, formed when Chinese sumac aphids parasitise *Rhus chinensis* leaves, has been found to be effective in inhibiting demineralization and enhancing remineralization, increasing the efficacy of fluoride, besides inhibiting biofilm development [Cheng *et al.,* 2008, 2010; Cheng *et al.,* 2011; Cheng *et al.,* 2015; Xie *et al.,* 2008]. Hesperidin, a citrus flavonoid, is another phytochemical that suppresses acid-dependent demineralization and enhances remineralization even under fluoride-free conditions [Hiraishi *et al.,* 2011; Islam *et al.,* 2012]. While fluoride will continue to remain the cornerstone of dental caries prevention, natural products with remineralizing ability could potentially be used to design oral care products with lower fluoride concentrations, intended for use in young children to minimize their risk of developing dental fluorosis.

Natural products can also contribute to caries prevention by offering a source of non-cariogenic natural sweeteners. With sucrose recognised as the “arch criminal” in the carious process [Newbrun, 1969], reducing sugar intake and replacing it with non-fermentable sweeteners is an useful approach to caries prevention. Although some commercially available non-nutritive artificial

sweeteners are considered safe for teeth (e.g. aspartame or acesulfame potassium), these synthetic compounds have been associated with other health issues. Recent research is oriented towards the discovery of non-cariogenic natural sweeteners, with over 100 plant-derived sweet compounds being reported [Kim and Kinghorn, 2002]. Among them, the extracts of *Stevia rebaudiana* (sweetleaf) have been approved for use in sweetening foods and beverages in several countries, where they are used as sugar substitutes for caries-active and diabetic patients [Ferrazzano *et al.,* 2015]. There is now robust evidence showing that bioactive *Stevia* phytochemicals (stevioside and rebaudioside A), besides being non-fermentable highly intense sweeteners (200-400 times sweeter than sucrose), also have the ability to interfere with biofilm metabolism [Brambilla *et al.,* 2014; Das *et al.,* 1992; Gamboa and Chaves, 2012; Giacaman *et al.,* 2013].

NATURAL PRODUCTS: CHALLENGES AND RESEARCH DIRECTIONS

From the literature, there appears to be considerable promise for the use of therapeutically relevant natural products for dental caries control and prevention. However, a number of challenges have to be overcome for the potential of natural products to be translated into clinically relevant anti-caries agents. To start with, it will be important to ascertain how the cariostatic effects of natural products can be applied clinically through daily-use products such as dentifrices or mouthwashes. A key challenge is the formulation of lipophilic natural products, to address issues such as phase separation and stability. Many phytochemicals are water insoluble and highly volatile, making them difficult to formulate into aqueous vehicles, while others are not chemically stable, degrading in the presence of heat, humidity, light, or oxygen, negatively impacting their clinical utility [Turek and Stintzing, 2013]. Likewise, some natural products have bitter or alkaloid tastes or aftertastes, which means they must be balanced with intense sweeteners. There is little point using a phytochemical whose effective concentration is either unpalatable, astringent or irritant, since compliance will be poor [Walsh, 2013].

Another key challenge is to improve the retention time of natural agents that are applied into the oral cavity. Dental plaque biofilms have to be exposed to effective concentrations of the chemoprophylactic agent for a sufficiently long time for them to be able to alter the microbial ecology and/or the demineralization-remineralization balance. Micellar drug delivery systems and various polymeric delivery systems have been shown to increase retention times [Chen *et al.,* 2010; Gaffar *et al.,* 1997], and could possibly be used for oral care products containing natural products. Nanotechnology can also be a useful tool for this purpose with nano-sized calcium phosphate

particles incorporating tea polyphenols having shown potential as a caries preventive agent [He *et al.,* 2015]. Cariostatic natural products could also be delivered using varnishes, wafers, gums, or even confectionery.

When considering using natural products for caries control, one must also take into account the effect geographic location and seasonal influences have on phytochemical composition [Jeon *et al.,* 2011]. The variability often associated with natural products makes it essential that natural agents used for caries prevention have well-documented origins and composition, and that the active compounds are known and standardized, as much as that is possible [Freires *et al.,* 2015a]. It is necessary to have reproducible, consistent, and optimised botanical sources from which to isolate cariostatic bioactive phytochemicals, if they cannot be manufactured more conveniently by chemical synthesis.

An additional issue that is often ignored is the safety profile of oral care natural products. While many food-derived phytochemicals have been classified as GRAS (Generally Recognised as Safe) compounds and are not expected to have acute toxicological problems [Bakkali *et al.,* 2008; Freires *et al.,* 2015b], this does not mean that all natural products are completely safe for human use. For example, the use of sanguinarine in dentifrices and mouthwashes was discontinued after controversy over its association with oral leucoplakia [Vlachojannis *et al.,* 2012]. Among the natural products suggested for use in dentistry, there is very limited information on issues such as their product quality, safety, and possible interactions with other medicines [Groppo *et al.,* 2008]. Comprehensive animal and human safety studies, evaluating acute, sub-chronic, and chronic toxicity, especially with regards to brief daily exposures in topical applications, are needed before the widespread use of natural products for oral care can be recommended.

The ultimate success of therapeutic products containing cariostatic natural products will depend on them being able to clearly show an appreciable reduction in individual caries increment. Well-designed RCTs are needed to establish the effectiveness of natural products in preventing dental caries. As seen in Table 1, most of the cariostatic effects of natural products have been shown in *in vitro* studies, rather than in clinical trials. Recent reviews indicate that just over a tenth of the studies of natural products are in Phase IV clinical trials, while even fewer have led to commercially successful products [Freires *et al.,* 2015a; Freires and Rosalen*,* 2016]. Further research is also required to know the precise cariostatic mechanisms of action of natural products and to establish their most effective dosing regimen.

In conclusion, phytochemicals hold considerable potential for use as caries preventive agents, once the technical issues outlined above are resolved. With traditional antimicrobial measures increasingly falling out of favour, virulence-targeted therapies are gaining increased interest. From an ecological perspective, cariostatic natural products that inhibit bacterial adhesion, glucan synthesis, acidogenicity, and aciduricity could potentially be more beneficial for caries prevention than agents with broad growth inhibitory effects. Future research on cariostatic natural products needs to focus on cross-disciplinary approaches, using well-characterized agents and testing them in relevant laboratory and clinical models, both alone and in combination with fluoride and other agents, to ensure that any synergistic or antagonistic interactions are recognised.

2.2.2 REVIEW PAPER 4

Cranberry polyphenols: Natural Weapons against Dental Caries

This paper presents an overview of the oral health benefits of cranberry polyphenols with particular focus on dental caries prevention. The paper details the cariogenic virulence properties that can be potentially targeted by specific cranberry flavonoids.

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Dent J 2019; 7(1): 20-24.

This chapter is comprised of the publication as it stands in print.

ABSTRACT

Bioactive polyphenol components of cranberry (*Vaccinium macrocarpon*) are known to have virulence attenuating effects against several cariogenic virulence properties responsible for dental caries pathogenesis. In particular, the unique cranberry A-type proanthocyanidins and flavonols have demonstrated potent inhibitory effects against cariogenic virulence targets such as bacterial acidogenicity, aciduricity, glucan synthesis, and hydrophobicity. Cranberry phenols have the ability to disrupt these cariogenic virulence properties without being bactericidal, a key quality essential to retain the benefits of the symbiotic resident oral microbiome and prevent emergence of resistant microbes. This review discusses the cariostatic mechanisms of specific cranberry phytochemicals and their potential use as therapeutic agents against cariogenic bacteria in the prevention and control of dental caries.

There has been an increasing global interest in the potential therapeutic uses of plant-derived natural products to prevent oral diseases. Phytodentistry is based on long-standing evidence showing population groups that habitually consume polyphenol-rich diets have significantly better oral and dental health [Yoo *et al.,* 2011]. Traditional chemotherapeutic approaches to oral diseases using synthetic oral biocides like chlorhexidine are becoming less popular because of the growing concerns of allergic reactions and the emergence of microbial resistance [Kampf, 2016; Moka *et al.,* 2015]. Furthermore, the realization of the key health benefits of having an oral microbiome that is in a symbiotic relationship with the host has resulted in virulence-targeted therapies being preferred over broad-spectrum antimicrobials. This is especially vital for biofilm-mediated diseases like dental caries, where reversing the microbiome dysbiosis responsible for caries development is crucial for long-term disease control rather than simply eliminating the dental plaque biofilm [Philip *et al.,* 2018].

The Global Burden of Diseases report highlighted that dental caries is the most common human disease condition worldwide [Kassebaum *et al.,* 2015]. Newer and more effective preventive measures that go beyond using fluoride dentifrices and conventional mechanical plaque removal are clearly needed to tackle this ubiquitous human disease. In this regard, a number of cariostatic natural products have shown the potential to be useful for dental caries prevention [Jeon *et al.,* 2011]. Phytochemicals present in the cranberry fruit are particularly promising, having been shown to exert biological effects against critical virulence properties of cariogenic bacteria, besides having other well-established benefits to human health.

ORIGIN AND HEALTH BENEFITS OF CRANBERRY

Cranberry is a small red fruit that is cultivated almost exclusively in the cooler climes of the American Northeast, Canada, and Chile. Four species have been described: *Vaccinium macrocarpon* (large cranberry, American cranberry), *Vaccinium microcarpom* (small cranberry), *Vaccinium oxycoccus* (common cranberry or northern cranberry), and *Vaccinium erythrocarpum* (southern mountain cranberry) [Feghali *et al.,* 2012]. The American cranberry has received the most attention for its beneficial effects on human health, dating back to the 17th century. Native Americans used cranberries as a meat preservative and a medicine to relieve diverse ailments from scurvy to stomach and liver problems. Today, cranberry is commonly recognized as having a preventive effect on urinary tract infections and gastric ulcers because its high-molecular weight phytochemicals inhibit the adhesion of uropathogenic P-fimbriated *Escherichia coli* to the mucosa of the urinary

tract and *Helicobacter pylori* to the gastric mucosa [Burger *et al.,* 2000; Howell *et al.,* 2005]. These anti-adhesive properties of cranberry phenols are unique and are not found with the polyphenols present in other fruits like grapes, apples and oranges [Johnson-White *et al.,* 2006]. Other purported health benefits of cranberry include lowering cardiovascular and neurological disease risk, modulation of inflammatory responses, and inhibiting cancer cell proliferation [Pappas and Schaich, 2009; Shabrova *et al.,* 2011].

With regards to oral health, *in vitro* studies have shown cranberry constituents can exert beneficial effects on gingival and periodontal health by inhibiting the host inflammatory response, suppressing bacterial biofilm formation, and reducing the activity of periopathogenic proteolytic enzymes [Bodet *et al.,* 2008; Feghali *et al.,* 2012; Labrecque *et al.,* 2006]. Specific flavonoids present in cranberry were also shown to disrupt key virulence factors responsible for dental caries pathogenesis [Duarte *et al.,* 2006; Feng *et al.,* 2013; Gregoire *et al.,* 2007]. This narrative review will explore the potential therapeutic targets and the specific cranberry phenols reported to be involved in disrupting virulence traits of cariogenic bacteria.

POTENTIAL CARIOGENIC VIRULENCE TARGETS

Dental caries is a polymicrobial multifactorial disease that develops when the acidogenic and aciduric members of the resident oral flora obtain a selective ecological advantage over other commensal microorganisms, disrupting the homeostatic balance of the plaque biofilm, and initiating the disease process [Marsh, 2003]. While the disease is polymicrobial in nature, mutans streptococci (MS) function as keystone pathogens, as they are principally responsible for the initial assembly of exopolysaccharide (EPS) glucan-rich scaffold matrix. The ability of MS (predominantly *Streptococcus mutans* and *Streptococcus sobrinus*) to synthesize copious amounts of insoluble glucans is critical for caries pathogenesis [Hajishengallis *et al.,* 2017]. The first part of this review discusses the cariogenic virulence traits that cranberry flavonoids can potentially target in order to reverse the dental plaque dysbiosis responsible for caries development.

Glucan synthesis

The *S. mutans* glucosyltransferases (Gtfs) are exoenzymes that are primarily responsible for orchestrating the virulent plaque biofilm matrix. In the presence of sucrose, different Gtfs (i.e. B, C and D) synthesize extracellular carbohydrate polymers called glucans. These glucans confer structural integrity and bulk to plaque biofilms, enhance bacterial adhesion and accumulation, and

provide a readily metabolizable carbohydrate source for sustained acid production [Paes Leme *et al.,* 2006]. The glucan-rich EPS matrix also creates a diffusion limiting barrier that not only protects plaque microflora from topical antimicrobial agents, but also helps maintain acidic microenvironments critical to enamel dissolution [Bowen and Koo, 2011]. As the different Gtfs acting in concert are a primary virulence property of *S. mutans*, targeting Gtf enzyme activity should significantly disrupt the development of dental caries [Koo and Bowen, 2014].

Acidogenicity

An important attribute of cariogenic microorganisms is their proficiency to produce large amounts of organic acids as a by-product of carbohydrate metabolism. These acids (particularly lactic acid) lower the pH of the plaque fluid, causing demineralization of the tooth structure and lesion initiation. Sustained low pH within the plaque biofilm will favour the survival and growth of acidogenic and aciduric bacteria, which start to dominate the plaque microbial community, displacing many of the health-associated commensal oral microflora [Philip *et al.,* 2018]. Disrupting bacterial metabolism and acid production is thus key to not only preventing tooth demineralization, but also to restoring the ecological balance of dental plaque.

Aciduricity

The ability to survive and thrive in low pH environments is a key characteristic of MS. Proton translocating F_1F_0-ATPases play a major role in protecting the bacterial glycolytic and sugar transport system enzymes from the environmental stress caused by biofilm acidification. Inhibiting the F_1F_0-ATPase proton pump can result in cytoplasmic acidification and thus affect the optimal functioning of the intracellular acid-sensitive glycolytic enzymes.

Bacterial Adhesion

The *S. mutans*-derived Gtfs (particularly GtfC) are adsorbed onto salivary pellicle-coated tooth surfaces and rapidly generate an amorphous glucan layer that facilitates initial bacterial adhesion. Gtf exoenzymes (particularly GtfB) also have great affinity for cell surfaces of other microorganisms, converting them into de facto glucan producers in the presence of sucrose. The glucans formed *in situ* provide enhanced binding sites for MS, resulting in further bacterial aggregation, and favouring cohesive microcolonies [Xiao and Koo, 2010]. While the Gtf-derived glucans are mainly responsible for MS adhesion and accumulation, cell hydrophobicity can also play a role in the initial bacterial adhesion to the salivary pellicle. The hydrophobicity of *S. mutans* is believed to be mainly associated with its cell surface proteins [McBride *et al.,* 1984]. Targeting Gtf

exoenzymes and bacterial surface hydrophobicity could therefore impede microbial adhesion and lower the virulence of dental plaque.

CARIOSTATIC EFFECTS OF CRANBERRY POLYPHENOLS

The cranberry fruit is a unique and rich source of bioactive polyphenol compounds. Among the different classes of polyphenols, the flavonoid family has the largest variety of compounds with pharmacological activity. The three major flavonoids found in whole cranberries are the flavonols, anthocyanins, and the flavanols that include monomeric flavan-3-ols and the polymeric flavan-3-ols called proanthocyanidins [Cunningham *et al.,* 2004]. Very minute quantities of other polyphenols may also be found in cranberries including flavanones (e.g. prunin), non-flavonoids such as phenolics acids (e.g. benzoic acid), and anti-oxidant stilbenes (e.g. resveratrol) [Pappas and Schaich, 2009]. Table 2 summarizes the bioactive phytochemicals in cranberry fruit and their reported cariostatic effects. The role specific cranberry flavonoids play in disrupting cariogenic virulence is discussed further below.

Table 2. Cranberry polyphenols and their cariostatic effects

Polyphenol class	Cariostatic effects
Flavonoids	
1. Flavonols • e.g. quercetin, myricetin, kaempferol	Inhibits Gtf, F_1F_0-ATPase enzyme activity and acid production
2. Flavanols	
(a) Monomeric flavan-3-ols • e.g. catechin, epicatechin, epigallocatechin, epigallocatechin gallate	No reported effects
(b) Polymeric flavan-3-ols • e.g. A-linked proanthocyanidins	Inhibits Gtf, F_1F_0-ATPase enzyme activity and acid production
3. Anthocyanins • e.g. cyanidin, peonidin, malvidin, delphinidin	Insignificant effects against cariogenic virulence factors
4. Flavanones • e.g. prunin	No reported effects
Nondialyzable Material	Interferes with bacterial hydrophobicity and initial stages of plaque development
Phenolic acids • e.g. benzoic acid, ellagic acid	No reported effects
Stilbenes • e.g. resveratrol	No reported effects

Flavonols

Cranberry flavonols occur mainly as glycosylated forms of quercetin, myricetin, and kaempferol [Vvedenskaya *et al.,* 2004]. The total flavonols in cranberry has been quantified to be approximately 300-500 mg/kg of the fruit [Wang *et al.,* 2017]. The content and nature of cranberry flavonols are unique in character and are not usually seen in flavonols of other fruits. Cranberry flavonols are believed to be responsible for many of the systemic health benefits associated with cranberry consumption [Pappas and Schaich, 2009].

Cranberry flavonols, especially the most abundant quercetin glycosides, were found to be significant inhibitors of GtfB, GtfC, F_1F_0-ATPase, and of cytoplasmic glycolytic enzymes within *S. mutans* [Duarte *et al.,* 2006; Gregoire *et al.,* 2007]. Flavonol-mediated Gtf inhibition appears to be 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 [Duarte *et al.,* 2006]. Quercetin is also known to be a non-competitive inhibitor of the proton-translocating F_1F_0-ATPase activity that is critical to the survival of acidogenic bacteria in the low pH biofilm environment they create [Zheng and Ramirez, 2000].

Proanthocyanidins (PACs)

PACs (also referred to as condensed tannins) are oligomers or polymers of flavan-3-ols that are distinctive in terms of their size, high molecular weight, and free hydroxyl groups. These structural properties determine their bioactivity, including the ability to precipitate or denature polypeptides and proline-rich proteins [Bennick, 2002]. Binding of PACs to salivary proteins is responsible for the astringent taste of cranberries and other tannin-rich foods. PAC subunits (catechin, epicatechin, epigalloatechin, epigalloatechin gallate) are linked most often via a single B-type bond, and less often by a double interflavan A-type linkage (Fig. 4). The A-linked PACs (A-PACs) are found in high concentrations in cranberry and can also be found in peanut skins and cinnamon [Foo *et al.,* 2000; Singh *et al.,* 2009]. The cranberry A-PACs are mainly composed of epicatechin monomer subunits with at least one A-type interflavan bond and the type of PAC linkage is closely related to its biological effects. The A-PACs appear to be more biologically active than B-type PACs due to the conformational rigidity afforded by the double interflavan linkages [Foo *et al.,* 2000; Howell *et al.,* 2005]. For instance, A-PACs demonstrated significant anti-adhesion effects against *E. coli* and *H. pylori*, whereas B-type PACs were devoid of anti-adhesion properties.

Figure 4. Structure of cranberry proanthocyanidins with A-linkage

Among the different cranberry flavonoids, the A-PACs have exhibited the most potent effects against bacterial glycolytic, Gtf and F_1F_0-ATPase enzymes [Duarte *et al.,* 2006; Koo *et al.,* 2010]. The ability of PACs to form protein-polyphenol complexes possibly affects the enzyme activity by irreversibly binding to the catalytic and glucan-binding domains once complexed [Koo *et al.,* 2010]. The precise mechanisms by which cranberry A-PACs inhibit *S. mutans* enzyme activity are likely to be related to their unique rigidity conferring A-type linkages, as PACs from non-cranberry fruits that lacked A-type linkages did not affect these enzymes [Feng *et al.,* 2013; Gregoire *et al.,* 2007; Koo *et al.,* 2010].

The PACs in cranberry are predominantly found in oligomeric forms (up to 13 monomeric units), although smaller PAC dimers are also seen. The bioactivity of the A-PACs are also strongly associated with their degree-of-polymerization (DP). A-PACs with DP 4 and DP 8 to 13 are optimal for interaction and inhibition of Gtfs and glucan-mediated adhesion [Feng *et al.,* 2013]. On the other hand, inhibitory activity against bacterial acid production is mostly related to a low molecular weight A-PAC dimer called procyanidin A_2 [Gregoire *et al.,* 2007]. 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 [Yoo *et al.,* 2011].

Cranberry A-PACs have been shown to significantly reduce incidence and severity of smooth surface caries in an animal caries model, suggesting they may have value as a supplement to fluoride for dental caries prevention [Koo *et al.,* 2010]. Overall, the evidence does indicate that A-PACs have the most effective cariostatic properties amongst the different cranberry flavonoids, with the potential to be developed as natural agents for dental caries prevention.

Anthocyanins (ACYs)

ACYs such as cyanidin and peonidin are present at concentrations of approximately 100-900 mg/kg of the fruit and are responsible for the distinctive red colour of the fruit [Vorsa *et al.,* 2003]. While ACYs may a have a number of systemic health benefits, their ability to inhibit bacterial cariogenic virulence properties are relatively low. A study comparing the effects of different cranberry fractions on virulence traits of *S. mutans* found that A-PACs exhibited the greatest biological activity, followed by the flavonols, while the ACY extract did not have any significant effects [Duarte *et al.,* 2006].

Nondialyzable Material (NDM)

A number of studies have reported beneficial effects from a high molecular weight fraction of cranberry that remains after dialysis of concentrated cranberry juice using membranes with a molecular weight cut off of 12000-15000 [Pappas and Schaich, 2009]. Chemical analysis of the NDM fraction suggests it is a heterogeneous mixture of phenols containing about 65% PACs and trace amounts of ACYs [Bodet *et al.,* 2006].

A mouthwash supplemented with NDM has been found to significantly reduce salivary MS counts following 6 weeks of usage [Weiss *et al.,* 2004]. *In vitro* experiments have revealed that NDM inhibits adhesion of *S. mutans* and *S. sobrinus,* and promotes desorption of the bacteria from biofilms [Steinberg *et al.,* 2005; Weiss *et al.,* 2004; Yamanaka *et al.,* 2004]. This is likely because NDM components may interact with bacterial cell surface hydrophobic proteins, reducing hydrophobicity and thus initial bacterial adhesion to tooth surfaces [Yamanaka *et al.,* 2004]. Cranberry juice itself may not be suitable for dental caries prevention as it has a high content of enamel eroding organic acids (e.g. quinic, citric and malic acids) compared to juices from apple, grape or blueberry. However, the potential of using the non-fermentable NDM fraction from cranberry juice concentrate in oral care products needs to be explored [Bodet *et al.,* 2008].

Cranberry vs. other natural products

Cranberry polyphenols may have a number of advantages over other suggested cariostatic natural products. A comparison of three dark-coloured berries (cranberry, wild blueberry, and strawberry) showed that cranberry extracts had the greatest effects against *S. mutans* biofilm virulence properties [Philip *et al.,* 2019a]. While tea (*Camellia sinensis*) also contains high levels of biologically active phenolic compounds, studies have shown cranberry polyphenols have the ability to effect a more powerful disruption of cariogenic virulence factors [Yoo *et al.,* 2011]. The greater cariostatic effects of cranberry could partly be attributed to the presence of the unique cranberry A-PACs that are not present in other polyphenol-rich plants.

Isolated phytochemicals like xanthorrrhizol from Javanese turmeric (*Curcuma xanthorrhizha*) and macelignan from nutmeg (*Myristica fragrans*) have also shown significant growth inhibitory effects against oral bacteria [Chung *et al.,* 2006; Hwang *et al.,* 2000; Philip *et al.,* 2019c]. However, they lack the ability to discriminate between health- and disease-associated plaque microorganisms, which is key for long-term control over biofilm-mediated oral diseases like dental caries [Philip *et al.,* 2019c]. Cranberry polyphenols, while lacking bactericidal effects, have the potential to modulate cariogenic virulence allowing them to possibly reverse the microbiome dysbiosis responsible for dental caries, and still retain the key benefits of the resident oral microbiome.

A common problem with using natural products as therapeutic agents is their compositional variability. 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].

CONCLUSIONS

Cranberry polyphenols have enormous potential for development as adjunctive anti-caries agents. However, the cariostatic effects of cranberry have mostly been demonstrated in laboratory studies. There is a need for well-designed clinical trials to evaluate whether the proposed cariostatic effects of cranberry phenols can actually translate into preventing dental caries in high-risk individuals and population groups. The combination of virulence-attenuating standardized cranberry extracts and remineralizing agents in a single oral care product could hold great promise in the global fight against dental caries.

2.3 LITERATURE REVIEW: NON-FLUORIDE REMINERALIZING AGENTS

2.3.1 REVIEW PAPER 5

State of the Art Enamel Remineralization Systems: The Next Frontier in Caries Management

This paper discusses the rationale for using non-fluoride remineralization systems like CPP-ACP in caries management and the mechanisms and evidence behind the latest advances in enamel remineralization technologies.

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This chapter is comprised of the publication as it stands in print.

ABSTRACT

The principles of minimally-invasive dentistry clearly recommend the need for clinically effective measures to remineralize enamel caries lesions. While fluoride-mediated remineralization is the cornerstone of current caries management philosophies, a number of new remineralization strategies have been commercialized or are under development that claim to promote deeper remineralization of lesions, reduce the potential risks associated with high-fluoride oral care products, and facilitate caries control over a lifetime. These non-fluoride remineralizing systems can be broadly categorized into biomimetic enamel regenerative technologies and the approaches that repair caries lesions by enhancing fluoride efficacy. This paper discusses the rationale for non-fluoride remineralization and the mechanism of action, challenges, and evidence behind some of the most promising advances in enamel remineralization therapies.

Dental caries pathophysiology is not simply a continual cumulative loss of tooth minerals, but rather a dynamic process characterized by alternating periods of demineralization and remineralization. Lesion progression or reversal depends on the equilibrium between demineralization-favouring pathological factors (cariogenic bacteria, fermentable carbohydrates, salivary dysfunction) and the protective factors (antibacterial agents, sufficient saliva, remineralizing ions) that tip the balance towards remineralization [Featherstone and Chaffee, 2018]. Remineralization can occur as a natural repair process where plaque/salivary calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions are deposited into crystal voids of the demineralized tooth structure, resulting in net mineral gain. The presence of free fluoride (F^-) ions in the oral environment can drive the incorporation of Ca^{2+} and PO_4^{3-} ions into the crystal lattice, with the ensuing fluorapaptite mineral significantly more resistant to a subsequent acid challenge [ten Cate, 1999].

A better understanding of regenerative and physiochemical mechanisms has influenced the development of a number of innovative remineralization technologies that go beyond fluoride-mediated remineralization. While traditional fluoride-based remineralization remains the cornerstone for caries management with the highest level of supporting evidence, additional remineralizing agents to enhance fluoride effects are often needed in high caries-risk individuals and population groups [Amaechi and van Loveren, 2013; Fontana, 2016]. The first International Conference on Novel Anticaries and Remineralizing Agents (ICNARA) had suggested that the broad aim of new remineralization therapies should be to "facilitate caries control over a lifetime using evidence-based, clinically effective, multifactorial prevention to keep the caries process in balance" [Pitts and Wefel, 2009]. This paper discusses the rationale for using non-fluoride remineralization systems and the mechanisms, challenges, and evidence underpinning some of the technological advances in enamel remineralization therapies.

NON-FLUORIDE REMINERALIZATION SYSTEMS: ARE THEY NEEDED?

Natural remineralization alone is not sufficient

The remineralization potential of saliva is well-documented [Stookey, 2008], having evolved to deliver Ca^{2+} and PO_4^{3-} ions in a bioavailable form for hard tissue development and maintenance throughout life [Cochrane and Reynolds, 2012]. At physiological pH, saliva is supersaturated with phosphoprotein-stabilized Ca^{2+} and PO_4^{3-} ions, ensuring that the ions remain bioavailable to diffuse into mineral deficient lesions [Cochrane *et al.,* 2010]. However, longitudinal studies that followed the natural progress of white spot lesions (WSL) found that although some WSL get smaller, the

majority are largely unaffected even after two years [Mattousch *et al.,* 2007; van der Veen *et al.,* 2007]. Moreover, net salivary remineralization is a slow process [Dowd, 1999], with a tendency for mineral gain only on the surface of the WSL due to the low ion concentration gradient from saliva into the lesion [Silverstone, 1972]. Fluoride-mediated salivary remineralization is also seen to be restricted to the outer 30 µm of the tooth [Schmidlin *et al.,* 2016]. This surface-only remineralization neither improves the aesthetics or the structural properties of the subsurface lesion [Cochrane *et al.,* 2010]. The presence of additional extrinsic sources of stabilized Ca^{2+} and PO_4^{3-} ions could augment the natural remineralization potential of saliva by increasing diffusion gradients favouring faster and deeper subsurface remineralization.

Fluoride – Improving its efficacy and safety

The pivotal discovery of fluoride as an agent that could prevent dental caries was one of the most important landmarks in dentistry [ten Cate, 2015]. The dramatic decline in caries prevalence rates of developed countries from the latter half of the 20th century has been largely attributed to the widespread use of oral care products containing fluoride [Fejerskov, 2004]. Fluoride remains the gold standard for arresting caries lesions with multiple systematic reviews confirming the role of fluoride products in preventing dental caries [Benson *et al.,* 2013; Marinho *et al.,* 2016; Marinho *et al.,* 2003; Marinho *et al.,* 2015; Shahid, 2017]. However, emerging epidemiological data are showing a worrying trend, with caries experience plateauing or even increasing in some population groups, despite the regular use of fluoride dentifrices in these countries [Agustsdottir *et al.,* 2010; AIHW, 2018; Dye *et al.,* 2017; Haugejorden and Birkeland, 2005]. This raises questions on whether the earlier reduction in caries prevalence rates has continued into this century [Gimenez *et al.,* 2016]. The reported pause in the decline of dental caries has been attributed to the fact that diets across the world are changing to include more processed and sugar-laden foods, limiting the repair potential of fluoride [Duggal *et al.,* 2001]. While under normal physiological conditions, fluoride and salivary homeostatic mechanisms are often enough to remineralize early lesions, these are often not adequate in highly cariogenic oral environments. Other at-risk population groups (xerostomia patients, elderly individuals at risk of root caries) can also benefit from boosters to improve the remineralizing and preventive efficacy of fluoride [Fontana, 2016].

An obvious approach to increase the remineralizing potential of fluoride would be to just add more fluoride to oral care products. Dentifrices with 5000 ppm fluoride have been found to be more efficacious for remineralization of root caries lesions than 1000-1500 ppm fluoride dentifrices

[Wierichs and Meyer-Lueckel, 2015]. A dose-response relationship of decreasing caries incidence with increasing dentifrice fluoride concentration has also been observed [Walsh *et al.,* 2010]. However, the recent classification of fluoride as a chemical nuerotoxicant could raise safety concerns among the general public regarding the use of high concentration fluoride products [Grandjean and Landrigan, 2014]. More pertinent, are the growing concerns that children today are exposed to fluoride from multiple sources, potentially increasing their risk of developing dental fluorosis [Zohoori and Maguire, 2018]. This "halo" effect of fluoride probably accounts for the increased prevalence of permanent teeth mottling being seen in western countries [McGrady *et al.,* 2012; Pendrys, 2000]. The increased risk of dental fluorosis has led the World Health Organisation (WHO) to recommend the need to assess total fluoride exposure of the population before introducing any additional fluoridation for caries prevention [Baez and Marthaler, 2014]. Besides fluorosis in children, the surface-only remineralization that often occurs in the presence of high topical fluoride concentrations can increase the incidence of occult caries ("fluoride syndrome") across all age groups [Ball, 1986]. Considering the narrow "dose-gap" between caries reduction benefit and fluoride side-effects, regulatory authorities have limited the fluoride concentration in non-prescription toothpastes to within 1000-1500 ppm, while for children below 6 years this dose is even lower and probably suboptimal for effective remineralization of early lesions.

Evidently, there is a need for new-age remineralization technologies with an ability to complement fluoride, close the gap in its remineralizing efficacy, and effect a fuller consolidation of carious lesions [Lynch and Smith, 2012]. Effective non-fluoride remineralization systems can also potentially allow dental products to be designed with lower fluoride concentrations, to allay the safety concerns associated with consumer oral care products containing high fluoride concentrations.

Modern caries management

Enamel caries presents as a progressive subsurface demineralization that if not reversed will result in mechanical failure and cavitation, often leading to a vicious restoration cycle. Despite long-standing recommendations for adopting a biological approach to caries management [Pitts, 2004], a significant proportion of dentists continue with the restorative-only model that has failed both clinically and economically [Innes and Schwendicke, 2017; Pitts and Zero, 2016]. There is a global consensus that principal approach to modern-day caries management should be to "preserve the tooth structure and restore only when necessary" [Ismail *et al.,* 2013]. New remineralization

systems that either regenerate lesion body structure (e.g. biomimetic peptide scaffolds) or provide ions favouring subsurface mineral gain (e.g. calcium phosphate systems), can significantly reduce the need for traditional restorations and preserve tooth structure.

Disease detection is also increasingly shifting from the conventional decayed missing filled teeth criteria of the WHO to the use of the International Caries Detection and Assessment System (ICDAS), where non-cavitated enamel lesions (ICADS 1 and 2) are also included. This has increased the proportion of individuals diagnosed with dental caries, providing a significant opportunity for secondary prevention and non-operative care using regenerative medicine-based dental approaches. Modern-day dentistry clearly needs such minimally-invasive remineralization measures, not just to enhance clinical outcomes, but also to improve patient experience and well-being [Pitts and Wright, 2018].

NON-FLUORIDE ENAMEL REMINERALIZING SYSTEMS

The development of novel enamel remineralization systems has significantly progressed in the recent years with many of them already in clinical use, while others are in various stages of development. These remineralizing technologies are briefly summarized in Table 3, and can be broadly classified into: (i) biomimetic regenerative systems; and (ii) approaches that synergize fluoride efficacy. The mechanisms, advantages and challenges of these remineralizing technologies are discussed further below.

Table 3. Non-fluoride enamel remineralizing technologies

Technology	Commercial Product
A. Biomimetic Systems	
1. Dentin phosphoprotein 8DSS peptides	Not available
2. P11-4 peptides	Curodont Repair/ Curodont Protect
3. Leucine-rich amelogenin peptides	Not available
4. Poly(amido amine) dendrimers	Not available
5. Electrically accelerated and enhanced remineralization	Not available
6. Nanohydroxyapatite	Apagard/ Desensin
B. Fluoride Boosters	
1. Calcium phosphate systems	
Stabilized calcium phosphates	
• Casein phosphopeptide-amorphous calcium phosphate	Tooth Mousse/ MI Paste crèmes Recaldent/ Trident White gum MI Paste One toothpaste
Crystalline calcium phosphates	
• Functionalized β-tricalcium phosphate	ClinPro toothpaste
• Calcium sodium phosphosilicate (NovoMin™ technology)	Oravive toothpaste
Unstabilized calcium phosphates	
• Amorphous calcium phosphate (Enamelon™ technology)	Enamelon toothpaste
2. Polyphosphate systems • Sodium trimetaphosphate • Calcium glycerophosphate • Sodium hexametaphosphate	Oral-B Pro Expert toothpaste
3. Natural Products • *Galla chinensis* • Hesperidin • Gum Arabic	Not available

A. Biomimetic Remineralization

Oral care products containing fluoride are effective in remineralizing enamel but do not have the potential to promote formation of organized apatite crystals [Ruan and Moradian-Oldak, 2015]. Presently, there is an attempt to shift from reparative to regenerative biomineralization therapies, wherein diseased dental tissues are replaced with biologically similar tissues [Alkilzy *et al.,* 2018a]. Enamel regeneration is however particularly challenging as mature enamel is acellular and does not resorb or remodel itself unlike bone or dentin [Moradian-Oldak, 2012]. Advances in tissue engineering methods has yielded biomimetic methods that have demonstrated a strong potential for regenerating the hierarchical enamel microstructure.

Dentin Phosphoprotein-derived 8DSS Peptides

Dentin phosphoprotein (DPP) is the most abundant non-collagenous extracellular matrix component in dentin and is known to play a critical role in tooth mineralization [Hsu *et al.,* 2011]. Human DPP contains numerous repetitive aspartate-serine-serine (DSS) nucleotide sequences that are believed to promote hydroxyapatite (HA) formation, with studies showing that DPP can generate HA crystals in calcium phosphate solutions [George *et al.,* 1996; Prasad *et al.,* 2010]. Several short functional peptides based on DPP have been designed as they offer a number of advantages over full-length DPP such as higher purity and better conformational fit on enamel, while avoiding allergies and immunogenicity often associated with animal proteins [Hsu *et al.,* 2011]. Among the DPP-derived peptides, the octuplet repeats of aspartate-serine-serine (8DSS) are the most active in promoting biomineralization [Yarbrough *et al.,* 2010].

8DSS peptides have essentially two mineral-binding surfaces and can strongly bind not only to free Ca^{2+} and PO_4^3 ions but also to the HA surface [George *et al.,* 1996; Yarbrough *et al.,* 2010]. Applying these peptides to enamel can prevent dissolution of Ca^{2+} and PO_4^3 ions into the surrounding medium while promoting the capture of these ions from solution. 8DSS peptides thus appear to have a dual mechanism in the mediation of biologically directed mineral deposition. First, they limit the dissolution of Ca^{2+} and PO_4^3 ions from demineralized dentin, and second, they promote the capture of these ions to form new mineral deposits on demineralized enamel [Hsu *et al.,* 2011; Yang *et al.,* 2014]. The newly grown mineral had uniform deposition of small apatite crystals with significantly improved properties such as reduced surface roughness, and higher hardness and elastic modulus [Chung *et al.,* 2012; Hsu *et al.,* 2011]. A recent *in vitro* study also provided strong evidence that the biomimetic 8DSS peptide, besides inhibiting enamel demineralization on its own, could significantly potentiate the ability of fluoride to do the same [Yang *et al.,* 2016]. This synergistic interaction can be useful to lower fluoride concentration for caries prevention in young children reducing their risk of dental fluorosis.

To date, the proof of concept of 8DSS peptides have been shown only *in vitro* systems and is likely to present some challenges when used clinically. For example, it is not known whether these peptides can survive enzymatic action in the oral cavity, although being short peptides should make them relatively difficult targets for hydrolytic enzymes. Another drawback is that because 8DSS binds calcium strongly it could lead to calculus formation if not controlled. However, if future *in vivo*

studies can confirm the clinical promise of 8DSS and overcome the challenges, it holds great promise as non-fluoride biomineralizing agent [Yang *et al.,* 2014].

Self-assembling P11-4 Peptides

An ideal enamel regenerative approach would involve substituting the degraded enamel matrix with a biomimetic matrix that favours in-depth remineralization of enamel lesions [Alkilzy *et al.,* 2018]. An exciting development in this field is a monomeric peptide consisting of 11 amino acids called P11-4. This rationally-designed peptide self-assembles into hierarchical 3D fibrillar scaffolds in response to local conditions such as high ionic strength and acidic pH found in the lesion body [Kirkham *et al.,* 2007]. The P11-4 fibrillar matrix has a high affinity for Ca^{2+} ions and acts as a nucleator for de novo HA formation resulting in remineralization of the lesion body [Kind *et al.,* 2017; Kirkham *et al.,* 2007]. Analysis of *in vitro* data showed that the presence of P11-4 fibers in the lesion body resulted in faster HA formation, yielding tangentially arranged needle-shaped crystals, with increased microhardness of the remineralized subsurface lesion [Schmidlin *et al.,* 2016; Sousa *et al.,* 2017; Takahashi *et al.,* 2016].

P11-4 has shown promising results as a biomimetic mineralization agent in *in vivo* and clinical trials. This includes the ability to reverse early occlusal and proximal lesions that are more resistant to fluoride remineralization than smooth surface lesions [Alkilzy *et al.,* 2018; Alkilzy *et al.,* 2015; Brunton *et al.,* 2013; Schlee *et al.,* 2014; Schlee *et al.,* 2018]. The low viscosity isotropic P11-4 when applied on the initial carious lesion rapidly diffuses into the lesion body, where it transforms to an elastomeric nematic gel in the presence of cations and pH<7.4, leading to the 3D fiber matrix assembly and subsequent biomineralization of the lesion [Brunton *et al.,* 2013]. The P11-4 treated carious lesions showed significantly improved visual appearance and increased radiographic opacity, remaining stable even 6-12 months after treatment [Schlee *et al.,* 2014; Schlee *et al.,* 2018]. A recent randomised controlled trial (RCT) demonstrated that biomineralization facilitated by P11-4 in combination with fluoride is safe and more effective than the present clinical gold standard of fluoride treatment alone [Alkilzy *et al.,* 2018a].

As P11-4 relies on natural remineralization driven by saliva, its effectiveness will depend on the individual's quality of saliva especially its mineral content, pH, and flow rate [Schlee *et al.,* 2018]. This could reduce its efficacy in xerostomia patients. Undoubtedly, P11-4 therapy is a significant step towards the elusive goal of guided enamel regeneration, but more long-term controlled studies

are needed to confirm and quantify these findings, as well as to identify additional factors that can potentiate the repair process.

Amelogenin

The amelogenin-rich enamel organic matrix plays a critical role in regulating the growth, shape and arrangement of HA crystals during enamel mineralization. However, mature enamel lacks matrix proteins and cannot regenerate the mineral loss caused due to dental caries or erosion [Ruan and Moradian-Oldak, 2015]. Recently, several promising strategies have been proposed to replicate the complex enamel microstructure using synthetic amelogenin-based systems. Recombinant porcine amelogenin (rP172) was found to stabilize calcium phosphate clusters and promote the growth of hierarchically-arranged enamel crystals on acid-etched lesions, significantly improving its hardness and elastic modulus [Fan *et al.,* 2009; Ruan *et al.,* 2016; Ruan *et al.,* 2013]. This biomimetic regrowth of HA crystals also generated a robust interface between the newly formed layer and native enamel ensuring efficacy and durability of restorations.

An excellent low-cost and safer alternative to the full-length amelogenin is a leucine-rich amelogenin peptide (LRAP) that is comprised of only 56 amino acids. The non-phosphorylated LRAP contains only the N- and C-terminal domains of the parent amelogenin, with these domains known to be responsible for directing mineral growth and binding [Le Norcy *et al.,* 2011]. *In vitro* studies have shown treatment of enamel lesions with LRAP reduced lesion depth and allowed biomimetic reconstruction of enamel by promoting linear growth of mature enamel crystals along the *c*-axis [Bagheri *et al.,* 2015; Mukherjee *et al.,* 2016; Shafiei *et al.,* 2015]. The addition of mineralization inhibitors such as inorganic pyrophosphate or matrix metalloproteinase to synthetic amelogenin assemblies was able to better regulate size, shape, and orientation of a strongly adherent new mineral layer, while preventing undesirable protein occlusion within newly formed crystals [Kwak *et al.,* 2017; Prajapati *et al.,* 2018].

A disadvantage of amelogenin-mediated enamel regeneration is that not only is the protein difficult to extract and store, but the growth of the repaired enamel layer also takes an extended amount of time, making it potentially unsuitable for clinical use. Furthermore, while amelogenin has been seen to promote apatite nucleation *in vitro*, there is as yet no direct evidence that similar biomineralization occurs *in vivo* [Ruan and Moradian-Oldak, 2015].

Poly(amido amine) (PAMAM) Dendrimers

PAMAM dendrimers are highly branched polymers characterized by the presence of internal cavities, a number of reactive end groups, and well-defined size and shape [Chen *et al.,* 2013]. These amelogenin-inspired dendrimers have been referred to as “artificial proteins” as they can mimic the functions of organic matrices in modulating the biomineralization of tooth enamel. Several *in vitro* studies have demonstrated that amphiphilic, carboxyl-terminated, and phosphate-terminated PAMAM dendrimers exhibited strong tendency to self-assemble into hierarchical enamel crystal structures [Chen *et al.,* 2013; Chen *et al.,* 2015; Chen *et al.,* 2014; Wu *et al.,* 2013; Yang *et al.,* 2011]. The new crystals created by the PAMAM organic templates had the same structure, orientation and mineral phase of the intact enamel, with the HA nanorods closely paralleling the original prisms [Chen *et al.,* 2013].

The synthetic PAMAM dendrimers have the potential to act as amelogenin analogues for biomineralization, overcoming the difficulty associated with extracting, purifying, and storing the natural protein. However, they are still far from clinical translation with *in vivo* studies so far limited to only animal experiments. Furthermore, like amelogenin, PAMAM-mediated enamel remineralization is also a time consuming process and unless this can be potentiated their clinical application may not be practical. Recently, there have been suggestions that lasers could be used to speed up the biomineralization process and control the crystal growth precisely where needed [Sun *et al.,* 2017].

Electrically Accelerated and Enhanced Remineralization (EAER)

EAER is a recently developed remineralization technology targeted at initial and moderate enamel lesions with the treatment objectives of preserving all healthy tissue, restoring the full depth of the caries lesion, and improving mechanical properties of the treated enamel [Pitts and Wright, 2018]. It utilizes iontophoresis to accelerate the flow of remineralizing ions into the deepest part of the subsurface caries lesion. This creates an environment that favours remineralization of the lesion that then matures to give the repaired lesion optimal hardness and mineral density. Unlike the biomimetic peptides, EAER does not “regenerate” lost enamel via matrix proteins or the organic capture of Ca^{2+} and PO_4^{3} ions. However, the developers claim that the EAER-treated lesions have a very similar appearance to healthy enamel, with no broken rods or degraded prisms visible under scanning electron microscopic examination [Pitts and Wright, 2018]. An advantage that the EAER technology will have over synthetic biomimetic peptides is that it proposes to utilize tools and

chemicals commonly available in most dental practices. The early *in vitro* results using the EAER technology are promising, although a thorough evaluation of its remineralization potential will depend on results from *in vivo* studies, as well as studies independent of the technology developers.

Nanohydroxyapatite (nHA)

Synthetic nHA is considered one of the most biocompatible and bioactive materials having similar morphology, structure, and crystallinity to the apatite crystal within enamel [Hanning and Hanning, 2010]. The nano-sized particles can strongly bind to enamel surfaces and with fragments of plaque and bacteria. The small size of the particles that compose nHA considerably increase its surface area for binding as well as allowing it to act as a filler to repair small holes and depressions on the enamel surface [Pepla *et al.,* 2014]. *In vitro* dynamic pH-cycling experiments have shown that nHA had the potential to remineralize initial enamel lesions with a comparable or even superior efficacy to that of fluoride [Huang *et al.,* 2011; Huang *et al.,* 2009; Najibfard *et al.,* 2011; Tschoppe *et al.,* 2011]. Another *in vitro* study found that nHA gel had significant potential for enamel remineralization around restoration margins [Juntavee *et al.,* 2018]. The mechanism of nHA biomimetic function is not clear with some researchers suggesting that it promotes remineralization through the creation of a new layer of synthetic enamel around the tooth or by depositing apatite nanoparticles in the enamel defects [Li *et al.,* 2008; Pepla *et al.,* 2014]. However, others have proposed that nHA acts as calcium-phosphate reservoir maintaining a state of supersaturation with respect to enamel minerals, thereby inhibiting demineralization and enhancing remineralization [Huang *et al.,* 2011].

Although nHA products have been available since the 1980s, there are as yet no well-designed RCTs that prove its superior efficacy to fluoride toothpastes. Moreover, under neutral conditions, nHA is seen to promote preferential remineralization of the outer enamel caries lesion, with full remineralization of the lesion not observed [Huang *et al.,* 2011]. Further evidence is required before clinicians can recommend nHA oral products as a substitute to fluoride dentifrices or mouthwashes.

B. Fluoride Boosters

1. *Calcium Phosphate systems*

Biomimetic guided enamel regeneration could well be the future of non-fluoride remineralization, however, their widespread clinical application is still a few years away. Presently,

the need to enhance the remineralizing efficacy of fluoride in high caries-risk patients is largely met by calcium-phosphate systems. The bioavailability of Ca^{2+} and PO_4^{3-} ions is often the limiting factor for net remineralization to occur on topical fluoride application, and this is especially exacerbated under hyposalivation conditions [Reynolds *et al.,* 2008; Vogel *et al.,* 2008]. The presence of extrinsic sources of Ca^{2+} and PO_4^{3-} ions can increase diffusion gradients and augment the F^- ion-mediated remineralization. A number of unique calcium-phosphate remineralization systems have been commercialized in recent years and Cochrane *et al*. [2010] categorized them into three types: (i) stabilized amorphous calcium-phosphate systems; (ii) crystalline calcium-phosphate systems; and (iii) unstabilized amorphous calcium-phosphate systems (Table 3).

Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP)

This remineralization system was developed based on the idea that the tryptic digestion of milk caseinate produced multi-phosphorylated casein phosphopeptides (CPP), substantially increasing the milk protein's solubility and ability to stabilize Ca^{2+} and PO_4^{3-} ions [Reynolds, 1987]. CPP is a saliva biomimetic but with a significantly greater calcium-stabilizing capacity than salivary proteins due to the higher content of its phosphoseryl residues [Cochrane and Reynolds, 2012]. CPP-amorphous calcium phosphate (ACP) nanocomplexes are readily soluble in saliva, creating a diffusion gradient that allows them to localize in supragingival plaque. Low pH conditions that arise during a cariogenic attack facilitate the release of Ca^{2+} and PO_4^{3-} ions, inhibiting demineralization and favouring the remineralization of the incipient lesion by precipitation of the released ions [Reynolds, 2009]. The subsurface remineralization pattern produced by CPP-ACP has been shown to significantly improve the aesthetics, strength, and acid resistance of the remineralized WSL [Cochrane *et al.,* 2010; Mayne *et al.,* 2011].

CPP-ACP is probably the most studied non-fluoride remineralizing agent, although there is considerable variability in the reported results. Many RCTs have demonstrated significantly better remineralizing and anti-caries effects for CPP-ACP products compared to a placebo or a fluoride containing product [Bailey *et al.,* 2009; Guclu *et al.,* 2016; Heravi *et al.,* 2018; Juarez-Lopez *et al.,* 2014; Krithikadatta *et al.,* 2013; Llena *et al.,* 2015; Morgan *et al.,* 2008; Rao *et al.,* 2009; Robertson *et al.,* 2011]. However, other RCTs contradict the above studies as they did not report any superior added effect for CPP-ACP [Beerens *et al.,* 2010; Brochner *et al.,* 2011; Huang *et al.,* 2013; Plonka *et al.,* 2013; Singh *et al.,* 2016; Sitthisettapong *et al.,* 2015; Sitthisettapong *et al.,* 2012]. Published literature and systematic reviews also reach conflicting conclusions, with some reviews suggesting

that CPP-ACP had significant remineralizing and caries preventive effects [Llena *et al.,* 2009; Wang *et al.,* 2017; Yengopal and Mickenautsch, 2009], while others conclude that the evidence to support its long-term remineralizing or synergistic effect with fluoride is limited [Azarpazhooh and Limeback, 2008; Fontana, 2016; Li *et al.,* 2014; Raphael and Blinkhorn, 2015; Zero, 2009].

The reasons for the conflicting results from CPP-ACP remineralization studies can partly be due to a poor understanding of CPP-ACP technology. Many RCTs that concluded that CPP-ACP did not provide any superior remineralization to fluoride have not accounted for the fact that remineralization patterns produced by CPP-ACP and fluoride are different. CPP-ACP enhanced remineralization of enamel subsurface lesions compared to predominantly surface-only remineralization produced by fluoride alone products [Shen *et al.,* 2011]. Fully remineralized WSL not only have better aesthetics and strength, but are more resistant to a subsequent acid challenge. Furthermore, the ability of CPP-ACP to provide high concentrations of stabilized Ca^{2+} and PO_4^{3-} ions could be especially important in highly cariogenic environments (e.g. xerostomia, >6 sugar exposures/day), where fluoride and salivary homeostatic mechanisms alone will not be enough to repair developing lesions. Reasons for inconsistent conclusions from systematic reviews can be attributed to inclusion of studies with inadequate statistical power and possible conflict of interest between competing product manufacturers. Clearly, there is a need for more independent long-term longitudinal studies focussing on high-risk population groups to demonstrate whether CPP-ACP therapy can effect superior remineralization of early lesions compared to fluoride-based products [Gonzalez-Cabezas and Fernandez, 2018].

Functionalized β-*Tricalcium Phosphate (fTCP)*

Crystalline β-tricalcium phosphate (β-TCP) was modified by coupling it with carboxylic acids and surfactants to yield *f*TCP [Karlinsey *et al.,* 2010]. The purpose of functionalizing β-TCP was to create barriers preventing premature fluoride-calcium interactions, thereby allowing it to act as a targeted low-dose delivery system when applied to teeth via dentifrices or mouthwashes [Karlinsey and Pfarrer, 2012]. It was designed primarily to boost F^- ion activity on the tooth surface, with remineralization driven mostly by salivary Ca^{2+} and PO_4^{3-} ions.

Although already available as a commercial product, data on its remineralizing efficacy is sparse, and limited to *in vitro* studies that do not fully reflect the complex biological process involved in lesion remineralization. Purely based on the mechanisms involved, it does seem that CPP-ACP will have a significant advantage over *f*TCP in remineralizing early lesions. While the pH-responsive CPP-

ACP nanocomplexes can deliver stabilized Ca^{2+} and PO_4^{3-} ions over an extended time, *f*TCP appears to supply only a small amount of unbound ions during the short period of brushing before being expectorated from the mouth [Walsh, 2009]. Clinical recommendation on using *f*TCP products will be premature without evidence from well-designed RCTs.

Calcium Sodium Phosphosilicate (CSPS)

CSPS is a bioactive glass material originally developed as a biocompatible bone regenerative agent. When introduced into the aqueous oral environment, it releases Na^{+}, Ca^{2+} and PO_4^{3-} ions, which then interact with saliva and deposit crystalline hydroxycarbonate apatite layer that is structurally and chemically similar to tooth mineral [Burwell *et al.,* 2009]. CSPS was initially incorporated into a dentifrice for the treatment of dentin hypersensitivity but there have been suggestions it could be useful for enamel remineralization too [Wefel, 2009]. However, evidence from *in vitro* and *in situ* data is weak and contradictory [Parkinson *et al.,* 2017; Wang *et al.,* 2016], while there is no clinical data from RCTs to prove its remineralizing efficacy.

Amorphous Calcium Phosphate (ACP)

ACP is an unstabilized calcium-phosphate system that has been incorporated into a dual-chamber fluoride toothpaste with the intention of separately delivering Ca^{2+} and PO_4^{3-} ions into the mouth [Tung and Eichmiller, 2004]. On brushing, the intraoral mixing of Ca^{2+} and PO_4^{3-} ions results in the immediate precipitation of ACP or amorphous calcium fluoride phosphate (ACFP). Both ACP and ACFP are unstable and rapidly transform into more stable HA or fluorhydroxyapatite. Before their phase transformation, the Ca^{2+} and PO_4^{3-} ions should be transiently bioavailable for subsurface lesion remineralization [Cochrane *et al.,* 2010].

Evidence for the ACP technology is available only from a single RCT in radiation patients where it was found to be superior to a conventional fluoride dentifrice in lowering root caries increment, although there were no significant differences in its ability to control coronal caries [Papas *et al.,* 2008]. One of the main concerns with using an unstabilized calcium-phosphate system is that it can promote dental calculus deposition on teeth. Moreover, ACP also tends to rapidly sequester free F^{-} ions in the oral environment, reducing their availability for lesion remineralization. Considering the limited evidence and better alternatives available, oral products based on the ACP remineralization technology have limited clinical applicability.

2. *Polyphosphates*

Sodium Trimetaphosphate (STMP)

One way to reduce the potential risk of fluorosis while maintaining the anticaries efficacy of conventional dentifrices is to partly replace fluoride with polyphosphate salts like STMP, calcium glycerophosphate, or hexametaphosphate [da Camara *et al.,* 2016; Takeshita *et al.,* 2016; Zaze *et al.,* 2014]. Among the polyphosphates, STMP is seen to be the most effective anticaries agent with an ability to not only inhibit demineralization but also enhance remineralization [Freire *et al.,* 2016; Takeshita *et al.,* 2011].

STMP ($Na_3P_3O_9$) is a condensed inorganic phosphate that is able to strongly bind to phosphate sites on enamel surface and remain adsorbed for a longer time compared to other phosphates [McGaughey and Stowell, 1977]. This leads to the formation of a protective layer on the enamel surface that limits acid diffusion of ions during a cariogenic challenge [McGaughey and Stowell, 1977]. The fact that STMP can minimize mineral loss even in the presence of low fluoride concentrations has been confirmed in several *in vitro* and *in situ* studies [Danelon *et al.,* 2014; Favretto *et al.,* 2013; Takeshita *et al.,* 2015; Takeshita *et al.,* 2011]. The protective barrier against acid diffusion created by the adsorption of STMP on enamel does not seem to hinder the diffusion of Ca^{2+} and F^- ions into the enamel. *In situ* models have shown that supplementation of a low-fluoride product with STMP produced similar remineralization effects to 1100 ppm fluoride formulation [Danelon *et al.,* 2013; Takeshita *et al.,* 2016], while the addition of STPM to conventional fluoride dentifrices and varnishes significantly enhanced their remineralization of artificial caries lesions [Danelon *et al.,* 2015; Manarelli *et al.,* 2015].

While earlier clinical trials evaluating caries preventive effects of STMP produced conflicting results [O'Mullane *et al.,* 1997; Stadtler *et al.,* 1996], a recent 18-month double-blinded RCT showed that a 500 ppm low-fluoride dentifrice supplemented with STMP was significantly superior to a 1100 ppm fluoride dentifrice in lowering the caries increment of children [Freire *et al.,* 2016]. There is a need for additional clinical studies to ascertain whether STMP can influence the reversal of non-cavitated lesions.

3. *Natural Products*

An interesting addition to remineralizing agents are plant-derived natural products that have demonstrated the ability to beneficially shift the de-/remineralization caries equilibrium. Among the most promising is *Galla chinensis*, a leaf gall produced by parasitic aphids, which has been found to

be effective in inhibiting demineralization, enhancing remineralization, and increasing the efficacy of fluoride [Cheng *et al.,* 2008, 2010]. The mechanisms are still not fully clear, but it is hypothesized that polyphenols present in *Galla chinensis* interact with and stabilize the organic matrix remnants, thereby blocking the ion diffusion pathways, and slowing demineralization [Huang *et al.,* 2017; Zhang *et al.,* 2015]. *Galla chinensis* remineralization is believed to be mediated through different polyphenol compounds that act as a Ca^{2+} ion carriers into the lesion body [Cheng *et al.,* 2015]. Hesperidin, a citrus flavonoid, and Gum arabic, an *Acacia* exudate, are other natural products that have been found to suppress acid-dependent demineralization and boost remineralization even under fluoride-free conditions [Islam *et al.,* 2012; Onishi *et al.,* 2008].

Natural remineralizing agents could find greater acceptability among the general public compared to fluoride-based remineralizing systems. However, chemical characterisation and standardization of the natural products will be required before further application in clinical trials.

CONCLUSIONS

The era of preventive and minimally-invasive dentistry clearly dictates the need for developing newer approaches to remineralize enamel caries lesions. While fluoride-mediated natural repair of early lesions can occur by influencing oral hygiene and diet, this is dependent on variables such as saliva quality and patient compliance. Non-fluoride remineralization systems are less reliant on such factors and can also significantly improve the structure, aesthetics, and acid resistance of the remineralized lesion. Furthermore, effective non-fluoride remineralizing strategies can prevent a non-cavitated lesion from being subjected to a “death spiral of restorations” due to secondary caries at the enamel-restoration interface [Qvist, 2008]. Currently, most commercially available non-fluoride remineralizing systems are aimed at enhancing fluoride efficacy and minimizing the potential risks associated with fluoride. However, a biomimetic strategy for enamel regeneration may well be the future, where organized enamel apatite crystals with robust attachment to the tooth surface is grown to replace demineralized tissue. Guided enamel regeneration is the holy grail of remineralizing therapeutic approaches and some of the biomimetic technologies discussed here are bringing us a step closer to the reality of growing artificial enamel.

Although highly promising, the currently available clinical evidence for most of the non-fluoride enamel remineralizing systems is either poor, equivocal, or limited to a few early studies. Well-designed RCTs are vital to clarify whether these new-age remineralizing approaches provide

any additional benefit over traditional fluoride remineralization, and these studies are especially needed for the products already in the market.

2.3.2 REVIEW PAPER 6

The Potential Ecological Effects of Casein Phosphopeptide-Amorphous Calcium Phosphate in Dental Caries Prevention

This review principally discusses the proposed ecological mechanisms of CPP-ACP and presents the current evidence for its effects on the oral microbiome ecology.

The paper was published on 21st February 2019 in *Australian Dental Journal*, the official publication of the Australian Dental Association (ADA).

Aust Dent J 2019; 64(1): 66-71.

This chapter is comprised of the publication as it stands in print.

ABSTRACT

Contemporary caries prevention protocols recommend not only effective remineralizing agents but also ecological measures to reverse the dental plaque dysbiosis responsible for the disease pathogenesis. There is a high-level of evidence supporting the remineralizing efficacy of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) from studies around the world. Evidence is also emerging that CPP-ACP may also have a beneficial influence on the dental plaque microbial ecology and homeostasis. The ecological cariostatic effects of CPP-ACP are believed to be mediated predominantly through its anti-adhesion, buffering, and biofilm disrupting actions. This review principally discusses the ecological mechanisms of CPP-ACP and presents the current evidence for its effects on the oral microbiome ecology.

The progress of dental caries lesions to cavitation depends on the equilibrium between demineralization, caused by dental plaque dysbiosis, and remineralization, which is contingent on the presence of calcium, phosphate, and fluoride in the oral environment [Featherstone *et al.,* 2018]. Ever since the cariostatic effects of fluoride were discovered, caries prevention has largely relied on the ability of fluoride ions to inhibit enamel dissolution and enhance remineralization of incipient lesions. More recently, several calcium-phosphate remineralization technologies have been developed aiming to further boost the remineralizing efficacy of fluoride [Fontana, 2016; Philip, 2019]. However, one consequence of accepting the ecological plaque hypothesis is the recognition that current approaches to caries management do not necessarily challenge the underlying aetiology of the disease [Burne, 2018; Philip *et al.,* 2018a]. Caries lesions develop due to a catastrophic ecological shift in the plaque biofilm microflora, triggering an imbalance in the physiologic equilibrium between tooth mineral and biofilm fluid. This ultimately tips the caries balance towards demineralization and lesion formation [Kleinberg, 2002; Marsh, 2003]. Environmental pressures (e.g. frequent sugar exposures, xerostomia etc.) can create an extended period of low pH in dental plaque, favouring acidogenic/aciduric bacteria over commensal resident species, and thereby increase caries-risk [Marsh, 2018]. The pH-driven shifts in composition and metabolism of oral microflora have been shown to be responsible for enamel demineralization and caries initiation [Bradshaw and Marsh, 1998; Bradshaw *et al.,* 1989]. The need to reverse this microbiome dysbiosis has prompted suggestions to include ecological measures to complement the physiochemical preventive effects of fluoride [Philip *et al.,* 2018].

Ideally, the remineralizing agents themselves should be able to influence the cariogenic virulence traits responsible for the microbial plaque dysbiosis. Several studies have shown that under appropriate conditions fluoride ions can inhibit the acidogenicity, aciduricity, and glucan synthesis of cariogenic bacteria [Domon-Tawaraya *et al.,* 2013; Pandit *et al.,* 2015; Pandit *et al.,* 2013; Takahashi and Washio, 2011]. However, it is unclear as to how much the antimicrobial mechanisms of fluoride contribute to its caries preventive effects. A recent study demonstrated that the brief fluoride exposure from toothpastes or mouthwashes could not sustain anti-acid production activity, with the biofilms recovering acidogenicity over time, even when high fluoride concentrations were used [Dang *et al.,* 2016].

In this regard, a naturally derived protein-based remineralizing technology called casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has been shown to not only significantly

improve remineralization of enamel subsurface lesions but may also hold great promise in effecting a beneficial shift in the dental plaque microbial ecology. CPP-ACP is probably the most studied non-fluoride remineralizing technology with a large number of randomised controlled trials (RCTs) and systematic reviews from across the globe having assessed its remineralizing efficacy [Philip, 2019]. However, the literature is relatively sparse on the ecological influences of CPP-ACP on dental plaque. This brief overview aims to discuss the proposed mechanisms and the evidence for its effects on the oral microbiome ecology.

CPP-ACP: DEVELOPMENT AND CARIOSTATIC MECHANISMS

The caries protective effects of milk and milk products like cheese have for long been attributed to casein phosphoproteins and the calcium and phosphate components present in them [Harper *et al.,* 1986; Reynolds and del Rio, 1984]. The tryptic digestion of milk caseinate has been shown to produce multi-phosphorylated casein phosphopeptides (CPPs), substantially increasing its ability to stabilize calcium phosphate in solution, through the binding of amorphous calcium phosphate (ACP) clusters with their multiple phosphoserine residues [Reynolds, 1987]. CPP acts as a salivary protein biomimetic but with significantly greater calcium-stabilizing capacity due to the higher content of its phosphoseryl residues [Cochrane and Reynolds, 2012]. Moreover, CPP exhibits ten times greater anti-cariogenicity on a weight basis than the intact milk protein, without the associated problems of unpalatability or allergenicity [Reynolds and Walsh, 2005]. This natural process of calcium stabilization, transport, and delivery was used to develop the CPP-ACP remineralisation technology. Both CPP-ACP and a fluoride-incorporated version (CPP-ACFP) are now used globally in a number of oral care products like chewing gums, topical crèmes, and toothpastes.

CPP-ACP nanocomplexes are readily soluble in saliva creating a diffusion gradient that allows them to localize in dental plaque. CPP-ACP can thus act as biological calcium phosphate delivery vehicles that significantly boost levels of bioavailable calcium and phosphate ions in saliva and plaque fluid without causing indiscriminate precipitation of the calcium salts [Reynolds *et al.,* 2003]. Low pH conditions that arise during a cariogenic attack facilitate the release of calcium, phosphate, and fluoride ions from the CPP-ACP/CPP-ACFP complexes, inhibiting demineralization and favouring remineralization by precipitation of the released ions [Reynolds, 2009]. The ability of CPP-ACP to readily provide supersaturated concentrations of stabilized calcium and phosphate ions significantly augments the remineralisation potential of saliva/plaque and enables deeper subsurface lesion remineralisation. The remineralization pattern produced by CPP-ACP is considered superior to the

predominantly surface-only remineralization mediated by fluoride [Shen *et al.,* 2011]. Fully remineralized enamel lesions not only have better aesthetics and strength but are also more resistant to a subsequent acid challenge [Cochrane *et al.,* 2008; Mayne *et al.,* 2011].

CPP-ACP: ECOLOGICAL EFFECTS ON DENTAL PLAQUE

The majority of the studies on the cariostatic effects of CPP-ACP have focussed on its remineralization capabilities. However, there is growing awareness that the daily application of CPP-ACP can have beneficial effects on the ecology of the oral plaque microflora. The existing evidence on its possible dental plaque effects are summarized in Table 4 and the proposed mechanisms underpinning the ecological influences of CPP-ACP are detailed further below.

Table 4. Scientific evidence for the ecological effects of CPP-ACP/CPP-ACFP

Author [year]	Study Design	Test Agent	Results/Conclusions
Anti-adhesion Effects			
Schüpbach *et al*. [1996]	*In vitro* incubation of bovine enamel discs with unstimulated saliva	CPP/CGMP	Significant reduction in adherence of *S. mutans*
Guggenheim *et al*. [1999]	*In vivo* animal caries model study	CPP/micellar casein	Inhibition of caries lesions and *S. sobrinus*
Rose [2000a,b]	*In vitro* calcium binding experiments	CPP-ACP	Binds to streptococcal plaque with twice the affinity of free Ca^{2+} ions and lowers the degree of calcium bridging between the pellicle and adhering bacteria
Reynolds *et al*. [2003]	Immunolocalization in plaque	CPP-ACP	Strong affinity of to bacterial cell surface molecules and plaque extracellular matrix
Subramanian and Naidu [2009]	RCT in children aged 13-18 years	CPP-ACFP	Reduced plaque *S. mutans* counts more than a fluoride-chlorhexidine gel
Beerens *et al*. [2010]	RCT in orthodontic patients	CPP-ACFP	Lowered dental plaque numbers of *S. mutans* and *Lactobacillus* but not significantly more than a fluoride-free control paste
Pukallus *et al*. [2013]	Longitudinal RCT in infants from birth to 24 months	CPP-ACP	Fewer MS-positive infants at 24 months in the CPP-ACP group with a dose-response effect observed with CPP-ACP usage
Plonka *et al*. [2013]	Longitudinal RCT in infants from birth to 24 months	CPP-ACP	Fewer MS-positive infants at 12 months in the CPP-ACP group
Yetkiner *et al*. [2014]	RCT in children with white spot lesions	CPP-ACP	Salivary *S. mutans* counts decreased at end of3-month experimental period
Emamieh *et al*. [2015]	RCT in students aged 20-25 years	CPP-ACP	CPP-ACP chewing gums significantly reduced salivary *S. mutans* counts even more than a xylitol chewing gum

Table 4. Continued

Author [year]	Study Design	Test Agent	Results/Conclusions
Buffering Effects			
Reynolds and Riley [1989]	*In vitro* protein dissimilation study	CPP	Enzymatic breakdown of CPP elevated plaque pH rise through production of NH_3
Caruana *et al*. [2009]	Randomised cross-over trial	CPP-ACP	Application of CPP-ACP crème reduced plaque pH drop after a sucrose challenge
Marchisio *et al*. [2010]	RCT in orthodontic patients		Significantly increased salivary pH levels but plaque pH changes were equivocal
Heshmant *et al*. [2014a]	Randomised cross-over trial	CPP-ACFP	Clinically beneficial in increasing plaque pH but no effect on salivary pH
Heshmant *et al*. [2014]	RCT in teenage patients	CPP-ACP/ CPP-ACFP	Prior application of CPP-ACFP kept plaque pH high for up to 96 h after a sugar challenge compared to 48 h for CPP-ACP
Biofilm Effects			
Rahiotis *et al*. [2008]	*In situ* biofilm study	CPP-ACP	Delayed intraoral biofilm formation
Dashper *et al*. [2016]	*In vitro* biofilm study	CPP-ACP	Inhibited *S. mutans* biofilm development and disrupted architecture of established biofilms

CPP-ACP: casein phosphopeptide-amorphous calcium phosphate; CPP-ACFP: casein phosphopeptide-amorphous calcium fluoride phosphate; CGMP: caseinoglycomacropeptide; RCT: randomised controlled trial; MS: mutans streptococci

Inhibition of Bacterial Adhesion

Early *in vitro* studies have reported a rapid saturation of saliva-coated hydroxyapatite surfaces by casein complexes inhibiting the adherence of *Streptococcus mutans* and *Streptococcus sobrinus* [Reynolds and Wong, 1983; Schüpbach *et al.,* 1996; Vacca-Smith *et al.,* 1994]. This was confirmed in an experimental animal caries model study where rats fed cariogenic diets containing micellar casein/CPP showed a significant reduction in caries lesions and numbers of *S. sobrinus* colonizing the teeth of these animals [Guggenheim *et al.,* 1999]. Further studies by Rose revealed that CPP-ACP binds both to dental plaque (with a strong affinity for *S. mutans*) and to pellicle macromolecules adsorbed on the tooth surface [Rose, 2000a; Rose, 2000b]. This was corroborated by electron micrographs of immunocytochemically stained sections of dental plaque showing CPPs bound to bacterial cell surfaces as well as to the extracellular matrix [Reynolds *et al.,* 2003]. It has been suggested that major bonds localizing the CPP-ACP on bacterial cell surfaces are not mediated by the acid-labile calcium ions or ACP cross-linking but were predominantly between the peptides and cell surface molecules [Reynolds *et al.,* 2003].

The exact anti-adhesion mechanisms for CPP-ACP are not clearly defined but it has been shown that CPP-ACP competes with free calcium for plaque calcium binding sites, reducing the degree of calcium bridging between the pellicle and adhering cells and between the bacterial cells themselves [Rose, 2000a]. In addition, CPP molecules have hydrophilic and hydrophobic regions that can mask the bacterial cell surface hydrophobic proteins and thereby impede the initial bacterial adhesion to pellicle-coated tooth surfaces. Furthermore, localization of CPP-ACP on bacterial surfaces and plaque could prevent bacterial co-aggregation and accumulation in dental plaque. This interference with microbial attachment may prevent deleterious ecological shifts in dental plaque thereby inhibiting caries development.

Several clinical trials have shown that regular use of CPP-ACP/CPP-ACFP oral care products reduced mutans streptococci levels in saliva [Emamieh *et al.,* 2015; Plonka *et al.,* 2013; Pukallus *et al.,* 2013; Subramanian and Naidu, 2009; Yetkiner *et al.,* 2014]. Another RCT reported that while CPP-ACFP caused a reduction in percentages of *S. mutans* and aciduric lactobacilli in dental plaque samples, the plaque composition was not significantly different from that effected by a fluoride-free control paste [Beerens *et al.,* 2010]. The authors of this study suggested that poor compliance of subjects using the CPP-ACFP product could be one of the reasons why no advantage was detected. A limitation of these clinical trials is that a single bacterial species (usually *S. mutans*) was used as the surrogate marker to evaluate changes in the salivary/plaque microbial profile. However, assessing the dental plaque numbers of a range of cariogenic and health-associated bacteria would be more relevant. Clearly, more well-designed RCTs are required to establish whether the reported anti-adhesion effects of CPP-ACP can translate into altering the plaque microbial composition to one associated with health and not disease.

Buffering Effects

A sustained low pH environment in dental plaque not only favours enamel demineralization but also allows acidogenic/aciduric bacteria to gain an ecological advantage over health-associated oral bacterial species [Nascimento, 2018]. The buffering effects of CPP-ACP can alkalize the plaque biofilm and possibly prevent the detrimental microbial ecological shift from occurring. RCTs in orthodontic patients have shown that CPP-ACP crèmes could elevate plaque/salivary pH levels and improve the oral hygiene index [Heshmant *et al.,* 2014a; Marchisio *et al.,* 2010]. Prior application of CPP-ACP products was also seen to reduce the fall in plaque pH following a sucrose challenge, with

CPP-ACFP capable of keeping the pH high for up to 96 h compared to 48 h for CPP-ACP [Caruana *et al.,* 2009; Heshmant *et al.,* 2014].

The mechanisms by which the buffering influences of CPP-ACP are mediated may be varied. CPP-ACP does not necessarily impede bacterial acid production per se but rather acts as a reservoir of peptides and phosphate ions which offset any drop in pH [Reynolds, 1997]. While the enzymatic breakdown of CPPs by plaque peptidases and phosphatases reduces their capacity to stabilize calcium and phosphate ions [Cross *et al.,* 2005], the plaque pH rise that results from this hydrolysis is beneficial in favourably tilting the de-/remineralization equilibrium [Cochrane *et al.,* 2010]. For instance, the glutaminyl and asparaginyl residues of CPPs have been shown to be non-arginine sources of nitrogen for oral commensal bacteria to catabolize and effect a pH rise through the production of ammonia [Reynolds and Riley, 1989]. The CPP phosphoseryl sequences are relatively more resistant to hydrolysis but readily accept protons and neutralize plaque acids [Reynolds, 1987]. Evidently, CPPs and their breakdown products can have a positive impact on plaque biofilm homeostasis and thus provide a selective ecological advantage to non-aciduric microorganisms. This could, over time, increase the proportion of amino acid utilizing commensal bacteria in dental plaque while reducing the numbers of cariogenic bacteria.

Biofilm Disruption

Dental caries is the most common biofilm-mediated disease afflicting humans. The exopolysaccharide glucan-rich matrix of cariogenic plaque biofilms offers substantial protection against the inimical influences of remineralizing agents and antimicrobials. Cariostatic agents that can potentially delay biofilm development and disrupt its structural organisation should be beneficial in caries prevention. A recent *in vitro* study demonstrated that a glass ionomer cement containing 3% CPP-ACP significantly inhibited biofilm development by over 50% [Dashper *et al.,* 2016]. A single 10 min treatment with aqueous 1% CPP-ACP was also shown to significantly reduce biomass and thickness of established *S. mutans* biofilms, while severely disrupting the structural architecture of these biofilms [Dashper *et al.,* 2016]. This was consistent with an *in situ* study that showed treatment with CPP-ACP delayed intraoral biofilm formation [Rahiotis *et al.,* 2008]. The ability of CPP-ACP to mask pellicle and bacterial cell surface macromolecules possibly prevents the cell to cell adhesion essential for biofilm development. The CPP-ACP mediated disruption to the biofilm structural architecture would render it less capable of maintaining an acidic pH at the

attachment surface and allow diffusion of fluoride, calcium and phosphate ions to the plaque-tooth interface for remineralizing any incipient lesions.

Bacteriostatic/Bactericidal Effects

There have been suggestions that CPP-ACP by forcing the maintenance of high free calcium concentrations in dental plaque may indirectly have additional biocidal or bacteriostatic effects. Calcium concentrations greater than 1 mmol/L have been found to induce streptococcal membrane permeability and partial lysis [Trombe *et al.,* 1992], while the ATP-dependent calcium export system may also be unable to cope with high calcium concentrations [Kobayashi *et al.,* 1978]. Whether these mechanisms actually play a role in CPP-ACP reducing bacterial counts in dental plaque/saliva needs further elucidation.

CONCLUSIONS

CPP-ACP is a natural non-toxic anti-cariogenic agent that can safely be added to dentifrices, chewing gums, and food products to lower caries experience in high-risk individuals. The evidence presented in this paper does indicate that CPP-ACP may possibly have multiple modes of cariostatic action (inhibition of demineralization, enhanced remineralization, reduced bacterial adhesion, buffering action, biofilm disruption, and bacteriostatic/bactericidal effects). While there is a high-level of evidence supporting the remineralization potential of CPP-ACP, further research is needed on whether the suggested ecological mechanisms can effectively translate into beneficial microbial changes in dental plaque. Molecular techniques, based on the 16S rRNA real-time quantitative polymerase chain reaction or next-generation sequencing, can be used to confirm bacterial profile changes in dental plaque after using CPP-ACP oral care products. Three RCTs are currently registered on the Australian New Zealand Clinical Trials Registry (ACTRN12618000095268, ACTRN12615001227583, ACTRN12617000148370), evaluating whether dentifrices and chewing gums containing CPP-ACP can influence microbial compositional changes in dental plaque. Results from these clinical trials should provide more definitive evidence on the potential ecological effects of CPP-ACP.

2.4 LITERATURE GAPS

The following gaps have been identified from the review of the literature:

1. There have been no studies that have assessed whether natural products with antimicrobial activity can selectively inhibit the growth of caries-associated bacterial species without affecting the viability of health-associated bacterial species.

2. While isolated phytochemical compounds have exhibited potent activity against cariogenic virulence factors, there is a paucity of studies on whether commercially available standardized plant or fruit extracts can have similar influence.

3. The literature is lacking studies on whether the synergistic virulence of *Streptococcus mutans-Candida albicans* dual-species biofilms, implicated in contributing to the severity of early childhood caries, can be disrupted using antimicrobial natural products.

4. There is are no reports in literature on whether any of the natural products suggested to have cariostatic effects can influence beneficial microbial ecological changes.

5. The large majority of studies of CPP-ACP have been on its remineralizing effects, and there is a need for more research into its ecological effects.

6. The few clinical studies on the ecological effects of CPP-ACP have focussed on a single bacterial species in saliva. There is a need to study its ecological effects on a range of caries- and health-associated bacteria in dental plaque.

7. There is a need for well-designed randomised controlled trials to investigate the ecological effects of natural products and CPP-ACP.

3. HYPOTHESIS AND AIMS

3.1 RESEARCH HYPOTHESIS

The overarching hypothesis for this thesis is that natural products and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) can attenuate virulence in cariogenic biofilms and influence beneficial microbial ecological changes in the dental plaque.

3.2 RESEARCH AIMS

This research project has the following aims:

1. Identify natural products with growth inhibitory or virulence attenuating activity that could translate into beneficial microbial ecological effects against cariogenic biofilms.

2. Assess the virulence inhibitory and ecological effects of CPP-ACP on polymicrobial biofilms and the dental plaque microbiome.

3. Evaluate whether the addition of a cariostatic natural product to a CPP-ACP oral care product can enhance beneficial plaque ecological effects.

4. SCIENTIFIC PAPERS

4.1 SCIENTIFIC PAPER 1

Growth Inhibitory Effects of Antimicrobial Natural Products against Cariogenic and Health-associated Oral Bacterial Species

This study investigated whether selected natural products could specifically target the growth of a caries-associated bacterial species (*Streptococcus mutans*) without affecting the viability of a health-associated oral bacterial species (*Streptococcus sanguinis*). The study also determined the minimum inhibitory concentrations of the natural products that exhibited bacterial growth inhibitory effects.

The paper was accepted on 8th January 2019 for publication in *Oral Health and Preventive Dentistry*, the official publication of the European Society for Preventive Dentistry. It is currently being prepared for print.

This chapter is comprised of the publication as accepted.

ABSTRACT

Purpose: This study investigated whether selected natural products could specifically target the growth of a caries-associated bacterial species (*Streptococcus mutans*) without affecting the viability of a health-associated oral commensal bacterial species (*Streptococcus sanguinis*).

Materials and Methods**:** Agar diffusion assays were used to screen the natural products for bacterial growth inhibitory effects and the diameters of the inhibitory zones for the two bacterial species were compared. The minimum inhibitory concentrations (MIC) of the natural products that showed growth inhibitory effects were then determined using the broth microdilution method.

Results**:** Except for the fruit berry extracts (cranberry, wild blueberry, and strawberry), all the other selected natural products (peppermint, cinnamon, ginger, cinnamon, rosemary, liquorice, xanthorrrhizol, *tt*-farnesol, guaijaverin, and macelignan) exhibited varying degrees of bacterial growth inhibition. The MIC values ranged from as low as 4 µg/mL for xanthorrrhizol to 1000 µg/mL for guaijaverin. All the growth inhibitory natural agents tested showed similar inhibition for both *S. mutans* and *S. sanguinis*.

Conclusions**:** Although several natural products exerted significant antibacterial effects, none had selective inhibitory action on the growth of *S. mutans*.

INTRODUCTION

Dental caries remains among the most widespread global oral health problems, affecting quality of life and imposing a costly burden on health services [Kassebaum *et al.,* 2015]. An additional concern is that recent epidemiological surveys are indicating that dental caries prevalence may be increasing again even in developed countries [AIHW, 2018; Dye *et al.,* 2015]. Furthermore, the disease is not uniformly distributed with multiple population groups, including children and the elderly, at increased risk of developing the disease [Featherstone *et al.,* 2018].

In addition to the established caries preventive methods, adjunctive measures commensurate to individual caries-risk, are often needed to control the disease in high-risk populations. With dental caries known to be a disease of microbiome dysbiosis, preventive strategies that ecologically modify the dental plaque biofilm are being increasingly recommended [Philip *et al.,* 2018]. Health-associated microbiomes can deliver small but relevant benefits over a prolonged period and could be particularly useful for long-term dental caries control [Burne, 2018; Marsh, 2018]. With the aim of reducing levels of mutans streptococci (MS), a range of biocides (e.g. chlorhexidine, triclosan, cetylpyridinium chloride) and antibiotics (e.g. vancomycin) have been used in the past [Chen *et al.,* 2010; De Paola *et al.,* 1974; Holbeche *et al.,* 1976; Ribeiro *et al.,* 2007]. However, a concern with conventional antimicrobial agents is that they usually exert a broad-spectrum of antibacterial action, suppressing even health-associated oral microbial communities, and thus disrupting key health benefits of the resident oral microbiome [Yoo *et al.,* 2011]. Furthermore, the side-effects associated with synthetic antimicrobials limit their acceptability and the duration for which they can be used. Once the chemotherapeutic intervention stops, susceptible tooth surfaces are often repopulated with the same disease-associated microbiome that was eliminated.

Plant-derived natural products with bacterial growth inhibitory effects are an attractive alternative to traditional oral biocides for long-term caries prevention. Phytochemicals have been shown to reduce the development of dental plaque, influence bacterial adhesion, and reduce symptoms of oral diseases [Palombo, 2011]. For caries prevention, such natural agents should ideally suppress the growth of MS without significantly inhibiting the commensal health-associated plaque microflora. There is comparatively little information in the literature on whether any of the reported antimicrobial natural products can selectively inhibit the growth of cariogenic bacteria without affecting health-associated bacteria. Therefore, the aim of this study was to compare the

growth inhibitory effects of a range of natural products on the caries-associated *Streptococcus mutans* and the health-associated *Streptococcus sanguinis* bacterial species.

MATERIALS AND METHODS

Test Agents

The selection of test agents was based on a literature review that identified potential cariostatic natural products. High-quality supercritical CO_2 extracts of peppermint, cinnamon, ginger, rosemary, liquorice, and xanthorrrhizol were sourced from Flavex Naturextrakte (Rehlingen, Germany). Water soluble molecular extracts of cranberry, wild blueberry, and strawberry were sourced from Diana Food (Champlain, QC, Canada). In addition, phytochemicals found in bee-hive propolis (*tt*-farnesol) (Sigma-Aldrich, St. Louis, MO, USA), and those isolated from guava leaves (guaijaverin) and nutmeg (macelignan) (Seebio Biotech, Shanghai, China) were also selected. Stock solutions of all the natural products were prepared, with the berry extracts dissolved in phosphate buffered saline (PBS), while the other test agents were suspended in 2% dimethyl sulfoxide (DMSO, Sigma-Aldrich). Both PBS and the 2% DMSO were used as vehicle controls in the different experiments.

Bacterial Strains

Bacterial cultures were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). *S. mutans* ATCC 25175 and *S. sanguinis* ATCC 10556 were revived from freeze-dried vials and cultured on trypticase soy agar plates (Becton Dickinson, San Jose, CA, USA) supplemented with 5% defibrinated sheep blood and incubated in a 5% CO_2 atmosphere at 37°C for 72 h. The bacterial colonies were then subcultured in brain heart infusion (BHI, Merck, Darmstadt, Germany) and incubated overnight at 37°C The resulting bacterial cultures were centrifuged at 4000 x *g* for 5 min, washed twice with PBS, and resuspended in BHI. The bacterial cell count in the culture medium was spectrophotometrically adjusted to approximately 2 x 10^8 CFU/mL just before each assay.

Agar Diffusion Assay

A standard agar diffusion assay was used for the initial screening of growth inhibitory effects against *S. mutans* and *S. sanguinis* [Evans *et al.,* 2015]. Briefly, five wells of 5 mm diameter were punched into pre-prepared Mueller-Hilton agar petri dishes (MH agar, Thermo Fisher, Rockford, IL, USA). The wells were placed 30 mm apart and 20 mm from the outer edge of the petri dish. Each

well was loaded with a fine spiral of sterile filter paper (90 mm x 4 mm). Aliquots (50 µL) of the respective test agent solutions/controls were the carefully pipetted onto the filter paper. Each plate had three test agents, plus 0.2% chlorhexidine (CHX) as a positive control, and the appropriate vehicle control. For each plate, 5 mL aliquots of the standardized bacterial suspension was added to 5 mL of melted MH agar at 45°C, mixed thoroughly and poured evenly over the surface of the agar plates containing the wells loaded with test agents/controls. After incubation at 37°C for 24 h, the plates were assessed for bacterial growth or inhibition. The zones of inhibition were measured directly at their minimum diameter (Fig. 5). All experiments were performed in triplicate on three independent occasions.

Figure 5. Agar diffusion assay with arrows pointing to zones of bacterial growth inhibition

Broth Microdilution Assay

The minimum inhibitory concentration (MIC) of the test agents was determined using the broth microdilution method as specified by the Clinical and Laboratory Standards Institute [CLSI, 2015]. Briefly, twofold serial dilutions of the test agent stock solutions were prepared and pipetted into separate wells of 96-well microtiter plates (Costar 3596, Sigma-Aldrich, New York, NY, USA). The wells were then inoculated with the bacterial suspensions such that the final bacterial concentration in each well was approximately 5 x 10^5 CFU/mL. Triplicate samples were prepared for each concentration of the agent being tested (Fig. 6). Appropriate solvent control, growth control, and sterility control wells were also maintained in each microtiter plate. After incubation for 24 h at 37°C, the optical density (O.D.) was determined at 600 nm wavelength in a microplate

spectrophotometer (Tecan Infinite 200 Pro, Grodig, Austria) after correcting for the background absorbance of the test agent solutions. The MIC of each test agent against *S. mutans* and *S. sanguinis* was calculated from the adjusted O.D. values obtained from three independent experiments.

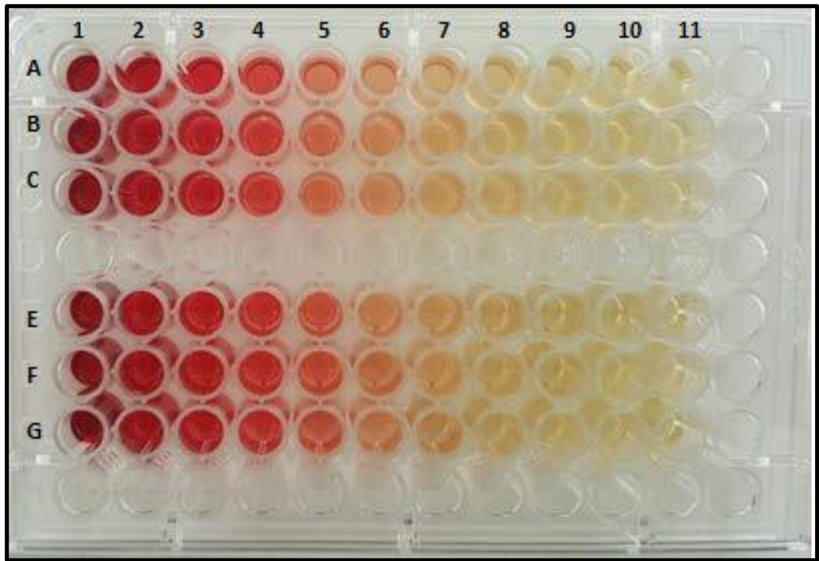

Figure 6. Microdilution assays for minimum inhibitory concentration determination

Statistical Analysis

After data sets were assessed for normality, an independent sample t-test was used to evaluate differences in the zones of growth inhibition between *S. mutans* and *S. sanguinis* bacterial species for each test agent. The level of significance was set at 5%. Statistical software SPSS version 24 (IBM, NY, USA) was used to perform the analysis.

RESULTS

Growth inhibitory effects

Except for the three berry extracts, all the other natural products inhibited the growth of both *S. mutans* and *S. sanguinis* in the agar diffusion assay. The highest inhibition of bacterial growth was seen for xanthorrrhizol, followed by macelignan and *tt*-farnesol (Table 5). The diameters of the zones of inhibition for *S. mutans* were not significantly different from that for *S. sanguinis* for any of the growth inhibitory natural products tested ($P > 0.05$).

Table 5. Growth inhibitory effects of natural products against *S. mutans* and *S. sanguinis*

	Zone of inhibition diameter (mm)		
Test Agent (concentration)	*Streptococcus mutans* (SM)	*Streptococcus sanguinis* (SS)	*P*-value (SM vs. SS)
Xanthorrrhizol (200 µg/mL)	13.2 ± 0.55	13.1 ± 0.64	N.S
Macelignan (200 µg/mL)	11.8 ± 0.43	12.2 ± 1.11	N.S
tt-Farnesol (200 µg/mL)	11.6 ± 0.82	11.3 ± 0.35	N.S
Liquorice (1 mg/mL)	10.8 ± 0.48	11.0 ± 0.77	N.S
Cinnamon (1 mg/mL)	9.8 ± 0.97	9.9 ± 0.71	N.S
Peppermint (1 mg/mL)	9.2 ± 0.60	9.4 ± 0.72	N.S
Ginger (1 mg/mL)	8.4 ± 0.69	8.3 ± 0.46	N.S
Rosemary (1 mg/mL)	8.1 ± 0.61	7.8 ± 0.52	N.S
Guaijaverin (1 mg/mL)	7.2 ± 0.34	7.2 ± 0.16	N.S
Cranberry (16 mg/mL)	0	0	-
Wild Blueberry (16 mg/mL)	0	0	-
Strawberry (16 mg/mL)	0	0	-
Vehicle control (2% DMSO/PBS)	0	0	-
Positive Control (0.2% CHX)	17.7 ± 0.62	18.0 ± 0.89	N.S

Diameter of inhibitory zones (mean ± S.D) from three independent triplicate experiments (n = 9). DMSO: dimethyl sulfoxide; PBS: phosphate buffered saline; CHX: chlorhexidine. N.S: no significant differences between *S. mutans* and *S. sanguinis* inhibition using the independent sample t-test ($P > 0.05$).

Minimum inhibitory concentrations

The MICs of the natural products that exhibited bacterial growth inhibition are presented in Table 6. The MIC values obtained from the microdilution assays followed the bacterial growth inhibitory pattern seen in the agar diffusion assay, with xanthorrrhizol showing the lowest MIC (4 µg/mL) against both *S. mutans* and *S. sanguinis* among all the natural products tested.

Table 6. MIC of natural products with bacterial growth inhibitory effects in µg/mL

Test Agent	*Streptococcus mutans*	*Streptococcus sanguinis*
Xanthorrrhizol	4	4-8
Macelignan	8	4
tt-Farnesol	8	8
Liquorice	80	100
Cinnamon	340	175
Peppermint	375	340
Ginger	275	475
Rosemary	800	800
Guaijaverin	1000	1000
CHX (control)	1	1

MIC: minimum inhibitory concentration calculated from three independent triplicate experiments (n = 9). CHX: chlorhexidine.

DISCUSSION

The results of this study provide insights into the growth inhibitory effects of selected natural products against *S. mutans* and *S. sanguinis* bacteria. *S. mutans* is one of the primary bacterial culprits responsible for caries pathogenesis due to its acidogenic, aciduric, and glucan synthesis properties. On the other hand, *S. sanguinis* can use its arginine deaminase system to neutralize acids and slow caries lesion progression. These bacterial species were thus chosen as being representative of those associated with dental caries and health.

None of the fruit berry extracts (cranberry, blueberry, and strawberry) demonstrated bacterial growth inhibitory effects against either *S. mutans* or *S. sanguinis* despite their high polyphenol content. This suggests that the bioactive phytochemicals present in these berry extracts may lack bactericidal effects at the concentrations tested. In contrast, all the other selected natural products inhibited the growth of both *S. mutans* and *S. sanguinis* with varying degrees of potency. Xanthorrrhizol, the bioactive compound in Javanese turmeric (*Curcuma xanthorrhiza*) showed the highest bacterial growth inhibition, followed closely by macelignan from nutmeg (*Myristica fragrans*), and *tt*-farnesol, a sesquiterpene isolated from bee-hive propolis. The MICs of the tested natural agents ranged from 4 µg/mL for xanthorrrhizol, up to 1000 µg/mL for the guaijaverin compound found in guava (*Psidium guajava*).

There were no significant differences observed between *S. mutans* and *S. sanguinis* growth inhibition for any of the natural products tested. Considering oral streptococci have common structural properties [Banas and Vickerman, 2003; Hamada *et al.,* 1980], it will be challenging to find an antimicrobial agent that can selectively suppresses the growth of cariogenic MS without also affecting health-associated oral streptococci such as *S. sanguinis*, *Streptococcus gordonii* or *Streptococcus mitis*/*oralis*. It would be worthwhile to investigate whether non-streptococcal health-associated plaque microflora (e.g. *Neisseria flavescens* or *Corynebacterium durum*), can remain relatively unaffected to the antimicrobial effects of these natural products. While previous studies have shown that plant-derived essential oils present in mouthrinses can reduce MS levels and total plaque [Fine *et al.,* 2000; Ouhayoun, 2003; Overholser *et al.,* 1990], their ability to discriminate between disease- and health-associated microflora requires further study.

The MICs of xanthorrrhizol and macelignan against *S. mutans* (4 µg/mL and 8 µg/mL respectively) found in this study were consistent with earlier reports on these phytochemicals [Chung *et al.,* 2006; Hwang *et al.,* 2000]. While MIC values of these phytochemicals may seem only slightly higher than the reported 1 µg/mL MIC of CHX, it is important to consider the molecular weight of these compounds when comparing the antimicrobial potency of various agents. For example, the 4 µg/mL MIC of xanthorrrhizol corresponds to 18.3 µmol/mL based on its molecular weight (MW) of 218.3 g/mol, while the similar MIC of CHX (MW 897.8 g/mol) is only 1.1 µmol/mL. Evidently, CHX is at least 16-fold more potent than xanthorrrhizol against these bacterial species. However, the demonstrated MICs of xanthorrrhizol (18.3 µmol/mL) and macelignan (24.4 µmol/mL) were much lower than the reported MICs of essential oils like menthol (3200 µmol/mL) and thymol (3329 µmol/mL) that are commonly used in mouthrinses for antibacterial effects. This suggests their excellent potential for incorporation into oral care products as alternatives to the currently used antibacterial essential oils in conditions where broad antimicrobial action is required. For the other natural extracts tested in this study, identification and isolation of their bioactive antimicrobial compounds could further improve their antibacterial effects.

CONCLUSIONS

Recent reports have underlined the symbiotic benefits a healthy oral microbiome affords the host [Devine *et al.,* 2015; Kilian *et al.,* 2016]. This has led to clinical recommendations for adopting ecological strategies as part of modern caries management [Philip *et al.,* 2018a]. Clearly, long-term control of caries risk should be based on successful "stewardship" of the plaque biofilm,

rather than simply focussing on eliminating it. This will allow health-associated microbial communities to dominate the dental plaque biofilm, and thereby lower its virulence potential. From an ecological perspective, it may thus be more beneficial to use natural products that disrupt cariogenic virulence properties (e.g. acidogenicity or glucan synthesis), rather than those that broadly affect bacterial viability and growth [Jeon *et al.,* 2011]. For example, specific phytochemicals like apigenin and cranberry proanthocyanidins were able to modulate virulence properties and inhibit dental caries *in vivo* despite lacking significant biocidal activity [Koo *et al.,* 2010; Koo *et al.,* 2005]. While this screening study could not identify antimicrobial natural products that specifically targeted the growth of cariogenic bacteria, further studies using natural agents with potential for inhibiting plaque virulence properties are needed. The vast therapeutic potential of phytodentistry in lowering caries-risk and preventing oral diseases remains to be fully exploited.

RAW DATA

Study 1: Agar diffusion assay

Zone of bacterial inhibition (mm)

Blueberry	*S. mutans*	*S. sanguinis*
EXPT. 1	0	0
	0	0
	0	0
EXPT. 2	0	0
	0	0
	0	0
EXPT. 3	0	0
	0	0
	0	0
Mean	0	0
S.D		

Cinnamon	*S. mutans*	*S. sanguinis*
EXPT. 1	9.1	10.1
	7.8	9.9
	10.3	9.9
EXPT. 2	10.4	11.0
	9.7	8.9
	9.3	9.3
EXPT. 3	10.0	9.8
	10.2	9.1
	11.2	10.8
Mean	9.8	9.9
S.D	1.0	0.7

Cranberry	*S. mutans*	*S. sanguinis*
EXPT. 1	0	0
	0	0
	0	0
EXPT. 2	0	0
	0	0
	0	0
EXPT. 3	0	0
	0	0
	0	0
Mean	0	0
S.D		

tt-Farnesol	*S. mutans*	*S. sanguinis*
EXPT. 1	12.4	11.3
	10.5	11.4
	11.8	12
EXPT. 2	10.6	11
	12.7	11.2
	11.1	10.9
EXPT. 3	10.8	11.2
	12.2	11.3
	11.9	11.8
Mean	11.56	11.34
S.D	0.82	0.35

Ginger	*S. mutans*	*S. sanguinis*
EXPT. 1	9.1	8.1
	9.5	8
	8.1	7.8
EXPT. 2	7.2	8.3
	8.5	8.4
	8.8	8.6
EXPT. 3	8	8
	7.9	9.3
	8.6	7.9
Mean	8.41	8.27
S.D	0.69	0.46

Guaijaverin	*S. mutans*	*S. sanguinis*
EXPT. 1	8.1	7.1
	7	7.3
	7.2	7.1
EXPT. 2	7.2	7.2
	7.1	7.2
	7.1	7.6
EXPT. 3	7	7.3
	7.3	7.3
	7.1	7.1
Mean	7.23	7.24
S.D	0.34	0.16

Liquorice	*S. mutans*	*S. sanguinis*
EXPT. 1	11.4	10.9
	11.2	11.8
	10.3	11
EXPT. 2	10.5	10.6
	11	10.9
	9.9	11.4
EXPT. 3	11.1	12.2
	10.9	9.5
	10.7	11.4
Mean	10.78	11.08
S.D	0.48	0.77

Peppermint	*S. mutans*	*S. sanguinis*
EXPT. 1	9.9	9.4
	8.9	9.7
	9.1	10.1
EXPT. 2	7.8	9.2
	9.3	8.6
	9.2	7.9
EXPT. 3	9	9.6
	9.5	9.9
	9.7	10
Mean	9.16	9.38
S.D	0.60	0.72

Macelignan	*S. mutans*	*S. sanguinis*
EXPT. 1	12.2	13.1
	11.9	11.3
	12	11.2
EXPT. 2	12	13.4
	11.5	10.3
	11.6	13.2
EXPT. 3	11.7	12.5
	12.6	12
	11.1	13.2
Mean	11.84	12.24
S.D	0.43	1.11

Rosemary	*S. mutans*	*S. sanguinis*
EXPT. 1	7.9	8
	8.5	7.7
	8.1	8.4
EXPT. 2	9	8.7
	8.6	7.3
	8.8	7.3
EXPT. 3	7.4	8
	7.3	7.3
	7.7	7.4
Mean	8.14	7.79
S.D	0.61	0.52

Strawberry	*S. mutans*	*S. sanguinis*
EXPT. 1	0	0
	0	0
	0	0
EXPT. 2	0	0
	0	0
	0	0
EXPT. 3	0	0
	0	0
	0	0
Mean	0	0
S.D		

Xanthorrrhizol	*S. mutans*	*S. sanguinis*
EXPT. 1	13	12.8
	13.3	13.7
	12.2	13.1
EXPT. 2	13.4	13.3
	14	12.8
	12.8	13.9
EXPT. 3	13.2	12.9
	13.2	11.6
	14.1	13.5
Mean	13.24	13.07
S.D	0.55	0.64

0.2% CHX	*S. mutans*	*S. sanguinis*
EXPT. 1	18.2	18.9
	17.4	19
	18.1	18.4
EXPT. 2	18.1	17.7
	18.6	16.6
	16.8	18
EXPT. 3	17.1	19.1
	18.2	17
	17.2	17.7
Mean	17.74	18.04
S.D	0.62	0.89

2% DMSO/PBS	*S. mutans*	*S. sanguinis*
EXPT. 1	0	0
	0	0
	0	0
EXPT. 2	0	0
	0	0
	0	0
EXPT. 3	0	0
	0	0
	0	0
Mean	0	0
S.D		

4.2 SCIENTIFIC PAPER 2

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

This study compared the influence of standardized extracts of polyphenol-rich fruit berry extracts on virulence properties and microbial viability of *Streptococcus mutans* biofilms.

The paper was published on 12th March 2019 in the *European Journal of Oral Sciences*, the official publication of the International Association of Dental Research Scandinavian Division (IADR-NOF).

Eur J Oral Sci 2019 127(2): 122-129.

This chapter is comprised of the publication as it stands in print.

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* 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 *S. mutans* biofilms. None of the berry extracts were bactericidal for *S. mutans*. The results indicate that the cranberry extract was the most effective in disrupting *S. 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.

INTRODUCTION

Dental caries is a polymicrobial disease caused by dysbiosis in the resident microbiome that can lead to progressive demineralization of dental hard tissues [Marsh, 2018]. 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 [Simon-Soro and Mira, 2015]. Local environmental stresses, such as frequent exposure to sugar in the diet 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* accounting for only a small fraction of the bacterial community implicated in disease pathogenesis [Gross *et al.,* 2012; Simon-Soro and Mira, 2015; Tanner *et al.,* 2016]. However, *S. 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) [Paes Leme *et al.,* 2006]. Among the different exopolysaccharides (EPS), the insoluble glucans are considered to be the prime building blocks of cariogenic biofilms, influencing adherence and the physical and biochemical virulence properties of dental plaque [Bowen and Koo, 2011]. 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 [Hajishengallis *et al.,* 2017; Klein, 2013]. *S. 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 [Burne, 2018; Hajishengallis *et al.,* 2017]. Hence, *S. mutans* still remains a suitable indicator organism for exploring 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 recognition of the key health benefits of a symbiotic oral microbiome has resulted in virulence-targeted therapies being preferred over broad-spectrum antimicrobials [Marsh, 2015; Marsh *et al.,* 2015]. 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 [Philip *et al.,* 2018]. 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 [Jeon *et al.,* 2011].

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 [Seeram, 2008]. Regarding oral health, specific phytochemicals isolated from cranberry have demonstrated inhibitory effects on virulence factors of *S. mutans* virulence factors such as hydrophobicity-mediated adhesion, acid production, aciduricity, and glucan synthesis [Duarte *et al.,* 2006; Gregoire *et al.,* 2007; Koo *et al.,* 2010; Yamanaka *et al.,* 2004]. 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 [Ben Lagha *et al.,* 2015; Bodet *et al.,* 2008; Feghali *et al.,* 2012].

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 *S. mutans* biofilms. Therefore, the null hypothesis of the study was that none of the selected berry extracts had inhibitory effects against *S. 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

[Ainsworth and Gillespie, 2007]. 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 was used to grow the *S. mutans* biofilms [Exterkate *et al.,* 2010]. Hydroxyapatite (HA) discs (9.5mm diameter x 2mm thick; Himed, New York, NY, USA) were attached to nylon clamps of the custom-made stainless steel lid of the AAA model (Fig. 7), such that each disc fits into one well of a standard polystyrene 24-well microtiter plate (Costar 3526; Sigma-Aldrich, New York, NY, USA).

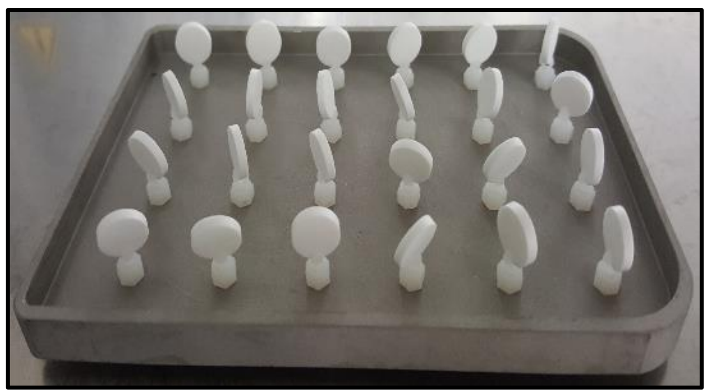

Figure 7. AAA biofilm model with the mounted HA discs

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 adult 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 (polyethersulphone low-protein binding filter with 0.22 µm-diameter pore size; 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 *S. mutans* (ATCC 25175) were grown for 24 h on the saliva-coated HA (sHA) discs as previously described [Exterkate *et al.,* 2010] with some modifications. Briefly, the inoculation medium was an overnight culture of *S. mutans* adjusted spectrophotometrically (Infinite 200 Pro; Tecan, Grödig, Austria) to a standard cell count (1 X 10^7 colony forming units (CFU)/mL) in brain heart infusion both (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 rpm to allow adhesion of bacteria. 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 concentration (500 µg/mL) to examine their influence on bacterial viability, biofilm biovolumes and structural organisation. 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 *S. 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.

1. *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 [Bandara *et al.,* 2016]. 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 into separate wells of a 96-well plate and absorbance at 492 nm measured with a microplate reader (Infinite 200 Pro, Tecan).

2. *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 [Exterkate *et al.,* 2014; Janus *et al.,* 2015].

3. *Biofilm Biovolumes and Structural Organisation: Confocal Microscopic Imaging*

A novel *in situ* labeling technique [Klein *et al.,* 2011] 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 Gtfs and becomes 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 µm 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 (volume of biomass/substratum surface area) of the bacterial and EPS biofilm components were quantified separately and structural architecture assessed qualitatively.

4. *Biofilm Bacterial Viability: Colony Forming Unit (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 [Bandara *et al.,* 2009]. The resultant suspension containing the detached biofilm cells was serially diluted (10^1-10^4) and a 50 µL 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% CO_2 incubator at 37°C for 48 h. Subsequently, the number of CFUs was counted with a digital colony counter and the CFU values were Log_{10} transformed prior to analysis.

Statistical Analysis

After normality of data distribution was checked using 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 performed for multiple comparisons of the treatment groups with 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 *S. mutans* biofilms are summarized in Table 7. 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 µg/mL (32% reduction, $P < 0.001$) and 250 µg/mL (29% reduction, $P < 0.001$), with the 125 µg/mL cranberry extract effecting a more modest decrease (14% reduction, $P = 0.005$) in metabolic activity of the *S. 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. The combination of the three berry extracts (Orophenol) showed significant reduction in metabolic activity at the 500 µg/mL (25% reduction, $P < 0.001$), 250 µg/mL (22% reduction, $P < 0.001$), and 125 µg/mL concentrations (18% reduction, $P = 0.001$).

Acid production

Significant reductions in lactic acid production were seen when the *S. mutans* biofilms were treated with the 500 µg/mL of cranberry (46% reduction, $P = 0.001$) and Orophenol extracts (40% reduction, $P < 0.001$) when compared to their respective control-treated biofilms (Table 7). 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, $P < 0.001$). 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.

Table 7. Berry extract effects on metabolic activity and acid production of *S. mutans* biofilms

Treatment group	Concentration (µg/mL)	Metabolic activity (% change) (OD_{492})	Lactic acid production (% change) (mM/L)
Cranberry	500	0.186 ± 0.027 (-32)*	6.4 ± 2.7 (-46)*
	250	0.194 ± 0.035 (-29)*	8.3 ± 2.9 (-29)*
	125	0.234 ± 0.029 (-14)*	9.1 ± 2.4 (-23)*
	62.5	0.261 ± 0.454 (-4)	10.4 ± 2.7 (-12)
	Control	0.272 ± 0.030	11.8 ± 2.7
Wild blueberry	500	0.303 ± 0.048 (-14)*	9.3 ± 1.2 (-24)*
	250	0.329 ± 0.050 (-6)	10.6 ± 1.1 (-13)
	125	0.340 ± 0.051 (-3)	11.6 ± 1.1 (-6)
	62.5	0.355 ± 0.041 (+1)	11.9 ± 1.8 (-3)
	Control	0.352 ± 0.044	12.3 ± 2.2
Strawberry	500	0.319 ± 0.038 (-1)	13.0 ± 1.6 (-4)
	250	0.332 ± 0.056 (+3)	13.4 ± 1.8 (-1)
	125	0.344 ± 0.042 (+7)	14.0 ± 1.2 (+3)
	62.5	0.339 ± 0.047 (+6)	15.0 ± 1.6 (+10)
	Control	0.321 ± 0.036	13.6 ± 1.8
Orophenol	500	0.271 ± 0.028 (-25)*	6.3 ± 0.6 (-40)*
	250	0.281 ± 0.041 (-22)*	7.6 ± 0.9 (-27)*
	125	0.296 ± 0.049 (-18)*	9.0 ± 0.8 (-14)*
	62.5	0.337 ± 0.064 (-7)	9.4 ± 0.6 (-10)
	Control	0.361 ± 0.057	10.5 ± 1.8

Metabolic activity represents mean XTT absorbance values ± SD from five independent triplicate experiments (n = 15) at optical density absorbance of 492 nm. Lactic acid production of biofilms in mM/L (mean ± S.D) is from three independent triplicate experiments (n = 9). Values given in round brackets after mean ± SD represent percentage change of metabolic activity/acid production compared to respective controls. * indicates significant difference ($P < 0.05$) compared to control-treated biofilms.

Biovolumes and structural organisation

Analysis of the confocal imaging data revealed that the cranberry and Orophenol extracts produced both quantitative (Table 8) and qualitative alterations (Fig. 8) in the *S. 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. 8).

Table 8. Quantitative analysis of *S. mutans* biofilm confocal images

	Biovolumes of biofilm components ($\mu m^3/\mu m^2$)	
Treatment group	**EPS**	**Bacteria**
Cranberry	1.3 ± 0.4*	1.9 ± 0.4*
Wild blueberry	1.9 ± 0.8	2.4 ± 0.4
Orophenol	1.4 ± 0.2*	2.0 ± 0.6*
Control	2.5 ± 1.5	3.0 ± 1.9

Biovolumes of bacterial cells and exopolysaccharides (EPS) are given as mean ± SD.
* Significant difference compared to control-treated biofilms.

Bacterial counts

The test agents did not significantly impact bacterial viability of the treated *S. mutans* biofilms as indicated by CFU counts (Fig. 9). 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, however none of them appeared to be bactericidal for *S. mutans* in the biofilms. In contrast, CHX severely affected the viability of *S. mutans* (> 3 Log decrease in CFU/mL).

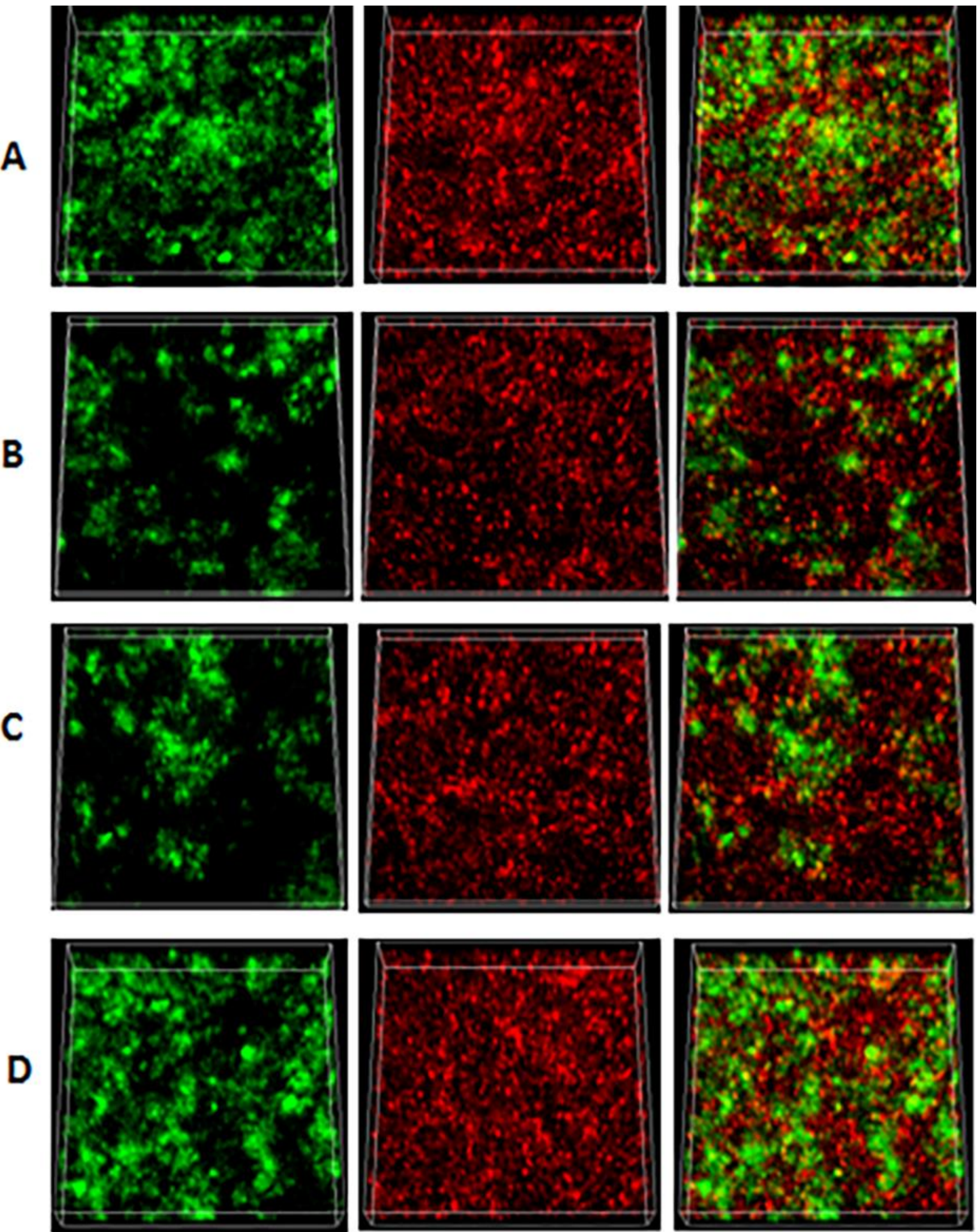

Figure 8. Representative 3D confocal image stacks of *S. mutans* biofilms

Structural organisation of 24 h *S. mutans* biofilms following treatment with (A) vehicle control, (B) cranberry, (C) Orophenol, and (D) wild blueberry. The structures depicted in green (SYTO 9; left column) are metabolically active bacterial cells, while the structures in red (Dextran, Alexa Fluor; middle column) represent the extracellular polymeric substances. Third column represents merged images of EPS and bacterial biofilm components. The biofilms treated with cranberry and Orophenol appear less compact than the control biofilms with areas of porosity.

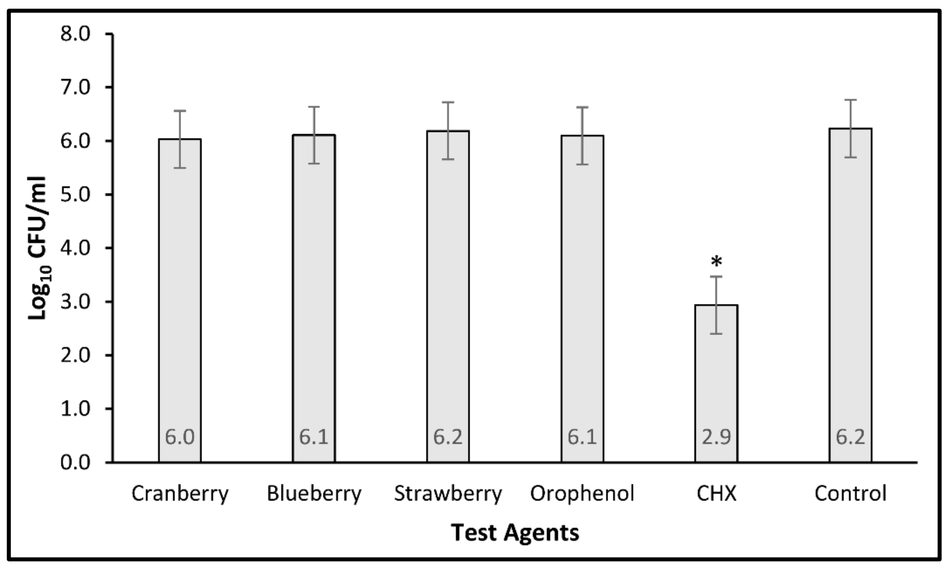

Figure 9. Average number of CFUs recovered from 24 h *S. mutans* biofilms

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 *S. mutans*.* indicates significant difference between CHX- and control-treated biofilms.

DISCUSSION

The results of this study indicate that certain dark-coloured berry extracts may have useful biofilm-inhibitory effects by reducing virulence of *S. mutans* without significantly affecting its 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 *S. mutans* virulence factors. Furthermore, because of the natural origin of these extracts, they may be more readily accepted for long-term control of biofilm-mediated diseases, such as 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 anti-caries agents [Maltz and Beighton, 2012]. 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 [Exterkate *et al.,* 2010; ten Cate, 2015]. 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 *S. 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 than in the blueberry and strawberry extracts, and, more importantly, to the different types of phytochemicals present in these extracts. For instance, the double interflavan A-linked proanthocyanidins (A-PACs) are found in high concentrations only in cranberry, with the PAC subunits in other berries and fruits usually linked via a single B-type bond [Cunningham *et al.,* 2004]. The A-PACs have been shown to have the most potent effects against *S. mutans* glycolytic enzymes and Gtfs when compared with other berry flavonoids [Duarte *et al.,* 2006; Gregoire *et al.,* 2007; Koo *et al.,* 2010]. 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 *S. 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_2 [Duarte *et al.,* 2006; Gregoire *et al.,* 2007]. 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 [Yoo *et al.,* 2011]. Quercetin is also known to be a non-competitive inhibitor of the proton-translocating F_1-F_0 ATPase activity that is critical to the survival of acidogenic bacteria in the low pH biofilm environment they create [Zheng and Ramirez, 2000]. 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 indicate that bioactive phytochemicals present in the cranberry and Orophenol extracts disrupted biofilm integrity and structural architecture. This

qualitative assessment was consistent with the quantitative data that showed biovolumes of both the EPS and bacterial components were significantly reduced (Table 8). Like acidogenicity, EPS are critical to *S. mutans* virulence as they provide structural integrity and bulk to dental biofilms, enhance bacterial adherence and co-aggregation, and can act as a substrate for sustained acidogenesis [Bowen and Koo, 2011]. The polyphenols responsible for the reduced EPS are believed to be the flavonols and A-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 [Duarte *et al.,* 2006]. For the PACs, those with degrees of polymerization between 4 and 12 were considered optimal for interaction and inhibition of Gtfs, as a higher degree of polymerization actually diminished inhibitory effects [Yoo *et al.,* 2011]. 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 the binding sites available 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 [Steinberg *et al.,* 2005; Yamanaka *et al.,* 2004]. 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 bacterial cells in contrast to the CHX control. 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 [Burne, 2018]. 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 [Koo *et al.,* 2010; Koo *et al.,* 2005].

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 bind irreversibly to proteins, forming protein-polyphenol complexes that can impair the activity of glycolytic and Gtf enzymes over a period of time [Duarte *et al.,* 2006; Gregoire *et al.,* 2007]. Furthermore, the binding of cranberry PACs to salivary proteins may confer improved substantivity beyond their brief period of exposure in the mouth [Koo *et al.,* 2006]. 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 been dependent on limiting exposure to dietary sugars and on 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 [Featherstone *et al.,* 2018]. 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 [Dang *et al.,* 2016]. 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 of time, and these could be particularly useful for caries control in high-risk populations [Burne, 2018; Marsh, 2018]. Cariogenic virulence-targeting natural products are one of the several ecological caries preventive approaches currently under investigation [Philip *et al.,* 2018]. 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 [Seeram, 2008], all actions that can potentially play a role in reducing virulence of *S. mutans*.

CONCLUSIONS

Prudency is required when interpreting the presented data to avoid over evaluation of the cariostatic effects. Our *in vitro* monospecies biofilms clearly do 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 *S. mutans* pathogenicity without necessarily killing the target bacteria, and is thus a promising candidate for development as an ecological caries preventive agent. It could act possibly 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 elucidate whether the biological effects shown can actually result in a favourable microbial ecological change in dental plaque.

RAW DATA

Study 2: XTT reduction assay on 24 h *S. mutans* biofilms

XTT absorbance values at 492 nm

CRANBERRY	500 µg/mL	250 µg/mL	125 µg/mL	62.5 µg/mL	Control
EXPT. 1	0.1845	0.1882	0.2195	0.2031	0.2907
	0.1688	0.2104	0.1835	0.2551	0.3289
	0.1927	0.1848	0.2389	0.2408	0.2836
EXPT. 2	0.1936	0.1834	0.2759	0.2077	0.2505
	0.1870	0.2006	0.2275	0.2405	0.2781
	0.2011	0.1938	0.2406	0.2304	0.2718
EXPT. 3	0.1727	0.2089	0.1976	0.2449	0.2276
	0.1576	0.2212	0.2002	0.3319	0.2482
	0.1886	0.1544	0.2042	0.2501	0.2260
EXPT. 4	0.2008	0.2798	0.2461	0.3211	0.2477
	0.1631	0.1999	0.2281	0.2495	0.2583
	0.2689	0.2295	0.2714	0.2338	0.3222
EXPT. 5	0.1713	0.1650	0.2358	0.2698	0.2692
	0.1614	0.1655	0.2627	0.3614	0.2984
	0.1768	0.1316	0.2738	0.2758	0.2837
Mean	0.186	0.194	0.234	0.261	0.272
S.D	0.027	0.035	0.029	0.045	0.030
% Redn.	32	29	14	4	0

OROPHENOL	500 µg/mL	250 µg/mL	125 µg/mL	62.5 µg/mL	Control
EXPT. 1	0.2638	0.2477	0.3017	0.3081	0.3418
	0.2674	0.2715	0.3145	0.3416	0.3981
	0.2729	0.2630	0.2941	0.2935	0.3938
EXPT. 2	0.2756	0.3269	0.3438	0.4865	0.3777
	0.2292	0.3064	0.2947	0.4002	0.4918
	0.2188	0.3285	0.2810	0.3081	0.3578
EXPT. 3	0.3061	0.2571	0.2529	0.3831	0.3626
	0.2706	0.2891	0.3777	0.3128	0.3501
	0.2978	0.3140	0.3297	0.3810	0.3782
EXPT. 4	0.2879	0.2993	0.2969	0.3562	0.3681
	0.3194	0.2656	0.2777	0.3478	0.3701
	0.2984	0.3487	0.3673	0.2235	0.2846
EXPT. 5	0.2617	0.2747	0.2049	0.2893	0.2445
	0.2428	0.2272	0.2113	0.3649	0.3971
	0.2554	0.1992	0.2927	0.2520	0.2927
Mean	0.271	0.281	0.296	0.337	0.361
S.D	0.028	0.041	0.049	0.064	0.057
% Redn.	25	22	18	7	0

BLUEBERRY	500 µg/mL	250 µg/mL	125 µg/mL	62.5 µg/mL	Control
EXPT. 1	0.3229	0.3231	0.3319	0.3011	0.3148
	0.3609	0.3189	0.2995	0.3711	0.3279
	0.3106	0.3635	0.3949	0.3174	0.3541
EXPT. 2	0.3259	0.3308	0.3347	0.2759	0.3092
	0.3251	0.2943	0.4046	0.3729	0.3191
	0.2682	0.2876	0.3689	0.3387	0.3989
EXPT. 3	0.2791	0.2478	0.3490	0.3306	0.3048
	0.2519	0.2510	0.2952	0.3476	0.3822
	0.3234	0.3882	0.2669	0.3195	0.3538
EXPT. 4	0.2958	0.4061	0.4106	0.3938	0.3276
	0.3216	0.4120	0.3559	0.3623	0.3882
	0.2882	0.3282	0.4151	0.3779	0.3386
EXPT. 5	0.2711	0.3402	0.2682	0.4041	0.3305
	0.3008	0.3447	0.3091	0.3958	0.4714
	0.2934	0.2950	0.2919	0.4206	0.3516
Mean	0.303	0.329	0.340	0.355	0.352
S.D	0.028	0.050	0.051	0.041	0.044
% Redn.	14	6	3	-1	0

STRAWBERRY	500 µg/mL	250 µg/mL	125 µg/mL	62.5 µg/mL	Control
PLATE 1	0.3094	0.3331	0.3755	0.3416	0.2857
	0.2645	0.3582	0.3002	0.3533	0.3338
	0.3766	0.3445	0.3967	0.3899	0.3241
PLATE 2	0.3174	0.2808	0.3347	0.2562	0.3295
	0.4012	0.3213	0.4030	0.3245	0.3112
	0.3212	0.3776	0.3600	0.3559	0.2998
PLATE 3	0.3291	0.3008	0.3265	0.3306	0.3158
	0.3011	0.2510	0.3849	0.4131	0.3265
	0.2923	0.2242	0.4007	0.3883	0.3748
PLATE 4	0.2788	0.3724	0.3106	0.3206	0.3244
	0.3131	0.4549	0.2875	0.3042	0.3882
	0.3215	0.3044	0.3005	0.2543	0.2266
PLATE 5	0.3119	0.3527	0.3682	0.3101	0.3197
	0.2966	0.3341	0.3241	0.3958	0.3204
	0.3453	0.3718	0.2919	0.3432	0.3332
Mean	0.319	0.332	0.344	0.339	0.321
S.D	0.038	0.056	0.042	0.047	0.036
% Redn.	1	-3	-7	-6	0

Study 2: Lactic acid production assay on 24 h *S. mutans* biofilms

Lactic acid concentrations in mM/L

CRANBERRY	500μg/ml	250μg/ml	125μg/ml	62.5μg/ml	control
Expt. 1	7.1	13.3	11.5	12.0	13.7
	6.6	11.0	10.1	14.2	15.8
	13.2	10.3	10.3	15.1	15.7
Expt. 2	5.2	9.6	7.4	8.3	8.4
	4.3	6.4	7.7	10.3	11.4
	4.7	4.3	7.6	8.3	9.4
Expt. 3	5.5	6.1	7.3	7.5	11.3
	5.6	7.0	7.1	9.1	10.3
	5.6	7.1	8.3	9.0	10.1
Mean	**6.4**	**8.3**	**8.6**	**10.4**	**11.8**
S.D	**2.7**	**2.9**	**1.6**	**2.7**	**2.7**
% Redn.	**46**	**29**	**27**	**12**	**0**

OROPHENOL	500μg/ml	250μg/ml	125μg/ml	62.5μg/ml	control
Expt. 1	6.8	8.4	9.7	10.0	11.2
	7.0	8.4	10.3	9.5	8.0
	6.4	9.0	9.0	10.0	14.0
Expt. 2	6.2	6.5	7.8	8.7	8.4
	6.6	6.3	9.4	9.1	11.4
	5.2	7.2	7.8	8.2	9.4
Expt. 3	6.0	6.8	8.7	9.5	11.3
	5.3	8.1	9.5	9.9	10.3
	6.8	7.6	9.0	9.7	10.1
Mean	**6.3**	**7.6**	**9.0**	**9.4**	**10.5**
S.D	**0.7**	**0.9**	**0.8**	**0.6**	**1.8**
% Redn.	**40**	**27**	**14**	**10**	**0**

BLUEBERRY	500μg/ml	250μg/ml	125μg/ml	62.5μg/ml	control
Expt. 1	7.6	9.9	10.1	10.2	11.2
	10.1	9.1	11.4	10.1	8.0
	8.4	9.7	9.6	11.0	14.0
Expt. 2	7.6	10.1	12.7	12.5	14.3
	9.4	12.4	11.3	9.8	10.1
	11.1	10.9	12.4	14.8	13.4
Expt. 3	9.9	10.6	12.0	13.3	14.2
	9.5	11.2	12.2	12.1	13.5
	10.0	12.0	12.6	13.8	11.9
Mean	**9.3**	**10.6**	**11.6**	**11.9**	**12.3**
S.D	**1.2**	**1.1**	**1.1**	**1.8**	**2.2**
% Redn.	**24**	**13**	**6**	**3**	**0**

STRAWBERRY	500μg/ml	250μg/ml	125μg/ml	62.5μg/ml	control
Expt. 1	12.6	14.8	15.9	16.3	13.7
	14.9	16.5	15.1	16.9	15.8
	16.1	14.9	14.5	17.0	15.7
Expt. 2	11.9	10.1	12.6	15.0	14.3
	12.2	12.4	13.9	12.5	10.1
	11.1	13.3	11.9	13.4	13.4
Expt. 3	12.4	12.7	14.3	15.7	14.1
	12.3	12.9	14.0	14.3	13.4
	13.8	13.3	13.5	13.9	11.8
Mean	**13.0**	**13.4**	**14.0**	**15.0**	**13.6**
S.D	**1.6**	**1.8**	**1.2**	**1.6**	**1.8**
% Redn.	**4**	**1**	**-3**	**-10**	**0**

Study 2: Colony Forming Unit (CFU) assay on 24 h *S. mutans* biofilms

CFU/mL

Column1	Cranberry	Blueberry	Strawberry	Orophenol	CHX	Control
Expt. 1	1.28E+06	2.04E+06	1.32E+06	1.16E+06	4.00E+04	2.26E+06
	7.60E+05	8.80E+05	2.42E+06	8.00E+05	2.00E+04	2.16E+06
	1.62E+06	1.18E+06	1.40E+06	1.28E+06	2.00E+04	1.24E+06
Expt. 2	1.18E+06	1.40E+06	8.80E+05	1.22E+06	2.00E+04	2.96E+06
	1.04E+06	7.00E+05	1.70E+06	1.10E+06	0.00E+00	2.02E+06
	1.04E+06	1.46E+06	1.90E+06	1.80E+06	4.00E+04	1.08E+06
Expt. 3	7.80E+05	1.58E+06	1.20E+06	1.98E+06	0.00E+00	2.12E+06
	1.20E+06	1.10E+06	2.04E+06	1.20E+06	0.00E+00	1.28E+06
	9.60E+05	1.82E+06	1.60E+06	1.04E+06	2.00E+04	1.18E+06
Mean	1.10E+06	1.35E+06	1.61E+06	1.29E+06	1.78E+04	1.81E+06
S.D	2.65E+05	4.33E+05	4.69E+05	3.72E+05	1.56E+04	6.45E+05

Study 2: CLSM quantitative assay on 24 h *S. mutans* biofilms

Biofilm biovolumes ($\mu m^3/\mu m^2$)

Cranberry	Bacteria	EPS
1	2.2	0.9
2	2.3	1.6
3	1.6	1.8
4	2.3	1.6
5	2.4	2.3
6	2.1	1.5
7	2.1	1.6
8	1.6	1.2
9	1.9	1.3
10	2.1	1.2
11	2.0	0.8
12	1.9	0.8
13	1.4	0.8
14	1.6	0.8
15	0.9	0.8
Mean	**1.9**	**1.3**
S.D	**0.4**	**0.4**

Orophenol	Bacteria	EPS
1	1.2	1.4
2	2.2	1.5
3	2.7	1.4
4	3.1	1.2
5	1.7	1.2
6	2.9	1.5
7	2.2	1.8
8	2.5	1.1
9	2.2	1.8
10	2.0	1.5
11	1.6	1.1
12	1.4	1.1
13	1.1	1.2
14	1.4	1.2
15	1.9	1.5
	2.0	**1.4**
	0.6	**0.2**

Blueberry	Bacteria	EPS
1	3.1	4.4
2	2.6	2.0
3	2.7	2.2
4	1.9	2.0
5	2.3	1.7
6	2.2	1.5
7	1.3	1.4
8	2.3	1.5
9	2.6	2.4
10	2.1	1.3
11	2.5	2.2
12	2.6	1.7
13	3.0	1.3
14	2.5	1.6
15	2.1	1.6
	2.4	**1.9**
	0.4	**0.8**

CONTROL	Bacteria	EPS
1	1.3	1.6
2	1.6	1.6
3	2.0	1.4
4	2.2	1.4
5	2.0	1.4
6	1.5	2.1
7	5.6	5.2
8	1.6	2.1
9	6.1	4.5
10	2.0	1.4
11	5.6	5.4
12	2.2	1.6
13	3.0	1.7
14	2.3	1.6
15	6.3	4.6
	3.0	**2.5**
	1.9	**1.5**

4.3 SCIENTIFIC PAPER 3

Polyphenol-rich Cranberry Extracts Modulate Virulence of *Streptococcus mutans-Candida albicans* Biofilms Implicated in the Pathogenesis of Early Childhood Caries

This study investigated the effects of polyphenol-rich cranberry extracts on cariogenic virulence properties of dual-species *Streptococcus mutans*-*Candida albicans* biofilms.

The paper was published on 16th February 2019 in *Pediatric Dentistry*, the official publication of the American Academy of Pediatric Dentistry (AAPD) and the College of Diplomates of the American Board of Pediatric Dentistry.

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The study images were featured on the cover of the journal issue.

This chapter is comprised of the publication as it stands in print.

ABSTRACT

Purpose: A synergistic alliance between *Streptococcus mutans* and *Candida albicans* has been implicated in contributing to the severity of early childhood caries. The purpose of this study was to investigate the effects of polyphenol-rich cranberry extracts on dual-species *S. mutans*-*C. albicans* biofilms.

Methods: *S. mutans*-*C. albicans* biofilms were grown on saliva-coated hydroxyapatite discs (s-HA) mounted on the high-throughput Amsterdam Active Attachment model. The s-HA discs were treated with the cranberry extracts/vehicle control for 5 min just before biofilm growth, and subsequently, for similar exposure times, after 12 h and 24 h of biofilm growth. The treated 24 h-old biofilms were then assessed for acidogenicity, metabolic activity, exopolysaccharide (EPS)/microbial biovolumes, structural organisation, and colony forming unit (CFU) counts.

Results: Treatment with 500-1000 µg/mL of the cranberry extracts produced highly significant reductions in acidogenicity and metabolic activity ($P < 0.001$) compared to the control-treated biofilms. A significant decrease in biovolumes of the EPS ($P = 0.003$) and microbial ($P = 0.007$) biofilm components was also seen. Qualitative assessment of confocal biofilm images revealed that the cranberry extract disrupted biofilm structural architecture. Finally, significantly fewer *S. mutans* ($P = 0.006$) and *C. albicans* ($P = 0.036$) CFUs were recovered from the cranberry-treated biofilms than from the control-treated biofilms.

Conclusions: Cranberry extracts inhibited cariogenic virulence properties of *S. mutans*-*C. albicans* dual-species biofilms in an *in vitro* model.

INTRODUCTION

Early childhood caries (ECC) is among the most common childhood diseases, disproportionately afflicting underprivileged and socially disadvantaged children, both in the U.S and globally [Dye *et al.,* 2015a; Kassebaum *et al.,* 2015]. The disease implications extend well beyond orofacial functions, as it can negatively affect the child's physical growth, education, socialization and overall well-being [Hajishengallis *et al.,* 2016]. Even after treatment, and despite a variety of preventive measures, there remains a high risk of future lesion recurrences, placing significant economic costs on patients and their families. While the existing paradigm of encouraging the early establishment of a "dental home" has met with limited success, there is clearly a need to consider additional measures to effectively tackle the ECC epidemic [Douglass and Clark, 2015].

Streptococcus mutans is considered the keystone bacterial pathogen in ECC, often exceeding 30% of the cultivable plaque biofilm flora in severe forms of the disease [Falsetta *et al.,* 2014]. *S. mutans*-derived glucosyltransferase (Gtf) exoenzymes assemble the exopolysaccharide (EPS) glucan-rich matrix scaffold, which confers structural integrity and bulk to plaque biofilms, enhances microbial adhesion and co-aggregation and provides a readily metabolizable carbohydrate source for sustained acid production [Bowen *et al.,* 2018]. Other acidogenic and aciduric bacteria such as lactobacilli, bifidobacteria, and *Scardovia* species also contribute to lesion development as the plaque biofilm matures, but these bacterial species lack the specific Gtfs that synthesize insoluble glucans [Hajishengallis *et al.,* 2017].

The fungus *Candida albicans* has also been frequently isolated from the dental plaque of children with ECC [de Carvalho *et al.,* 2006; Marchant *et al.,* 2001; Raja *et al.,* 2010; Yang *et al.,* 2012]. A recent systematic review and meta-analysis indicated that children colonized with oral *C. albicans* have >5 times higher odds of having ECC compared to those without *C. albicans* [Xiao *et al.,* 2018]. Mothers of children with severe ECC were also found to be infected with similar strains of *C. albicans,* suggesting that the parent is the likely primary source for *C. albicans* acquisition in these children [Xiao *et al.,* 2016]. *C. albicans* was initially regarded to neither bind well with *S. mutans* nor to effectively colonize teeth on its own. Rather, this opportunistic fungus was known to interact with commensal viridans streptococci and to form biofilms on mucosal/acrylic surfaces [Jenkinson *et al.,* 1990]. However, recent studies have demonstrated an extraordinary synergistic alliance between *S. mutans* and *C. albicans* mediated primarily through the influence of the bacterial Gtf exoenzymes [Falsetta *et al.,* 2014; Gregoire *et al.,* 2011; Kim *et al.,* 2017]. The symbiotic

relationship between these microorganisms is expressed in a number of ways. The ability of *S. mutans* to rapidly breakdown sucrose allows the fungus to utilize sucrose break-down products, with enhanced fructose metabolism known to promote *C. albicans* hyphal morphogenesis [Kim *et al.,* 2017]. Equally, the large surface area of *C. albicans* provides an increased number of binding sites for *S. mutans* to adhere to within dual-species biofilms. The *S. mutans*-derived Gtfs (particularly GtfB) also bind avidly to *C. albicans* cell surfaces, converting them into *de facto* glucan producers in the presence of sucrose [Gregoire *et al.,* 2011]. This significantly enhances virulence-enhancing insoluble glucans in the biofilm and assembles an EPS-rich matrix scaffold that is critical for further *S. mutans* accumulation [Falsetta *et al.,* 2014]. A contrary finding to this was a study that showed *S. mutans–C. albicans* biofilms suppressed EPS production by *S. mutans* [Sztajer *et al.,* 2014]. However, this was likely to be due to sucrose depletion in the co-culture rather than any inhibitory effect of *C. albicans* on *S. mutans*. Overall, the majority of the studies indicate that the mutualistic bacterial-fungal relationship amplifies biofilm virulence and led to rampant caries under *in vivo* experimental conditions conducive for ECC [Falsetta *et al.,* 2014; Gregoire *et al.,* 2011; Kim *et al.,* 2017].

The available evidence has prompted the suggestion of incorporating anti-*Candida* therapy into preventive programs that are designed to reduce the severity of ECC [Koo and Bowen, 2014]. While chlorhexidine (CHX) is bactericidal for *S. mutans* and fungicidal for *C. albicans*, susceptible oral niches are often repopulated with the same disease-associated microorganisms once the chemotherapeutic intervention with CHX stops, resulting in lesion recurrence and therapeutic failure. To avoid this, ecological caries preventive approaches that lower plaque virulence are being increasingly preferred over broad-spectrum antimicrobials for long-term caries control [Philip *et al.,* 2018]. In this regard, natural products that can disrupt cariogenic virulence properties and potentially reverse the microbiome dysbiosis are an attractive alternative to synthetic biocides such as CHX. Cariostatic natural products will also find better acceptance among the general public as they are less likely to be associated with adverse effects or emergence of resistant strains often seen with synthetic biocides and antifungals.

We have recently demonstrated that a highly-purified polyphenol-rich cranberry extract was able to significantly disrupt acidogenicity, metabolic activity, EPS/microbial biovolumes and structural organisation of *S. mutans* biofilms, without affecting bacterial viability [Philip *et al.,* 2019a]. Specific cranberry flavonoids, especially the A-linked proanthocyanidins (A-PACs), have been shown to be potent inhibitors of bacterial glycolysis and GtfB-mediated glucan synthesis in *S.*

mutans biofilms and were shown to prevent dental caries development *in vivo* [Duarte *et al.,* 2006; Gregoire *et al.,* 2007; Koo *et al.,* 2010; Koo *et al.,* 2006]. However, there have been no studies that have examined the effects of natural cranberry extracts on virulence properties of dual-species *S. mutans-C. albicans* biofilms. Hence, this study aimed to investigate the influence of polyphenol-rich cranberry extracts on the bacterial-fungal biofilms, given the critical role these microbes play in ECC pathogenesis.

MATERIALS AND METHODS

Cranberry Extracts

High-quality organic extracts of cranberry were sourced from Diana Food (Champlain, QC, Canada), and used to treat *S. mutans-C. albicans* biofilms. As per the manufacturer's data sheet, 80% of the polyphenols in these extracts were A-PACs, with modest amounts of different flavonols (e.g. quercetin, myricetin), anthocyanins, and phenolic acids making up the remainder. The cranberry extract was fully soluble in water, and was serially diluted in 0.15 M phosphate buffered saline, pH 7.2 (PBS; Gibco, Thermo Fisher, Paisley, UK) in concentrations ranging from 1000 to 125 µg/mL.

Biofilm Inoculum

Overnight cultures of *S. mutans* (ATCC 25175) and *C. albicans* (SC5314) were grown separately in brain heart infusion (BHI; Merck, Darmstadt, Germany). Cells were harvested, resuspended in BHI containing 0.2% (w/v) sucrose, and microbial counts adjusted to approximately 2 X 10^6 colony forming units (CFU)/mL for *S. mutans*, and 2 X 10^4 CFU/mL for *C. albicans*. This proportion of the microorganisms is similar to that found in saliva samples of children with ECC [de Carvalho *et al.,* 2006; Kim *et al.,* 2017].

Biofilm Preparation and Treatments

In order to mimic the biofilm formation on smooth surfaces of teeth, the *S. mutans-C. albicans* dual-species biofilms were grown on hydroxyapatite (HA) discs (9.5mm diameter x 2mm thick; Himed, New York, NY, USA). The HA discs were mounted on the high-throughput Amsterdam Active Attachment (AAA) model [Exterkate *et al.,* 2010]. To partially replicate the salivary pellicle, the HA discs were initially coated with filter-sterilized clarified whole human saliva for 1 h at 37°C as described previously.[Philip *et al.,* 2019a] The saliva coated HA discs (s-HA) were then moved to a flat-bottomed 24-well microtiter plate (Costar 3526, Sigma-Aldrich, New York, NY, USA) containing

2 mL/well of the different cranberry extract concentrations and treated for 5 min. The lid with the pre-treated s-HA discs was then gently shaken to remove any excess of treatment/control solutions and transferred to a 24-well plate containing 2 mL/well of the microbial inoculum and incubated at 37°C in an orbital shaker at 80 rpm. Subsequent treatments took place after 12 h and 24 h of biofilm growth, with the growth medium (0.2% sucrose-BHI) refreshed after the 12 h treatment period. All treatment exposures were for 5 min, with the biofilm-laden discs dip-rinsed 5 times in sterile PBS just before and after the 12 and 24 h treatments to remove non-adherent cells and any residual treatment solutions.

Biofilm Analyses

After the final treatment, the influence of the cranberry extracts on the 24 h-old *S. mutans-C. albicans* dual-species biofilms were assessed using different assays to estimate acidogenicity, metabolic activity, EPS/microbial biovolumes, structural organisation and CFU counts. All the biofilm assays were performed in triplicate and repeated in 3 independent experiments.

1. *Lactic Acid Concentrations: Acid Production Assay*

The treated 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% (w/v) sucrose and incubated anaerobically at 37°C. Acid formation was allowed to continue for 3 h under anaerobic conditions. The optical density (O.D) values of the BPW solutions were then measured at 340 nm and their lactate concentrations determined from lactic acid standard curves using an enzymatic (lactate dehydrogenase) method [Exterkate *et al.,* 2010].

2. *Biofilm Metabolic Activity: XTT Reduction Assay*

A standard XTT reduction assay was performed as previously described.[Philip *et al.,* 2018a] Briefly, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) salt solution (1 mg/mL in PBS) was filter-sterilized and stored at -80°C until required. Immediately before each assay, the reaction solution was prepared by mixing PBS, XTT solution, and freshly prepared 0.4mM menadione in a 79:20:1 volume ratio. The treated biofilm-coated HA discs were transferred to a 24-well plate containing the reaction solution and incubated in the dark for 3 h at 37°C. Thereafter, 100 µl of the cell-free supernatant from each well was pipetted to a 96-well microtiter plate and absorbance measured at a wavelength of 492 nm.

3. *Biofilm Biovolumes and Structural Organisation: Confocal Microscopy*

The biofilm treated with the 500 µg/mL cranberry extract was selected for confocal microscopic imaging to assess its EPS and microbial components. A novel *in situ* labelling technique that allows simultaneous visualization and quantification of EPS and microbial cells within intact biofilms was used [Klein *et al.,* 2011]. Briefly, 1 µM Alexa Fluor 647-dextran conjugate (647/668 nm; Thermo Fisher, Scoresby, Australia) was added to the sucrose-BHI growth medium to allow its incorporation into glucans during biofilm development. The microbial cells were labeled at the end of the biofilm treatment using 1 µM SYTO 9 (480/500 nm; Thermo Fisher). Imaging of the intact biofilm was done with a spectral spinning disc confocal microscope (Nikon, Tokyo, Japan). The biofilm was scanned at 5 randomly selected positions, with the *z*-series (512 by 512-pixel resolution) created by optical sectioning at 2.5 µm intervals. Three independent biofilm experiments were performed, generating a 15 image stacks each for the cranberry and control treated biofilms. The image-processing software IMARIS (Bitplane, Concord, MA, USA) was used to construct 3D renderings of the biofilm structural components (EPS and microorganisms). Biovolumes (volume of biomass/substratum surface area) of the EPS and microbial biofilm components were quantified separately. Structural architecture of the biofilms was assessed qualitatively based on the compactness of the biofilm components.

4. *Microbial Counts*

After treatment with 500 µg/mL cranberry extract, the dual-species biofilms were dispersed in 1 mL PBS by uniform vortexing at maximum speed for 1 min. The resulting microbial suspension was then serially diluted in PBS and a 50 µL aliquot of it plated separately on BHI agar plates supplemented with antifungal caspofungin (0.5 µg/mL) and sabouraud dextrose agar plates supplemented with antibacterial tetracycline (1 µg/mL) using a spiral plater. The plates were incubated in a 5% CO_2 incubator at 37°C for 48 h. The number of CFUs were totaled individually for *S. mutans* and *C. albicans* with a digital colony counter and the CFU values Log_{10} transformed prior to analysis.

Statistical Analysis

The normality of data distribution was checked with the Shapiro-Wilk test. For the non-parametric acid production assay data, the Kruskal-Wallis *H*-test and the Dunn-Bonferroni post hoc test was used for the multiple comparisons of the different cranberry concentrations to the control group. For the parametric XTT assay data, the one-way ANOVA and Dunnett's post hoc test was

applied for the multiple comparisons. The independent sample t-test was used for the parametric microbial biovolume confocal data, while the Mann-Whitney *U*-test was applied to the non-parametric EPS biovolume confocal data and CFU assay data. 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

Biofilm acidogenicity and metabolic activity

Effects of cranberry extract on lactic acid production and metabolic activity of the *S. mutans-C. albicans* biofilms are shown in Table 9. The results of the acid production and XTT metabolic assays were consistent with each other for the different cranberry concentrations tested. Biofilm treatment with 500 - 1000 µg/mL of the cranberry extracts produced highly significant reductions in acidogenicity and metabolic activity ($P < 0.0001$) compared to the control-treated biofilms, while no significant effects were seen after treatment with the 125-250 µg/mL cranberry extract concentrations ($P > 0.05$). The increased reduction of acidogenicity and metabolic activity with higher concentrations of the cranberry extract suggests a dose-response relationship.

Table 9. Effects of polyphenol-rich cranberry extracts on acid production and metabolic activity of *S. mutans-C. albicans* dual-species biofilms

Cranberry extract concentration (µg/mL)	**Lactic acid production** (% change) (mM/L)	**Metabolic activity** (% change) (OD_{492})
1000	6.2 ± 1.5 (-45)* $P < 0.0001$	0.277 ± 0.031 (-29)* $P < 0.0001$
500	8.1 ± 1.8 (-28)* $P < 0.0001$	0.314 ± 0.042 (-20)* $P < 0.0001$
250	10.5 ± 1.5 (-7) $P = 1.000$	0.362 ± 0.034 (-7) $P = 0.093$
125	11.1 ± 1.5 (-2) $P = 1.000$	0.380 ± 0.020 (-3) $P = 0.528$
Control	11.3 ± 2.4	0.390 ± 0.025

Lactic acid production of biofilms in mM/L (mean ± S.D) is from three independent triplicate experiments (n = 9). Metabolic activity represents mean XTT absorbance values ± S.D from three independent triplicate experiments (n = 9) at 492 nm optical density (OD) absorbance. Values given in round brackets represent percentage change of acid production/metabolic activity compared to respective controls. * indicates significant difference compared to control-treated biofilms. The Kruskal-Wallis H-test (with Dunn-Bonferroni post hoc) was applied for the lactic acid production data, while the one-way ANOVA (with Dunnett's post hoc) was applied for the XTT metabolic activity data.

Biofilm biovolumes and structural organisation

Confocal microscopic imaging revealed that topical treatment with the 500 µg/mL cranberry extract produced both qualitative and quantitative alterations within the dual-species biofilms. The

fluorescence images showed disruption of the architecture of the biofilms, with the cranberry-treated biofilms appearing less compact than the control biofilms (Fig. 10). There appeared to be less EPS (depicted in red) surrounding the bacterial-fungal microcolonies (depicted in green) in the cranberry-treated biofilms compared to the control-treated biofilms. Quantitative data confirmed significant reductions in the biovolumes of both the EPS (P = 0.003) and microbial (P = 0.007) components of the cranberry-treated biofilms (Table 10).

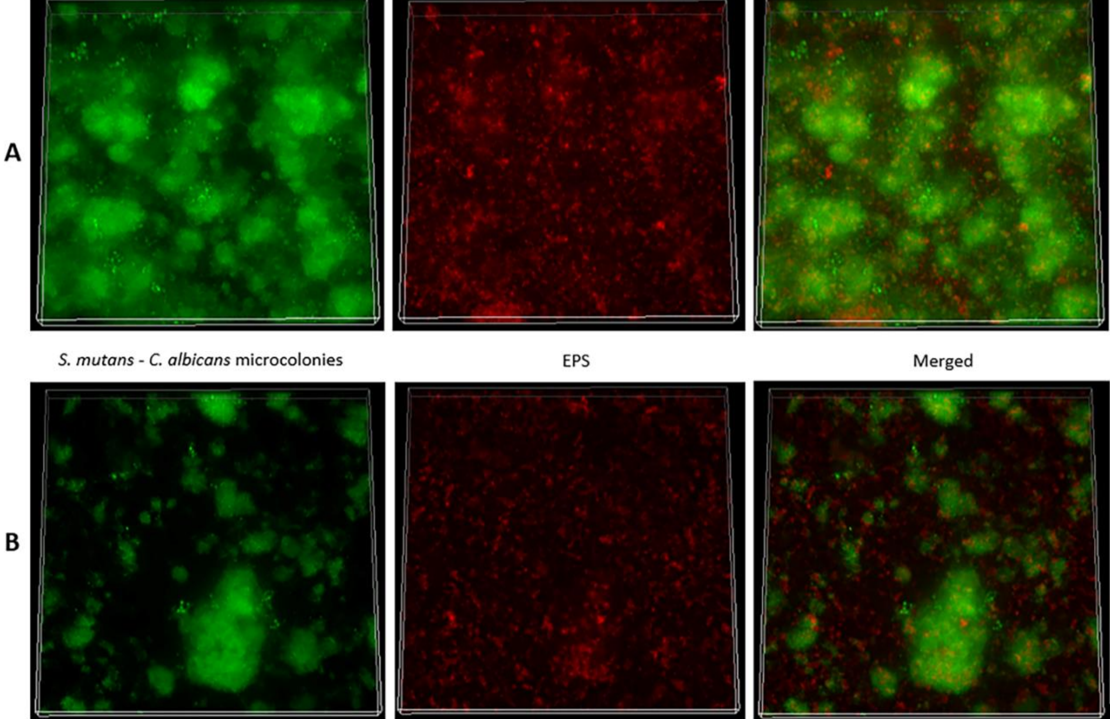


Figure 10. Representative 3D confocal image stacks of *S. mutans-C. albicans* biofilms

Structural organisation of dual-species *S. mutans-C. albicans* dual-species biofilms following topical treatment with A) vehicle control and B) 500 µg/mL of the cranberry extract. Microbial cells are depicted in green (SYTO 9) and EPS in red (Dextran, Alexa Fluor), with 20x magnification.

Table 10. Quantitative analysis of dual-species *S. mutans-C. albicans* biofilm confocal images

Treatment Group	**Biovolumes of Biofilm Components** ($\mu m^3/\mu m^2$)	
	EPS	**Microbial Cells**
Cranberry	2.3 ± 1.0* P = 0.003	4.8 ± 0.8* P = 0.007
Control	3.3 ± 1.4	6.3 ± 1.7

Biovolumes (mean ± S.D) of both EPS and total microbial cells (*S. mutans* and *C. albicans*) was quantified using IMARIS. The data are from 3 independent biofilm experiments (n = 15). * indicates statistically significant reduction of EPS/microbial biovolumes compared to control. The Mann-Whitney *U*-test (for EPS biovolumes) and the independent sample t-test (for microbial biovolumes) were used to test for significance.

Microbial counts

Pre-treatment of the sHA discs followed by treatment of the growing biofilms with the 500 µg/mL cranberry extract significantly reduced *S. mutans* (P = 0.006) and *C. albicans* (P = 0.036) populations in the dual-species biofilms (Fig. 11). The mean *S. mutans* cell numbers recovered from cranberry-treated biofilms were 1.1 x 10^8 CFU/mL compared to 1.6 x 10^8 CFU/mL for the control-treated biofilms. The *C. albicans* counts were 4 x 10^6 CFU/mL and 5.1 x 10^6 CFU/mL for cranberry and control-treated biofilms respectively.

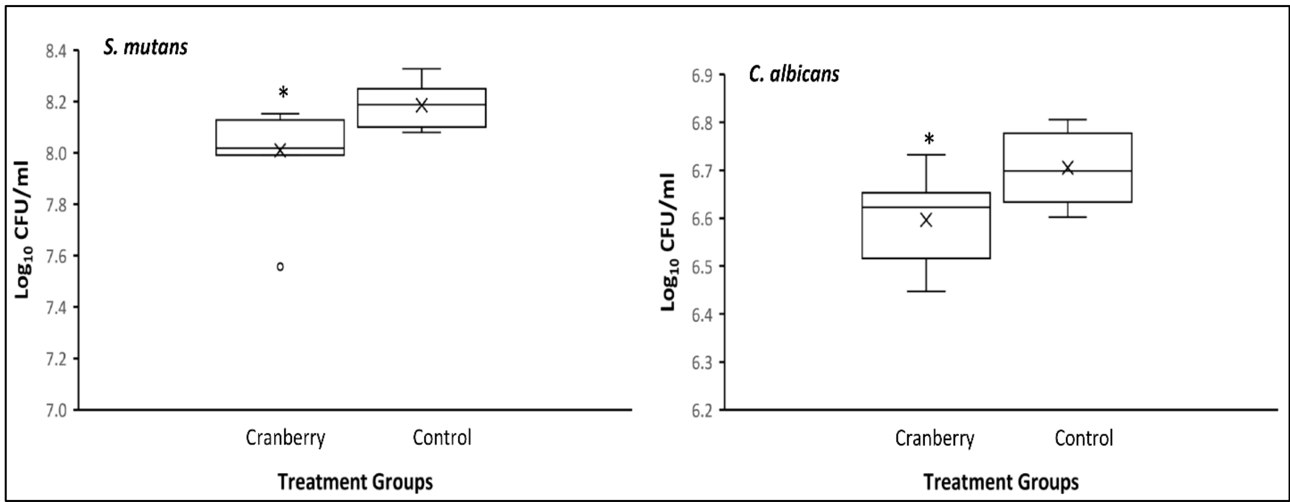


Figure 11. Box-plots of microbial colonies recovered from *S. mutans-C. albicans* biofilms

* indicates significantly different CFU values ($P < 0.05$) for the cranberry-treated biofilm compared to control-treated biofilms using the Mann-Whitney *U*-test (n = 9).

DISCUSSION

The results of the study demonstrate that polyphenols present in the cranberry extract can inhibit virulence properties and disrupt the structure of *S. mutans-C. albicans* dual-species biofilms. The polyphenols present in the cranberry extract are not only potent inhibitors of bacterial glycolytic and Gtf exoenzymes but can also affect microbial adhesion to pellicle-coated tooth surfaces, mechanisms that may impede the synergistic relationship between the bacterial keystone pathogen and the opportunistic fungus often seen at sites of ECC infection. An approach that involves targeting biofilm virulence factors with polyphenol-rich cranberry extracts could facilitate action of remineralizing agents and result in better control over aggressive cases of ECC. Such measures are in keeping with recent reviews that have highlighted the need for multi-targeted therapeutic strategies to disrupt the "pathogenic niche" by targeting the complex biofilm microenvironment [Koo *et al.,* 2017; Liu *et al.,* 2018].

In the present study, the reduction observed in the metabolism of the dual-species biofilm metabolism corresponded with a decrease in the biofilm acidogenicity. Both *S. mutans* and *C. albicans* have the ability to produce and tolerate acids, allowing them to outcompete non-cariogenic commensal species under low pH conditions [Metwalli *et al.,* 2013]. A low pH environment at the biofilm-tooth interface promotes enamel demineralization, leading to the clinical onset of dental caries. We had earlier shown that treating *S. mutans* biofilms with cranberry extracts at concentrations between 125-500 µg/mL resulted in significant reductions in metabolic activity and lactic acid production [Philip *et al.,* 2019a]. The same cranberry extract showed inhibitory activity against dual-species *S. mutans-C. albicans* biofilms, reducing metabolism and acidogenicity, although higher concentrations (500-1000 µg/mL) were required for significant effects. The greater biomass of EPS and microorganisms in dual-species *S. mutans-C. albicans* biofilms compared to monospecies *S. mutans* biofilms possibly explains why higher concentrations of the cranberry extract were needed to exert inhibitory effects. The anti-acidogenic mechanism of cranberry is possibly due to a low molecular-weight PAC dimer (procyanidin A_2) that has been shown to directly inhibit intracellular glycolytic enzymes, while larger PAC oligomers may have deleterious effects on membrane components of the glycolytic pathway [Gregoire *et al.,* 2007; Yoo *et al.,* 2011].

A 3D reconstruction of the confocal images revealed a partial impairment of the structural architecture of the cranberry-treated biofilms compared to the control-treated biofilms. This disruption to the biofilm structural organisation could render it less capable of maintaining acidic pH conditions at the attachment surface. An overall decrease in both EPS and microbial biovolumes was also observed in the confocal quantitative data, further corroborating the disruptive effect cranberry extracts had on biofilm structure. The biofilm disruptive effects of the cranberry extracts are believed to be related to specific flavonoids present in them. Cranberry phenols (especially the A-PACs) have pronounced inhibitory activity against the *S. mutans* Gtf exoenzymes responsible for glucan synthesis and EPS matrix assembly [Duarte *et al.,* 2006; Feng *et al.,* 2013]. The precise mechanisms by which A-PACs inhibit Gtf activity are not clear but are likely to be related to its unique rigidity conferring A-type linkages, as PACs from non-cranberry fruits that lack the double interflavan A-type linkages do not affect Gtfs [Koo *et al.,* 2010]. Inhibition of bacterial α-glucan synthesis not only affects the EPS biofilm matrix, but can also disrupt the bacterial-fungal interaction and accumulation in *S. mutans-C. albicans* biofilms. While *C. albicans* can also independently contribute to the biofilm matrix through the production of extracellular β-glucans, it is not known whether cranberry flavonoids affect fungal β-glucan production.

In the current study, there was a significant decrease in the numbers of bacterial and fungal cells recovered from the cranberry-treated biofilms. Previous studies have established that cranberry extracts and their purified compounds had no effect on growth and viability of either *S. mutans* [Duarte *et al.,* 2006; Feng *et al.,* 2013; Gregoire *et al.,* 2007; Koo *et al.,* 2010; Koo *et al.,* 2006; Philip *et al.,* 2019a] or *C. albicans* [Feldman *et al.,* 2012; Girardot *et al.,* 2014]. However, cranberry phenols are known to exert anti-adhesion effects against both *S. mutans* [Feng *et al.,* 2013; Weiss *et al.,* 2004; Yamanaka *et al.,* 2004] and *C. albicans* [Feldman *et al.,* 2012; Girardot *et al.,* 2014] which could account for the lower microbial populations observed in this study. The cranberry extracts may have exerted anti-adhesion effects in multiple ways. The exposure of the sHA discs to the cranberry extracts prior to biofilm growth could have impeded microbial adhesion at the early stages of biofilm growth, as cranberry polyphenols are known to irreversibly bind to proline-rich salivary proteins affecting pellicle-mediated adhesion [Bennick, 2002; Girardot *et al.,* 2014]. Moreover, cranberry A-PACs have been shown to markedly modify cell surfaces of both *S. mutans* [Weiss *et al.,* 2004; Yamanaka *et al.,* 2004] and *C. albicans* [Feldman *et al.,* 2012] from being highly hydrophobic to hydrophilic, thereby affecting their initial adhesion to pellicle-coated surfaces. However, the predominant anti-adhesion effects of cranberry phenols are likely to be mediated through their effective inhibition of the bacterial GtfB and GtfC exoenzymes. The Gtf-derived α1,6-linked glucans not only provide sites to which *S. mutans* cells can adhere to avidly but are also largely responsible for the physical interaction between *S. mutans* and *C. albicans* in dual-species biofilms [Falsetta *et al.,* 2014; Gregoire *et al.,* 2011]. It is reasonable to infer that anti-adhesion effects of the cranberry extract, rather than any biocidal activity, accounts for the decrease in the microcolonies recovered from the biofilms in the CFU assay, and the reduction in microbial biovolumes observed in the confocal imaging data.

Bioactive cranberry polyphenols have shown therapeutic potential against urinary tract infections, gastric ulcers, cardiovascular disease, and cancer cell proliferation [Pappas and Schaich, 2009]. They are now increasingly being advocated in preventing or managing dental caries and periodontal disease [Bonifait and Grenier, 2010; Feghali *et al.,* 2012; Koo *et al.,* 2010]. Among the different cranberry flavonoids, the unique A-PACs have shown the most potent effect against bacterial glycolytic, Gtf, and F_1F_0-ATPase enzymes that are critical to cariogenic virulence [Duarte *et al.,* 2006; Gregoire *et al.,* 2007]. The fact that the cranberry extract used in this study were rich in A-PACs (making up more than 80% of the total phenols) possibly explains the demonstrated inhibitory effects against virulence properties of *S. mutans*-*C. albicans* biofilms. Other advantages

of these sugar-free water soluble cranberry extracts are that they have standardized phytochemical concentrations, thereby overcoming the problem of compositional variability often associated with natural products.

Newer approaches are needed to tackle ECC especially with recent data from the National Health and Nutrition Evaluation Survey (NHANES) indicating that the disease remains the most common infectious disease among preschool children in the United States [Dye *et al.,* 2015]. While fluoride remains the gold standard among anti-caries agents, its cariostatic effects are predominantly due to its physicochemical influences on the de-/remineralization equilibrium [ten Cate and Cummins, 2013]. Recent studies have demonstrated that even high fluoride concentrations could not sustain antimicrobial activity against biofilm metabolism [Dang *et al.,* 2016; Souza *et al.,* 2018]. Moreover, considering that children at risk to ECC are often not able to use high concentration fluoride products (due to the risk of dental fluorosis), additional caries preventive measures are warranted in this high-risk population group. The present study indicates that polyphenol-rich cranberry extracts have the potential to disrupt the synergistic *S. mutans-C. albicans* alliance and can thus potentially play a role in influencing ECC pathogenesis. These commercially available natural extracts can possibly be incorporated into daily use oral care products to supplement the remineralizing effects of fluoride. However, our *in vitro* biofilm model clearly does not reflect the complex polymicrobial, ecological, and environmental influences encountered in the oral cavity. Well-designed clinical trials are warranted to demonstrate whether the beneficial properties shown under our assay conditions can translate into efficacious therapeutic approaches to control ECC.

CONCLUSIONS

The following conclusions can be made from this *in vitro* study:

1. Polyphenol-rich cranberry extracts significantly reduced the metabolic activity and lactic acid production of *S. mutans-C. albicans* dual-species biofilms.
2. Topical treatment of *S. mutans-C. albicans* biofilms with the cranberry extracts disrupted their structural architecture and lowered EPS/microbial biovolumes.
3. There was a significant reduction in the numbers of *S. mutans* and *C. albicans* microbial colonies recovered from the cranberry-treated *S. mutans-C. albicans* dual-species biofilms.

RAW DATA

Study 3: XTT reduction assay on 24 h *S. mutans-C. albicans* biofilms

XTT absorbance values at 492 nm

CRANBERRY	1mg/mL	500 µg/mL	250 µg/mL	125 µg/mL	Control
EXPT. 1	0.2387	0.3108	0.3959	0.3497	0.3824
	0.2935	0.3065	0.3677	0.3793	0.3979
	0.2600	0.3017	0.3893	0.3997	0.4278
EXPT. 2	0.2421	0.3681	0.2881	0.4012	0.3458
	0.2465	0.2415	0.3599	0.3872	0.3631
	0.3048	0.2706	0.3709	0.3919	0.3901
EXPT. 3	0.3170	0.3178	0.3704	0.3820	0.4188
	0.2815	0.3410	0.3865	0.3462	0.3872
	0.3123	0.3679	0.3280	0.3866	0.3990
Mean	**0.277**	**0.314**	**0.362**	**0.380**	**0.390**
S.D	**0.031**	**0.042**	**0.034**	**0.020**	**0.025**
% Redn.	**29**	**20**	**7**	**3**	**0**

Study 3: Lactic acid production assay on 24 h *S. mutans-C. albicans* biofilms

Lactic acid concentrations in mM/L

CRANBERRY	1mg/mL	500µg/mL	250µg/mL	125µg/mL	control
Expt. 1	3.7	10.0	10.5	11.1	14.6
	3.9	10.2	10.8	10.5	14.9
	3.8	10.3	10.9	13.3	15.2
	5.8	6.9	9.7	9.5	9.9
	6.2	7.0	9.8	13.4	9.6
	6.3	7.2	9.7	13.1	9.7
	7.4	5.1	11.8	9.6	10.6
	7.6	5.2	12.0	11.4	10.4
	7.7	5.4	12.0	10.3	10.3
Expt. 2	7.4	9.6	7.6	12.2	14.8
	7.7	9.7	9.4	10.6	15.6
	7.8	9.8	11.9	13.3	15.6
	5.7	6.2	12.1	12.3	7.9
	6.7	6.1	7.2	8.6	7.9
	6.7	6.2	8.6	11.2	8.0
	4.2	7.3	10.2	10.1	10.0
	4.2	7.6	12.7	8.5	10.3
	4.1	7.8	9.3	11.1	10.4
Expt. 3	4.8	9.0	11.1	12.5	11.2
	5.0	9.3	11.2	12.0	11.5
	5.0	9.6	13.4	10.9	11.8
	7.8	10.1	9.5	13.0	9.3
	7.9	10.5	9.7	9.4	9.3
	7.6	10.4	10.1	11.6	9.2
	7.4	7.9	10.9	11.2	12.6
	7.3	7.8	9.4	9.3	12.7
	7.4	7.9	12.5	9.5	12.8
Mean	**6.2**	**8.1**	**10.5**	**11.1**	**11.3**
S.D	**1.5**	**1.8**	**1.5**	**1.5**	**2.4**
% Redn.	**45**	**28**	**7**	**2**	**0**

Study 3: Colony Forming Unit (CFU) assay on 24 h *S. mutans-C. albicans* biofilms

CFU/mL

S. mutans	Cranberry	Control
Expt. 1	1.32E+08	1.64E+08
	1.42E+08	1.86E+08
	1.32E+08	1.28E+08
Expt. 2	9.80E+07	1.42E+08
	3.60E+07	1.70E+08
	9.80E+07	2.12E+08
Expt. 3	1.36E+08	1.24E+08
	1.00E+08	1.20E+08
	1.04E+08	1.54E+08
Mean	1.09E+08	1.56E+08
S.D	3.27E+07	3.08E+07

C. albicans	Cranberry	Control
Expt. 1	4.00E+06	5.00E+06
	2.80E+06	4.40E+06
	5.40E+06	6.20E+06
Expt. 2	4.60E+06	5.20E+06
	3.60E+06	4.20E+06
	3.00E+06	6.40E+06
Expt. 3	4.20E+06	5.00E+06
	4.20E+06	4.00E+06
	4.40E+06	5.80E+06
Mean	4.02E+06	5.13E+06
S.D	8.03E+05	8.60E+05

Study 3: CLSM quantitative assay on 24 h *S. mutans-C. albicans* biofilms

Biofilm biovolumes (µm³/µm²)

Cranberry	Microbes	EPS
1	5.3	2.6
2	5.3	1.8
3	4.3	1.9
4	4.3	1.5
5	6.2	2.0
6	5.0	2.0
7	3.7	1.7
8	4.8	1.4
9	4.9	2.7
10	4.8	4.2
11	4.3	1.5
12	5.1	5.1
13	3.3	1.9
14	6.3	2.5
15	4.8	1.5
Mean	**4.8**	**2.3**
S.D	**0.8**	**1.0**

Control	Microbes	EPS
1	6.6	7.4
2	5.0	4.6
3	3.7	2.8
4	8.0	2.3
5	4.9	3.3
6	6.9	2.1
7	7.0	2.4
8	5.0	2.4
9	3.9	2.0
10	5.7	2.6
11	8.1	4.1
12	9.9	4.5
13	6.9	2.8
14	7.0	2.9
15	5.6	4.0
Mean	**6.3**	**3.3**
S.D	**1.7**	**1.4**

4.4 SCIENTIFIC PAPER 4

Effect of Polyphenol-rich Cranberry Extracts on Cariogenic Biofilm Properties and Microbial Composition of Polymicrobial Biofilms

This study investigated the effects of polyphenol-rich cranberry extracts on saliva-derived polymicrobial biofilms growing in a cariogenic environment.

The paper was published on 30th March 2019 in the *Archives of Oral Biology*.

Arch Oral Biol 2019; 102:1-6.

This chapter is comprised of the publication as it stands in print.

ABSTRACT

Purpose: 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 *Prevotella 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.

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 anti-caries agents, has only limited antimicrobial actions, and is often not able to cope with the massive bacterial 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.,* 2018]. 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.,* 2019a]. 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 monospecies *Streptococcus mutans* biofilms [Duarte *et al.,* 2006; Feng *et al.,* 2013; Gregoire *et al.,* 2007; Philip *et al.,* 2019a]. 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 this study was that the cranberry extracts did not did not influence biofilm behaviour or its microbial composition.

MATERIALS AND METHODS

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.,* 2019a]. 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.

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].

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.,* 2019a]. The saliva-coated HA discs (sHA) 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 sHA 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 a 50-fold dilution of the pooled saliva-glycerol mixture in 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 any residual treatment solutions.

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.

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. 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].

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.

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.,* 2019a]. 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.

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*, *Prevotella 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).

Statistical Analysis

Depending on the normality of data distribution, either an independent sample 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.

RESULTS

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 11).

Cranberry extract inhibited polymicrobial biofilm lactic acid production

The cranberry extract caused a 44% reduction in lactic acid production (Table 11). 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).

Table 11. Inhibitory effects of polyphenol-rich cranberry extracts on biomass and acid production of polymicrobial biofilms

Treatment Group	**Biofilm biomass** (% change) (OD_{570})	**Lactic acid production** (% change) (mM/L)
Cranberry	0.089 ± 0.01 (-38)*	6.2 ± 1.9 (-44)*
Control	0.144 ± 0.02	11.2 ± 3.8

Biomass represents mean crystal violet absorbance values ± standard deviation (SD) at optical density (OD) of 570 nm. Lactic acid production of biofilms is in mM/L (mean ± SD). Data for both assays were obtained from three independent triplicate experiments (n = 9). Values in brackets represent percentage reduction of biofilm biomass/acid production compared to control. * indicates significant difference compared to vehicle control using independent sample-t-test/Mann-Whitney *U*-test at confidence interval of 95%

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 12), 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. 12). 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.

Table 12. Quantitative analysis of biovolumes of polymicrobial biofilms from confocal images

Treatment Group	EPS biovolume ($\mu m^3/\mu m^2$)	Microbial biovolume ($\mu m^3/\mu m^2$)	Total biofilm biovolume ($\mu m^3/\mu m^2$)
Cranberry	1.14 ± 0.15*	2.05 ± 0.55*	3.19 ± 0.63*
Control	2.64 ± 0.56	3.82 ± 0.73	6.46 ± 1.19

Biovolume data was quantified using IMARIS and represents mean values ± SD (n = 15) from 3 independent experiments. * indicates significant difference ($P < 0.05$) in cranberry-treated biofilms compared to control using independent sample t-test.

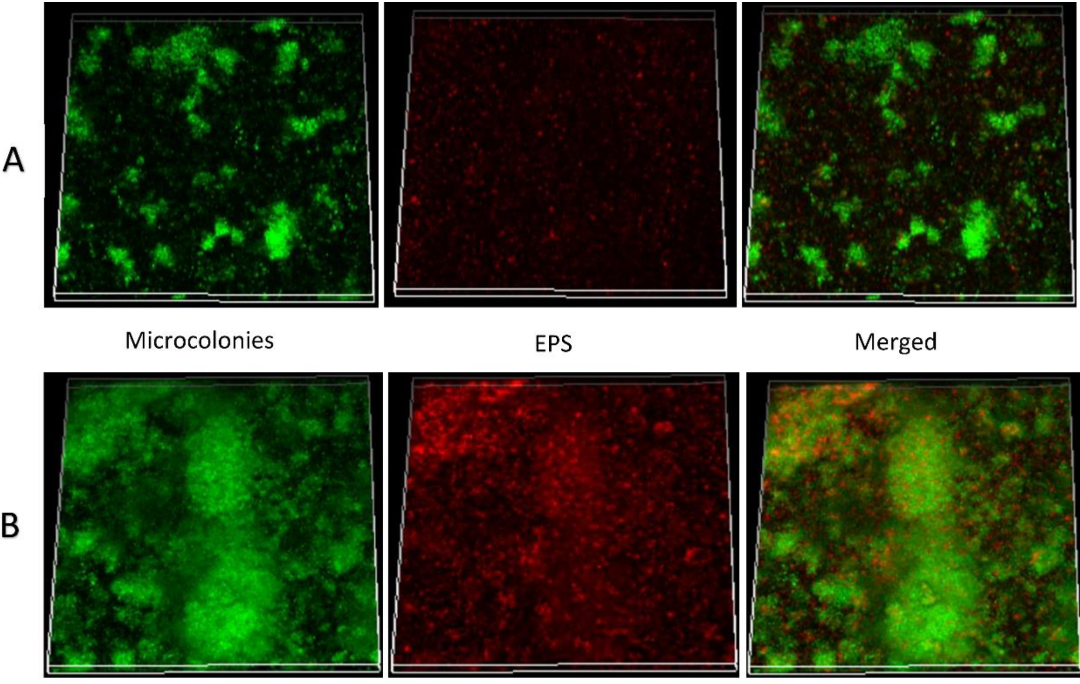


Figure 12. Representative 3D confocal image stacks of polymicrobial biofilms

Structural organisation of polymicrobial biofilms after treatment with A) 500 µg/mL of the cranberry extract and B) PBS control. Microbial colonies are depicted in green (SYTO 9) and EPS in red (Dextran, Alexa Fluor), with 20x magnification.

Cranberry extract lowered CFUs recovered from polymicrobial biofilms

Significantly fewer microbial colonies 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. 13).

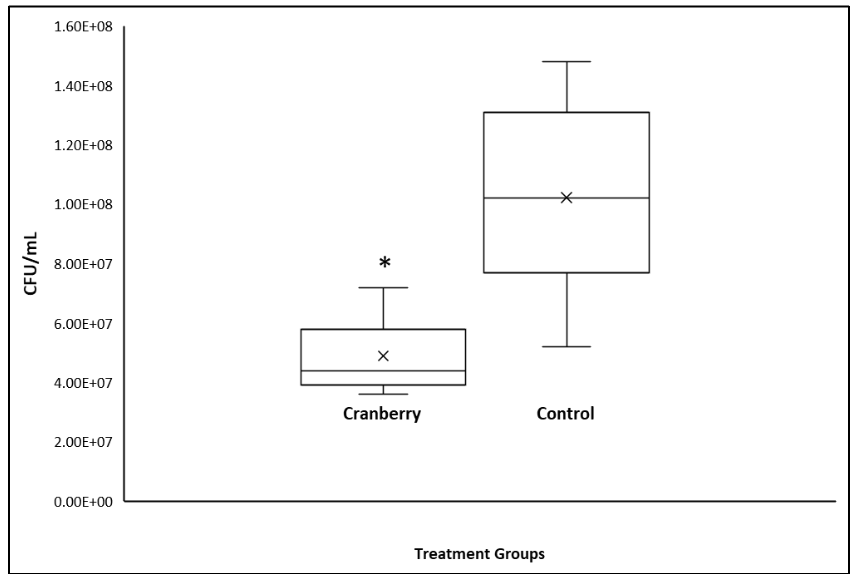
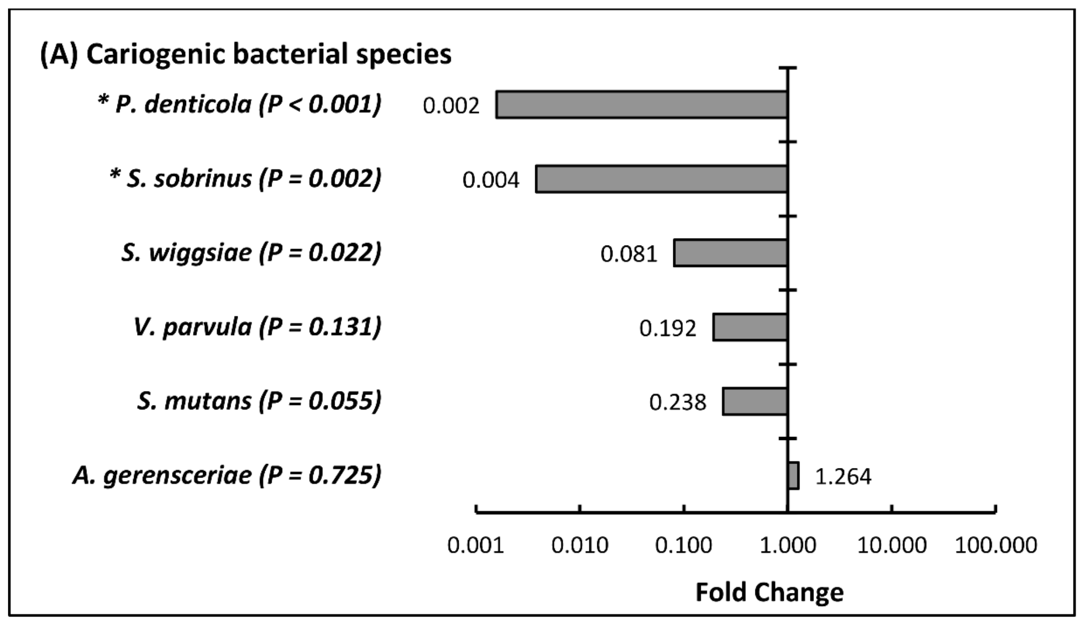


Figure 13. Box-plots of microbial colonies recovered from polymicrobial biofilms

* indicates significantly lower CFU counts ($P < 0.05$) for the cranberry-treated biofilm compared to control-treated biofilms using the Mann-Whitney *U*-test (n = 9).

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, $P < 0.001$) (Fig. 14A), and significantly greater abundance of the health-associated *S. sanguinis* (fold change 90.715, $P = 0.001$) (Fig.14B). 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, however these changes were not statistically significant (Fig. 14).



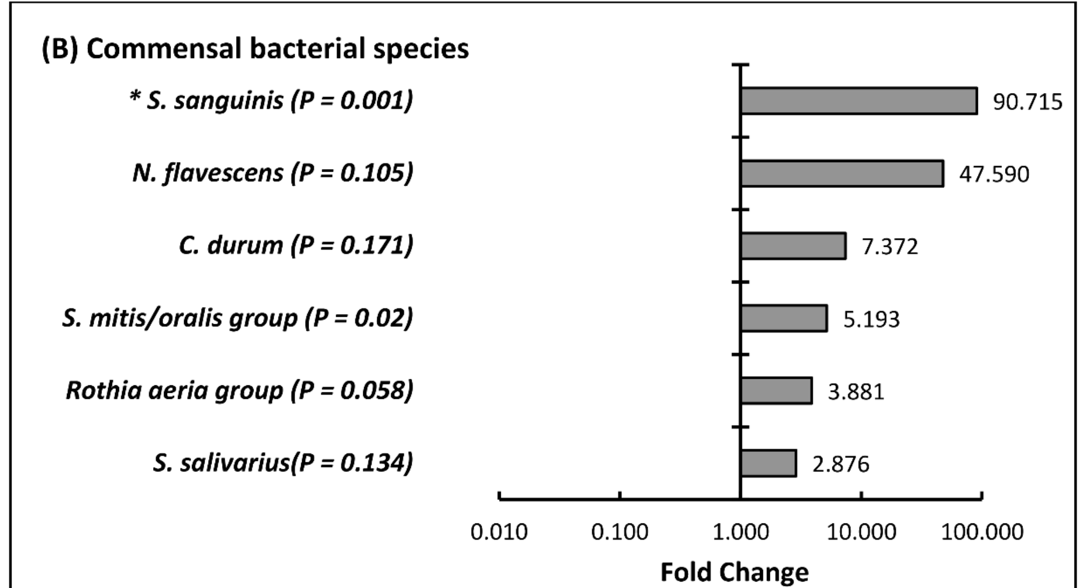


Figure 14 (A and B)**.** Fold change of bacterial species in cranberry- vs. control-treated biofilms

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.

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* monospecies biofilms [Duarte *et al.,* 2006; Feng *et al.,* 2013; Gregoire *et al.,* 2007; Philip *et al.,* 2019a]. 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 thereby 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.,* 2019a; Weiss *et al.,* 2004]. A likely reason for the present findings is 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 *Prevotella* 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).

CONCLUSIONS

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.

RAW DATA

Study 4: Crystal Violet (CV) biomass assay on 96 h polymicrobial biofilms

CV absorbance values at 570 nm

Column1	Cran 500 µg/mL	Control
EXPT. 1	0.0957	0.1207
	0.1058	0.1289
	0.0994	0.1158
EXPT. 2	0.0681	0.1308
	0.0980	0.1383
	0.0847	0.1628
EXPT. 3	0.0843	0.1683
	0.0918	0.1769
	0.0764	0.1498
Mean	**0.089**	**0.144**
S.D	**0.012**	**0.022**
% Redn.	**38**	

Study 4: Lactic acid production assay on 96 h polymicrobial biofilms

Lactic acid concentrations in mM/L

Column1	Cranberry 500 µg/mL	Control
Expt. 1	5.1	16.1
	5.1	16.6
	5.3	16.6
	7.9	16.3
	8.2	16.9
	8.5	16.6
	5.6	9.5
	5.6	8.7
	5.6	8.9
Expt. 2	3.8	6.3
	3.9	6.5
	3.9	7.1
	4.2	5.9
	4.2	6.1
	4.2	6.0
	4.8	8.9
	4.5	8.8
	4.8	8.8
Expt. 3	7.1	11.4
	7.2	11.6
	7.4	11.6
	8.6	13.2
	9.0	12.5
	8.8	13.3
	8.5	12.9
	8.2	13.1
	8.3	12.9
Mean	**6.2**	**11.2**
S.D	**1.9**	**3.8**
% Redn.	**44**	

Study 4: Colony Forming Unit (CFU) assay on 96 h polymicrobial biofilms

CFU/mL

Column1	Cranberry 500 µg/mL	Control
Expt. 1	4.40E+07	9.20E+07
	5.00E+07	1.08E+08
	3.60E+07	6.20E+07
Expt. 2	6.20E+07	1.24E+08
	4.00E+07	9.40E+07
	5.40E+07	1.48E+08
Expt. 3	7.20E+07	5.20E+07
	3.80E+07	1.02E+08
	4.40E+07	1.38E+08
Mean	4.89E+07	1.02E+08
S.D	1.20E+07	3.20E+07

Study 4: CLSM quantitative assay on 96 h polymicrobial biofilms

Biofilm biovolumes ($\mu m^3/\mu m^2$)

Cranberry 500 µg/mL	Bacteria	EPS
1	1.31	1.03
2	1.74	1.11
3	1.47	1.26
4	1.61	1.02
5	1.89	1.30
6	2.30	1.15
7	1.46	1.00
8	1.80	1.17
9	1.98	1.33
10	1.76	1.05
11	2.56	0.98
12	3.00	1.46
13	2.36	1.01
14	2.37	1.00
15	3.11	1.32
Mean	**2.05**	**1.14**
S.D	**0.55**	**0.15**

CONTROL	Bacteria	EPS
1	2.87	2.58
2	3.52	2.91
3	3.40	2.14
4	3.86	2.93
5	2.69	1.84
6	4.04	2.65
7	4.26	3.57
8	5.12	3.31
9	5.12	3.66
10	4.38	2.52
11	3.50	1.79
12	4.15	2.38
13	3.40	2.56
14	4.01	2.24
15	3.00	2.46
Mean	**3.82**	**2.64**
S.D	**0.73**	**0.56**

Study 4: qPCR assay on 96 h polymicrobial biofilms

Cycle threshold (Ct) values for each bacterial species

Treatment_group	Ct *A. gerencseriae*	Ct *C. durum*	Ct *L. Gasseri*	Ct *N. flavescens*	Ct *P. denticola*	Ct *Rothia spp*	Ct *Scardovia wiggsiae*	Ct *Streptococcus spp 3*	Ct *S. mitis*	Ct *S. mutans*	Ct *S. parasanguinis*	Ct *S. sanguinis*	Ct *S. sobrinus*	Ct *V. parvula*
Cranberry 500 μg/mL	26.85	32.54	40.00	40.00	36.28	31.45	40.00	21.43	24.93	28.48	40.00	17.53	38.16	36.31
Cranberry 500 μg/mL	31.08	40.00	40.00	40.00	40.00	27.71	40.00	22.33	30.56	28.86	40.00	17.92	35.57	30.55
Cranberry 500 μg/mL	27.22	25.04	40.00	22.68	34.19	24.87	40.00	21.28	23.37	23.77	40.00	15.91	28.81	24.99
Cranberry 500 μg/mL	27.77	26.06	40.00	23.56	35.82	28.84	40.00	21.46	27.02	29.06	40.00	18.30	36.24	26.76
Cranberry 500 μg/mL	27.92	26.19	40.00	22.64	34.97	27.15	40.00	20.52	25.00	25.66	40.00	17.78	36.84	25.56
Cranberry 500 μg/mL	28.46	25.14	40.00	21.6	40	25.46	40	18.79	23.05	21.07	40	15.41	25.4	22.51
Control	25.84	28.74	40.00	32.18	25.08	28.84	35.57	17.86	24.54	21.35	40.00	19.06	23.32	24.73
Control	26.87	29.50	40.00	35.35	25.97	30.75	35.01	22.27	27.88	24.53	40.00	23.60	27.55	27.16
Control	27.57	33.28	40.00	32.36	26.49	26.72	32.08	20.67	27.85	22.03	40.00	25.84	23.41	22.16
Control	24.82	26.68	40.00	29.55	24.04	25.90	34.69	21.77	23.95	23.49	40.00	18.09	22.54	21.10
Control	28.02	34.31	40.00	32.58	27.00	26.14	32.66	19.91	28.23	21.09	40.00	25.85	22.97	22.90
Control	25.36	26.92	40.00	29.06	23.99	26.03	35.37	19.63	22.91	19.15	40.00	16.59	20.16	21.50

4.5 SCIENTIFIC PAPER 5

Casein Phosphopeptide-Amorphous Calcium Phosphate Attenuates Virulence and Modulates Microbial Ecology of Saliva-derived Polymicrobial Biofilms

This study investigated the effects of CPP-ACP on saliva-derived- polymicrobial biofilms growing in a cariogenic environment.

The paper was published on 4th June 2019 in *Caries Research*, the official publication of the European Organisation for Caries Research (ORCA).

Caries Res 2019; DOI: 10.1159/000499869.

This chapter is comprised of the publication as it stands in print.

ABSTRACT

Background: Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) acts as a salivary biomimetic that provides bioavailable calcium and phosphate ions to augment fluoride-mediated remineralization of early caries lesions. However, there are indications that it may also have virulence attenuating and beneficial ecological effects on the oral microbiome.

Purpose: This *in vitro* study investigated whether CPP-ACP could influence acidogenicity, microbial counts, and relative abundance of specific caries-and health-associated bacterial species in polymicrobial biofilms.

Methods: Saliva-derived polymicrobial biofilms were grown for 96 h in a cariogenic environment and treated every 12 h with 2% CPP-ACP or vehicle control. Acidogenicity and colony forming units (CFUs) and were estimated from the treated biofilms. Microbial ecological effects of CPP-ACP were assessed based on the relative abundance of 14 specific caries- and health-associated bacterial species using a real-time quantitative PCR assay.

Results: CPP-ACP-treated biofilms showed relatively modest, but significant reductions, in acidogenicity (33% reduction, $P < 0.001$) and microbial CFUs (21% reduction, $P = 0.008$), compared to the control-treated biofilms. The CPP-ACP treated biofilms also exhibited significantly lower bacterial loads of cariogenic *Scardovia wiggsiae* (fold change 0.017, $P < 0.001$) and *Prevotella denticola* (fold change 0.005, $P < 0.001$), and higher bacterial loads of commensal *Streptococcus sanguinis* (fold change 30.22, $P < 0.001$), *Streptococcus mitis*/*oralis* (fold change 9.66, $P = 0.012$), and *Steptococcus salivarius*/*thermophilus* (fold change 89.35, $P < 0.001$) than the control-treated biofilms.

Conclusions: The results indicate that CPP-ACP has cariogenic virulence-attenuating attributes that can influence a beneficial microbial ecological change in the biofilm.

INTRODUCTION

A symbiotic oral microbiome delivers important health benefits and plays a critical role in preventing dysbiosis-mediated oral diseases like dental caries [Marsh, 2018]. Dental caries is now widely recognised as a disease triggered by dysbiosis in the plaque biofilm and maintaining microbial ecological balance in dental plaque is critical for long-term control over the disease [Burne, 2018; Philip *et al.,* 2018]. While acids produced from bacterial glycolysis of dietary carbohydrates can favour the dominance of acidogenic/aciduric bacteria in the plaque biofilm, health-associated plaque microflora can re-establish the homeostatic balance by producing alkali metabolites to neutralise the acid [Nascimento, 2018]. This evolving view of ecological battles and polymicrobial synergies has important implications for developing effective therapeutics against dental caries [Bowen *et al.,* 2018].

Fluoride is without doubt the gold standard among remineralizing agents used for caries prevention. However, recent clinical studies have shown that it has minimal effects on the microbiome ecology [Adams *et al.,* 2017; Koopman *et al.,* 2015; Reilly *et al.,* 2016; Reilly *et al.,* 2014]. This suggests the need for additional ecological measures to supplement the predominantly physicochemical cariostatic effects of fluoride, especially in high caries-risk individuals [Philip *et al.,* 2018]. Although biocides like chlorhexidine have been used in the past for caries control, their broad-spectrum of antimicrobial action tend to indiscriminately eliminate even health-associated commensals. A delicate balance has to be achieved to control oral microflora at levels compatible with health [Marsh *et al.,* 2015].

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), a naturally derived milk protein-based remineralizing technology, may hold greater promise in effecting a beneficial shift in the dental plaque microbial ecology. While there is a growing body of evidence that supports the use of CPP-ACP to augment remineralizing effects of fluoride [Philip, 2019], there have also been suggestions that it could potentially have ecological effects on dental plaque too [Philip and Walsh, 2019]. Several clinical trials have shown that regular use of CPP-ACP reduced mutans streptococci (MS) levels in saliva [Emamieh *et al.,* 2015; Plonka *et al.,* 2013; Pukallus *et al.,* 2013; Subramanian and Naidu, 2009; Yetkiner *et al.,* 2014]. This interference with cariogenic bacteria may beneficially modulate the virulence of dental plaque and enable health-associated commensals to thrive. However, these previous studies have in general used a single bacterial species (usually *Streptococcus mutans*) as the surrogate marker for dental plaque ecology. For ecological caries

control, it is more relevant to know the influence of CPP-ACP on a range of health- and disease-associated bacteria. Hence, this *in vitro* study was designed to investigate whether CPP-ACP could: 1) inhibit acidogenicity and microbial numbers of saliva-derived polymicrobial biofilms growing in a cariogenic environment; and 2) effect the relative abundance of specific caries- and health-associated bacterial species in these polymicrobial biofilms.

MATERIALS AND METHODS

Test Agent

A 15% aqueous solution of CPP-ACP was sourced from GC Corporation (Tokyo, Japan) and diluted to 2% for testing against the saliva-derived polymicrobial biofilms. The concentration was chosen to represent what would be achieved in the oral cavity with the dilution by saliva of a topical crème containing 10% CPP-ACP. As the test solution of CPP-ACP contained a sodium benzoate preservative, a 0.1% (w/v) sodium benzoate solution was used for control comparisons.

Saliva Collection

With institutional ethics approval (approval number 2017001492), parafilm-stimulated whole human saliva was collected from 16 caries-free healthy adult donors. The study participants did not brush their teeth the morning before saliva donation and abstained from food for 2 h prior to saliva donation. The donors and had no history of using antibiotics/mouthwashes in the previous 3 months. The collected saliva was pooled, diluted 2-fold in 60% sterile glycerol, dispensed into aliquots, and stored at -80°C.

Polymicrobial Biofilm Formation and Treatments

The high-throughput Amsterdam Active Attachment (AAA) model [Exterkate *et al.,* 2010] was used to grow the saliva-derived polymicrobial biofilms. The biofilms were grown for a total of 96 h on hydroxyapatite (HA) discs (9.5mm diameter x 2mm thick; Himed, New York, NY, USA). Briefly, the HA discs were fitted to the custom-made AAA model such that each HA disc fitted into one well of a 24-well flat-bottomed microtiter plate (Costar 3526, Sigma-Aldrich, New York, NY, USA). To partly replicate the salivary pellicle coating, the HA discs were initially conditioned with filter-sterilized clarified human saliva for 1 h at 37°C immediately prior to biofilm formation, as described previously [Philip *et al.,* 2019a]. The saliva-coated HA discs (sHA) were then moved to a 24-well plate containing 2 mL/well of 2% CPP-ACP or the vehicle control solution, and treated for 10 min. The pre-treated sHA 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 and incubated anaerobically at 37°C in an 80 rpm orbital shaker for a total of 96 h. The inoculation medium was a 50-fold dilution of the pooled saliva in the mucin-rich McBain medium [McBain *et al.,* 2005] supplemented with 1% (w/v) sucrose. The sucrose-containing growth medium was replenished every 8 h to provide a constant cariogenic challenge. Subsequent biofilm treatments took place every 12 h, until the end of the 96 h growth period. All treatment exposures were for 10 min, with the biofilm-laden HA discs dip-rinsed 5 times in phosphate buffered saline (PBS; Gibco, Thermo Fisher Scientific, Paisley, UK) prior to treatment to remove non-adherent cells. The biofilms were similarly rinsed post-treatment also to remove any residual treatment solutions.

Biofilm Assays

At the end of the biofilm growth period, the effect of CPP-ACP on the acidogenicity was estimated using a lactic acid production assay, while effects on microbial viability were assessed by counting the colony forming units (CFUs) recovered from the treated biofilms. These biofilm assays were performed in triplicate and repeated in three independent experiments. The ecological effects of CPP-ACP on the polymicrobial biofilms were assessed based on the relative abundance of certain specific caries- and health-associated bacteria obtained from three independent duplicate biofilm experiments using a real-time quantitative PCR (qPCR) assay.

For estimating biofilm acidogenicity, the biofilm-bearing HA discs were placed in a 24-well plate containing 2 mL/well of buffered peptone water (Merck, Darmstadt, Germany) 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 generated during this period was calculated by means of a standard curve colorimetric assay using a lactate dehydrogenase enzymatic method [Exterkate *et al.,* 2010].

To assess microbial populations, the HA discs with the biofilms were gently detached from the AAA model lid and transferred to sterile tubes containing 1 mL PBS. The biofilms were dispersed by uniform vortexing at maximum speed for 1 min and a series of dilutions made. A 50 µL aliquot of the microbial suspensions was plated on tryptic soy agar blood plates using an automatic spiral plater (Autoplate; Advanced Instruments, Norwood, MA, USA) and incubated anaerobically at 37°C for 48 h. Microbial colonies were counted from the dilution that allowed visualization of distinct colonies and the CFU/mL values calculated after correcting for the dilution factor.

For the qPCR assay, 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, Scoresby, Australia) and the amount of isolated DNA was quantified spectrophotometrically. The bacterial load of 14 bacterial species 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*, *Veillonella parvula*, and *Prevotella denticola*) and 6 health-associated commensal bacterial species (*Streptococcus sanguinis*, *Streptococcus mitis*/*oralis*, *Steptococcus salivarius*/*thermophilus*, *Corynebacterium durum*, *Rothia aeria*/*dentocariosa*, and *Neisseria flavescens*). The assay used the 16S rRNA gene of the relevant bacterium, with the proprietary probes designed using the GreenGene database for 16S sequences [DeSantis et al. 2006]. The DNA sample was mixed with a proprietary master mix and robotically dispensed into a 384-well plate (10µL, 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 were analysed using the sequence detection system software (QuantStudio v1.3; Thermo Fisher Scientific).

Statistical Analysis

For the lactic acid assay and CFU counts, depending on the normality of data distribution, an independent sample t-test or the Mann-Witney *U*-test was performed to detect any statistical difference between the CPP-ACP and control-treated biofilms. For the qPCR assay, the fold change of each bacterial amplicon in CPP-ACP- 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 CPP-ACP- versus control-treated biofilms was then calculated for each bacterial species as follows: $\Delta\Delta Ct = \Delta Ct^{\text{bacterial species (CPP-ACP)}} - \Delta Ct^{\text{bacterial species (control)}}$. 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 CPP-ACP- 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.

RESULTS

Biofilm acidogenicity

The polymicrobial biofilms treated with CPP-ACP demonstrated a significant reduction in acidogenicity compared to the control-treated biofilms (33% reduction, $P < 0.001$), with mean lactic acid concentrations of 3.6 ± 1.1 mM/L and 5.3 ± 1.2 mM/L respectively (Table 13).

Table 13. Effect of CPP-ACP on lactic acid production of polymicrobial biofilms

Treatment Group	**Lactic acid production** (% change) (mM/L)
CPP-ACP	3.6 ± 1.1 (-33)*
Control	5.3 ± 1.2

Lactic acid production data (mean ± SD) is from three independent triplicate experiments. Values in brackets represent percentage reduction in acid production compared to control. * indicates significant difference using independent sample t-test at 95% confidence interval.

Biofilm microbial counts

Total CFUs recovered from the CPP-ACP-treated biofilms were significantly lower ($P = 0.008$) compared to the microbial colonies obtained from the control-treated biofilms. The mean CFU counts were $7.9 \times 10^{7} \pm 1.2 \times 10^{7}$ and $1.0 \times 10^{8} \pm 1.1 \times 10^{7}$ for the CPP-ACP- and control-treated biofilms respectively (Fig. 15).

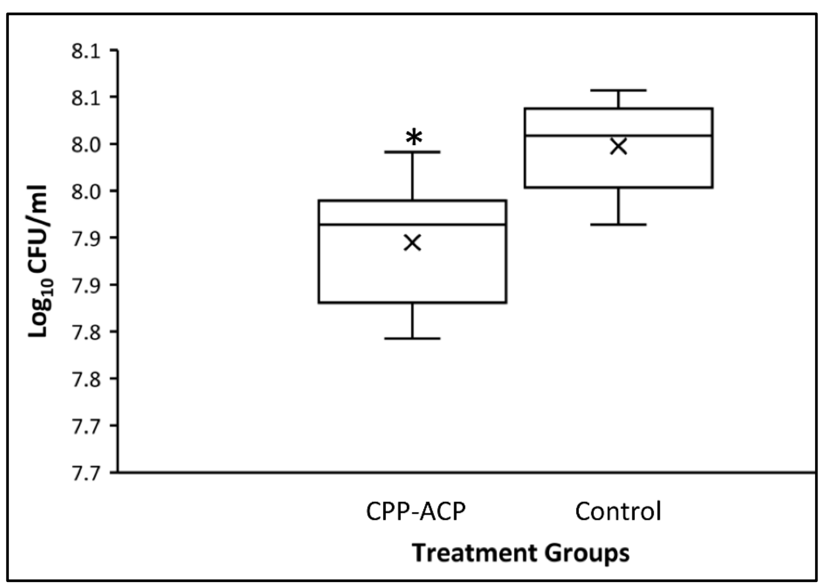
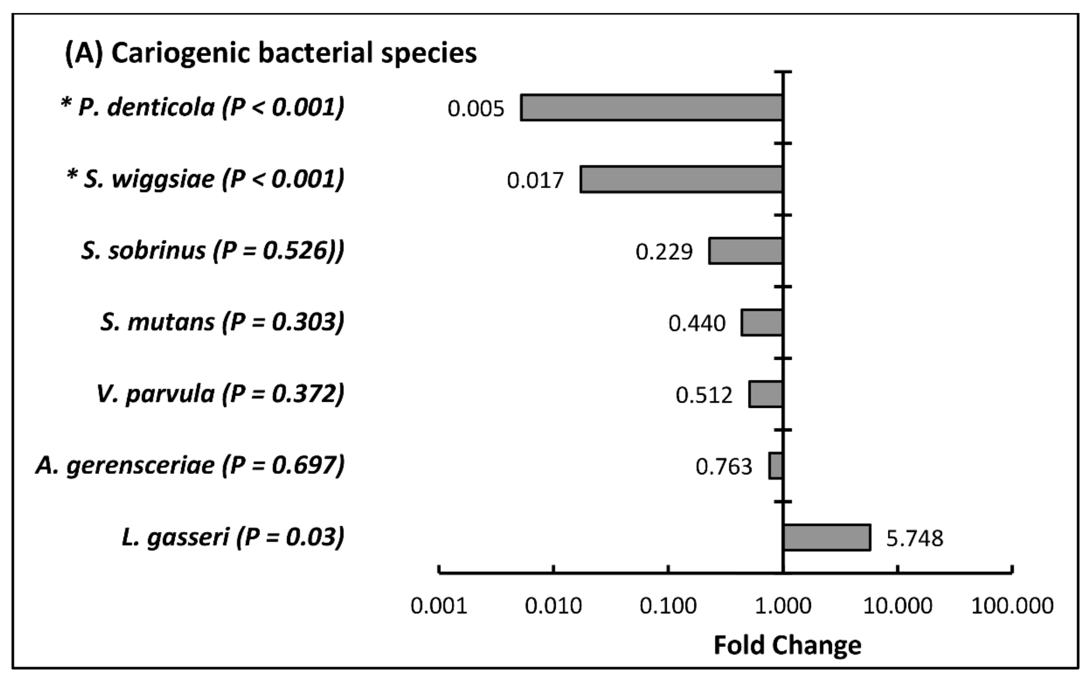


Figure 15. Box-plots of microbial colonies recovered from polymicrobial biofilms

* indicates significantly different CFU values ($P < 0.05$) for the CPP-ACP-treated biofilm compared to control-treated biofilms.

Biofilm ecological effects

Compared to the controls, the CPP-ACP-treated biofilms had significantly lower abundance for two caries-associated bacterial species: *P. denticola* (fold change 0.005, $P < 0.001$) and *S. wiggsiae* (fold change 0.017, $P < 0.001$). All the other caries-associated bacteria (except for *L. gasseri*) also showed fold decreases but this did not reach statistical significance (Fig. 16A). Among the health-associated commensals, *S. sanguinis* (fold change 30.22, $P < 0.001$), *S. mitis*/*oralis* group (fold change 9.66, $P < 0.012$), and the *S. salivarius*/*thermophilus* group (fold change 89.35, $P < 0.001$), were detected significantly more abundantly in the CPP-ACP-treated biofilms than in the control-treated biofilms (Fig. 16B). *S. parasanguinis* was not detected in any of the biofilm samples and was not included in the comparison.



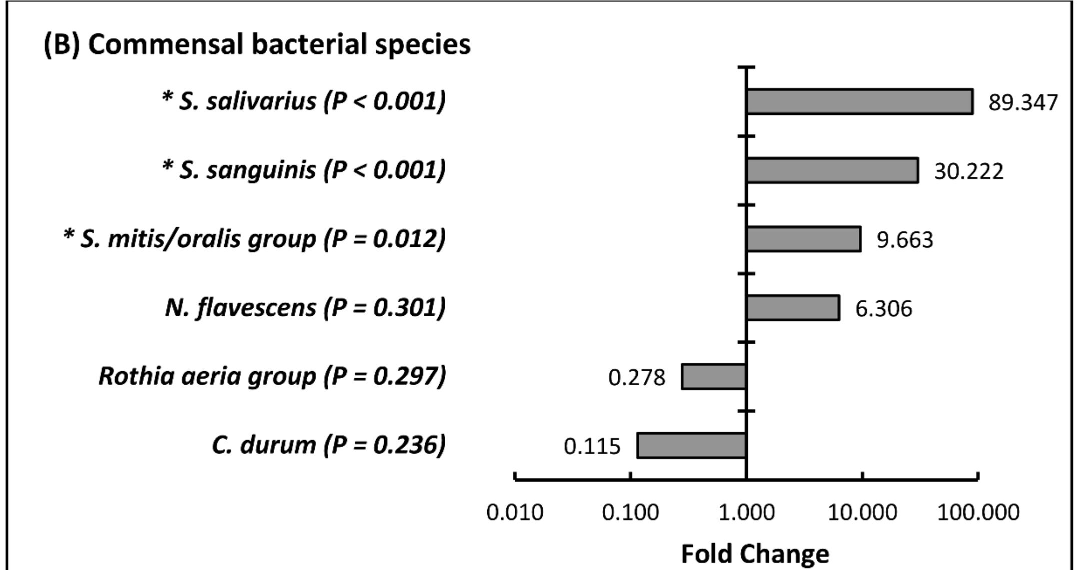


Figure 16 (A and B)**.** Fold change of bacterial species in CPP-ACP- vs. control-treated biofilms

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.

DISCUSSION

Dental caries is a polymicrobial biofilm-mediated disease triggered by environmentally-induced dysbiosis in the resident oral microbiome [Marsh, 2015]. If the dysbiosis is not reversed it can lead to bacterial acid-mediated demineralization of the dental hard tissues and ultimately result in clinical cavitation. Comprehensive caries prevention protocols should therefore encompass not only agents that can affect the de-/remineralization balance, but also include measures that can maintain or restore the ecological equilibrium in the oral microbiome. The current study provides

evidence of the beneficial ecological effects of CPP-ACP on complex polymicrobial biofilms and extends the recent findings of CPP-ACP ecological effects on multispecies biofilm comprised of 6 bacterial species growing in a highly cariogenic environment [Dashper *et al.,* 2018]. The present study also provides *in vitro* evidence that CPP-ACP can inhibit lactic acid production and microbial numbers in saliva-derived polymicrobial biofilms.

The AAA model used in this study has been shown to maintain reproducible, complex, and actively attached microbial biofilms in a habitat similar to dental plaque [Exterkate *et al.,* 2010; ten Cate, 2015]. Pooled saliva from several individuals was used as the biofilm inoculum to improve the microbial diversity of the *in vitro* polymicrobial biofilms. A cariogenic environment was created by regular replenishment of the sucrose-supplemented growth medium. The 10 min treatment time was chosen as CPP-ACP crèmes are intended to remain in the oral cavity until salivary clearance. Selection of the bacteria for the qPCR assay was based on profile studies of bacterial species commonly associated with dental caries and health [Aas *et al.,* 2008; Becker *et al.,* 2002; Burne and Marquis, 2000; Gross *et al.,* 2012; Kianoush *et al.,* 2014; Richards *et al.,* 2017; Takahashi, 2015; Tanner *et al.,* 2016].

The present study demonstrated a significant, though relatively modest, reduction in biofilm acidogenicity in the CPP-ACP-treated biofilms compared to the control-treated biofilms. It is possible that treating the biofilms for a longer duration than the 96 h of this study could have resulted in greater reductions in biofilm lactic acid concentrations. In fact, several clinical studies have shown that regular use of CPP-ACP had pronounced buffering effects on salivary and plaque pH [Caruana *et al.,* 2009; Heshmat *et al.,* 2014a; Heshmat *et al.,* 2014b; Marchisio *et al.,* 2010; Ozdas *et al.,* 2015; Peric *et al.,* 2015]. The buffering mechanisms of CPP-ACP are likely to be due to its ability to act as a reservoir of peptides and phosphate ions which can offset any drop in biofilm pH [Philip and Walsh, 2019]. The catabolism of certain CPP amino acids (e.g. glutamine, asparagine) by bacterial peptidases would buffer against a pH fall through the production of ammonia [Reynolds and Riley, 1989]. The CPP phosphoseryl residues that are more resistant to hydrolysis readily accept protons and neutralize plaque acids [Reynolds, 1987]. Furthermore, the inorganic phosphate (PO_4^{3-}) and organic phosphate ($-O-PO_3^{2-}$) ions in the CPP-ACP nanocomplex can also contribute to elevating salivary/plaque pH [Dashper *et al.,* 2018]. The buffering influence of CPP-ACP is thus an important virulence-attenuating attribute, creating a biofilm microenvironment that provides a selective ecological advantage to acid-sensitive commensal organisms.

A small but significant decrease in the total CFUs recovered from the CPP-ACP-treated polymicrobial biofilms was also seen in the current study. The lower microbial counts are likely to be due to the anti-adhesion effects of CPP-ACP, as the product is not known to have bactericidal or bacteriostatic effects. *In vitro* studies have demonstrated a rapid saturation of saliva-coated hydroxyapatite surfaces by casein complexes inhibiting the adherence of *S. mutans* and *S. sobrinus* [Erdem *et al.,* 2011; Reynolds and Wong, 1983; Schüpbach *et al.,* 1996; Vacca-Smith *et al.,* 1994]. CPP-ACP was also shown to bind both to dental plaque (with a strong affinity for *S. mutans*) and to pellicle macromolecules adsorbed on the tooth surface [Reynolds *et al.,* 2003; Rose, 2000b; Rose, 2000a]. The anti-adhesion effects of CPP-ACP may have a number of mechanisms. CPP-ACP is known to compete with free calcium for plaque calcium binding sites, reducing the degree of calcium bridging between the pellicle and adhering cells and between the bacterial cells themselves [Rose, 2000b]. CPP molecules can also mask the bacterial cell surface hydrophobic proteins and thereby impede the initial adhesion to pellicle-coated surfaces. The anti-adhesion effects of CPP-ACP could be the reason for the reduced levels of MS that have been observed in various clinical trials [Emamieh *et al.,* 2015; Plonka *et al.,* 2013; Pukallus *et al.,* 2013; Subramanian and Naidu, 2009; Yetkiner *et al.,* 2014]. Disruption of bacterial adhesion can impair the ability of cariogenic bacteria to recruit other key players during biofilm formation and prevent the establishment of a cariogenic biofilm [Simon-Soro and Mira, 2015].

The qPCR analysis provides evidence that CPP-ACP beneficially modulated the microbial ecology of the CPP-ACP-treated polymicrobial biofilms. In the present study, CPP-ACP was able to effect significantly lower relative abundance of *S. wiggsiae* and *P. denticola* compared to the control-treated biofilm. *S. wiggsiae* has been associated with severe early childhood caries and orthodontic white spot lesions [Tanner *et al.,* 2011; Tanner *et al.,* 2012], while *P. denticola* has been associated with initial enamel lesions [Chhour *et al.,* 2005; Tanner *et al.,* 2016; Torlakovic *et al.,* 2012]. *S. mutans* also showed a fold decrease in the CPP-ACP-treated biofilm, but this did not reach the threshold for statistical significance. It has been suggested that when "heavyweight" aciduric bacteria such as *S. wiggsiae* begin to dominate, the relative numbers of *S. mutans* tend to decrease, as the biofilm pH is driven to below what can be tolerated by even *S. mutans* [Burne, 2018]. This could be the reason why significant differences in relative abundance of *S. mutans* were not seen between the CPP-ACP- and control-treated biofilms.

The three health-associated bacterial species (*S. sanguinis*, *S. mitis*, *S. salivarius*) that showed significant fold increases in the CPP-ACP-treated biofilms possess systems actively engaged in biofilm pH homeostasis. *S. sanguinis* and *S. mitis* are known to express arginine deaminase system (ADS) activity that can effectively neutralize acids produced from carbohydrate metabolism [Burne and Marquis, 2000; Nascimento, 2018]. *S. salivarius* is among the relatively small number of oral bacteria that encode urease enzymes that also play a role in elevating resting plaque pH [Burne *et al.,* 2012]. Endogenous NH_3 production via ADS or ureases also provides bioenergetic advantages to health-associated bacterial species that are likely to influence plaque ecology and competitiveness of commensals [Burne, 2018].

CONCLUSIONS

A better understanding of oral microbial ecology, especially the importance of the balance between cariogenic and commensal microflora, has highlighted the need to develop strategies to modulate the microbial composition of dental plaque for caries prevention [Marsh *et al.,* 2015]. Measures that help maintain a symbiotic oral microbiome would be particularly beneficial for long-term control over dental caries in high caries-risk patients. CPP-ACP may hold particular promise in this regard as not only can it boost the remineralizing effects of fluoride, but the results of this study indicate that it may have beneficial microbial ecological effects too. Future clinical trials are required to ascertain whether these *in vitro* effects can translate to a clinical environment.

RAW DATA

Study 5: Lactic acid production assay on 96 h polymicrobial biofilms

Lactic acid concentrations in mM/L

Column1	CPP-ACP	Control
Expt. 1	3.5	6.8
	1.7	6.7
	4.9	4.8
	5.1	4.9
	2.8	6.5
	2.6	5.0
	5.3	3.2
	3.8	7.1
	3.5	5.2
Expt. 2	3.1	3.7
	2.2	3.2
	2.6	3.2
	1.6	4.1
	2.2	4.1
	3.8	4.1
	4.5	6.0
	4.6	5.6
	4.6	6.1
Expt. 3	2.5	5.1
	2.6	5.0
	2.6	5.2
	3.7	6.0
	3.7	6.1
	3.7	6.0
	5.0	7.3
	5.1	6.6
	5.1	6.2
Mean	**3.57**	**5.33**
S.D	**1.1**	**1.2**
% Redn.	**33**	

Study 5: Colony Forming Unit (CFU) assay on 96 h polymicrobial biofilms

CFU/mL

Column1	CPP-ACP	Control
Expt. 1	6.20E+07	8.40E+07
	7.40E+07	1.06E+08
	8.60E+07	9.60E+07
Expt. 2	6.20E+07	1.04E+08
	8.20E+07	1.14E+08
	7.80E+07	1.02E+08
Expt. 3	8.80E+07	1.12E+08
	9.80E+07	1.00E+08
	8.40E+07	8.20E+07
Mean	7.93E+07	1.00E+08
S.D	1.19E+07	1.11E+07

Study 5: qPCR assay on 96 h polymicrobial biofilms

Cycle threshold (Ct) values for each bacterial species

Treatment_group	*Ct A. gerencseriae*	*Ct C. durum*	*Ct L. Gasseri*	*Ct N. flavescens*	*Ct P. denticola*	*Ct Rothia spp*	*Ct Scardovia wiggsiae*	*Ct Streptococcus spp 3*	*Ct S. mitis*	*Ct S. mutans*	*Ct S. parasanguinis*	*Ct S. sanguinis*	*Ct S. sobrinus*	*Ct V. parvula*
CPP-ACP	25.95	31.44	40	40	33.77	30.74	40	13.59	23.25	23.88	40	20.75	27	27.35
CPP-ACP	25.93	32.11	40	40	34.52	32.41	40	15.87	23.71	26.87	40	20.73	28.09	27.6
CPP-ACP	30.22	40	40	30.51	36.7	24.21	40	13.42	27.51	21.46	40	21.73	37.6	24.96
CPP-ACP	30.34	40	40	35.63	36.89	26.97	40	16.07	27.59	23.35	40	24.29	29.24	27.26
CPP-ACP	27.58	33.95	40	26.49	30.83	28.79	35.79	18.07	26.53	26.5	40	22.7	30.28	29.34
CPP-ACP	29.55	40	40	32.06	40	25.05	40	16.91	27.83	25.81	40	21.99	27.58	25.88
Control	24.73	27.34	40	32.63	25.05	23.43	30.55	18.56	26.3	21.49	40	22.92	23.48	24.15
Control	27.43	33.45	35.74	32.7	26.01	25.91	31.8	19.36	27.43	22.98	40	25.63	23.11	23.25
Control	24.36	26.26	40	34.74	24.65	22.72	30.16	16.97	26.09	18.38	40	22.61	20.26	23.07
Control	24.35	26.41	40	35	24.81	22.69	30.2	16.67	26.77	19.04	40	22.81	20.18	22.91
Control	24.05	25.9	40	34.17	24.5	22.36	30.1	17.29	26.24	19.37	40	22.48	20.59	22.67
Control	22.91	40	40	32	22.78	20.58	28.42	24.58	23.83	20.1	40	25.85	40	21.15

4.6 SCIENTIFIC PAPER 6

Randomised Controlled Study to Evaluate Microbial Ecological Effects of Casein Phosphopeptide-Amorphous Calcium Phosphate and Cranberry on Dental Plaque

This clinical study investigated the microbial ecological effects of dentifrices containing CPP-ACP and polyphenol-rich cranberry extracts on dental plaque.

The paper was published in *JDR Clinical and Translational Research*, the official publication of the International Association of Dental Research (IADR).

JDR Clin Trans Res 2019; DOI: 10.1177/2380084419859871.

This chapter is comprised of the publication as it stands in print.

ABSTRACT

Introduction: Ecological approaches to dental caries prevention play a key role in attaining long-term control over the disease and maintaining a symbiotic oral microbiome.

Objectives: This study aimed to investigate the microbial ecological effects of two interventional dentifrices: a casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) dentifrice, and the same dentifrice supplemented with a commercial polyphenol-rich cranberry extract.

Methods: The interventional toothpastes were compared with each other and with an active control fluoride dentifrice in a double-blinded randomised controlled trial. Real-time quantitative PCR (qPCR) analysis was used to determine changes in the bacterial loads of 14 key bacterial species (8 caries-associated and 6 health-associated) in the dental plaque of trial participants after they used the dentifrices for 5-6 weeks.

Results: From the baseline to the recall visit, significant differences were observed between the treatment groups in the bacterial loads of two caries-associated bacterial species *S. mutans* (P < 0.001) and *V. parvula* (P < 0.001) and three health-associated bacterial species *C. durum* (P = 0.008), *N. flavescens* (P = 0.005), and *S. sanguinis* (P < 0.001). Compared to the fluoride control dentifrice, the CPP-ACP dentifrice demonstrated significant differences for *S. mutans* (P = 0.032), *C. durum* (P = 0.007) and *S. sanguinis* (P < 0.001), while the CPP-ACP – cranberry combination dentifrice showed significant differences with the control dentifrice for *S. mutans* (P < 0.001), *V. parvula* (P < 0.001), *N. flavescens* (P = 0.003) and *S. sanguinis* (P < 0.001). However, no significant differences were seen in the bacterial load comparisons between the CPP-ACP and combination dentifrices for any of the targeted bacterial species (P > 0.05).

Conclusions: Overall, the results indicate that dentifrices containing CPP-ACP and polyphenol-rich cranberry extracts can influence a species level shift in the microbial ecology of dental plaque resulting in a microbial community less associated with dental caries.

INTRODUCTION

The oral cavity harbours one of the most diverse microbiomes in the human body with over 700 different microbial species identified to date. When in equilibrium, the diversity of the endogenous oral microbial community not only prevents outgrowth of any single species or colonisation by exogenous pathogens, but also contributes to critical metabolic, physiological and immunological functions [Marsh, 2018]. Despite daily physical and chemical perturbations, a normal healthy microbiome exhibits remarkable long-term stability and is characterised by commensalism and mutualism with the host [Zaura *et al.,* 2014]. However, adverse local environmental conditions (e.g. frequent exposure to dietary sugars, poor oral hygiene or salivary dysfunction) can result in the breakdown of this symbiotic relationship and onset of diseases like dental caries.

Dental caries is a polymicrobial disease caused by dysbiosis in the dental plaque biofilm, with microbial community shifts driven by environmental acidification [Takahashi and Nyvad, 2008, 2011]. If the environmental acidic stress persists, acidogenic and aciduric bacteria will outcompete health-associated commensals and create multiple low-pH niches within the biofilm microenvironment. Health-associated commensal microorganisms can counter the acidic conditions through the production of alkaline compounds that attempt to maintain plaque pH values near neutrality [Bowen *et al.,* 2018; Nascimento, 2018]. The ecological battles between the opportunistic cariogenic pathogens and the health-associated commensals will determine whether incipient lesions caused by acid-induced mineral loss from susceptible tooth surfaces will progress to cavitation or can be remineralized.

Fluoride will continue to remain the gold standard for caries prevention. However, the cariostatic effects of fluoride are principally due its physiochemical effects on the de-/remineralization equilibrium, suggesting the need for additional ecological measures to complement fluoride effects on hard dental tissues. Mechanical plaque control using fluoride dentifrices alone is not likely to reverse the plaque dysbiosis responsible for the disease. Recent studies have shown that even high fluoride concentrations could not sustain antimicrobial activity against plaque biofilm metabolism [Dang *et al.,* 2016; Souza *et al.,* 2018]. Clinical trials have confirmed that fluoride alone was not able to effect significant changes in dental plaque microbial composition [Adams *et al.,* 2017; Koopman *et al.,* 2015; Reilly *et al.,* 2016; Reilly *et al.,* 2014]. This could be one of the reasons why caries persists in high-risk individuals and population groups despite the widespread use of fluoride products.

A variety of antimicrobial agents have also been used in the past for caries prevention. However, the use of broad-spectrum biocides (e.g. chlorhexidine) indiscriminately eradicates even commensal bacteria that are beneficial to health. Moreover, because of their adverse side-effects, such agents may not be suitable for daily use. Once the chemotherapeutic intervention stops, susceptible tooth surfaces are often repopulated with a microbiome similar in composition to the one that was eliminated [Burne, 2018]. Strategies that target cariogenic virulence properties within a pathogenic biofilm, rather than broadly affecting microbial viability, would thus be preferable to conventional antimicrobials for beneficially modulating and maintaining a healthy microbiome.

Comprehensive caries management protocols should therefore encompass agents that can influence the de-/remineralization balance as well as measures that have a moderating influence on acidogenic oral bacteria. In this regard, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is of interest as not only can it augment the remineralizing effects of fluoride, but it can also potentially exert a beneficial effect on the microbial ecology of dental plaque [Philip and Walsh, 2019]. Recent *in vitro* studies have demonstrated that CPP-ACP treatment was able to effect a decrease in the abundance of caries-associated bacteria and a concomitant increase in the abundance of health-associated bacteria in multi-species biofilms grown in a cariogenic environment [Philip *et al*. 2019; Dashper *et al.,* 2018]. Although majority of the clinical studies on CPP-ACP have been on its remineralizing effects, several clinical studies have also reported that that regular use of CPP-ACP reduced mutans streptococci (MS) levels in saliva [Emamieh *et al.,* 2015; Plonka *et al.,* 2013; Pukallus *et al.,* 2013; Subramanian and Naidu, 2009; Yetkiner *et al.,* 2014]. This interference with cariogenic bacteria may inhibit the virulence of dental plaque and enable health-associated commensals to thrive. However, the previous studies have mostly used salivary levels of a single bacterial species (usually *Streptococcus mutans*) as the surrogate marker. Of greater relevance is the possible influence of CPP-ACP on a range of health- and disease-associated bacteria in dental plaque.

In addition, the potential exists for synergism between the milk-derived CPP-ACP and other cariogenic virulence inhibiting natural agents. Cranberry flavonoids are of particular interest as its A-linked proanthocyanidins (A-PACs) have been shown to be potent disruptors of several cariogenic virulence factors without affecting bacterial viability [Duarte *et al.,* 2006; Feng *et al.,* 2013; Gregoire *et al.,* 2007; Philip *et al.,* 2019a, b]. The virulence attenuating effects of cranberry polyphenols have translated into reduced incidence and severity of carious lesions in an animal caries model [Koo *et*

al., 2010]. While cranberry phenols exhibit inhibitory effects against bacterial acidogenicity, aciduricity and glucan synthesis, CPP-ACP is known for its buffering and anti-adhesion effects. This suggests potential synergism if CPP-ACP and polyphenol-rich cranberry extracts are combined in a single oral care product. Hence, the objective of this study was to investigate the microbial ecological effects of a CPP-ACP dentifrice and a combination CPP-ACP – cranberry dentifrice with regards to species level changes of specific caries- and health-associated bacteria in the dental plaque of a high caries-risk group.

MATERIALS AND METHODS

Study Design and Sample Size

This parallel three-group double-blinded randomised controlled trial (RCT) was designed in accordance with the CONSORT guidelines. A study flow diagram based on the CONSORT guidelines is shown in Fig. 17. The trial was registered with the Australian New Zealand Clinical Trial Registry (ANZCTR 12618000095268). A clinical trial notification for using the interventional products of the study was obtained from the Australian Therapeutic Goods Administration (TGA, CT-2017-CTN-05069-1). As described previously (Lipták et al 2018), the G-POWER program tool was used to determine the sample size needed for a three-group one-way ANOVA test, assuming an alpha of 0.05, power of 80% and large effect size (f = 0.4). Based on these assumptions, the power analysis showed that a total sample size of 66 was needed. To ensure there was enough statistical power in case some of the aforementioned assumptions were not met, and to account for attrition (from drop-outs or poor compliance), 30 subjects were recruited into each of the three trial groups.

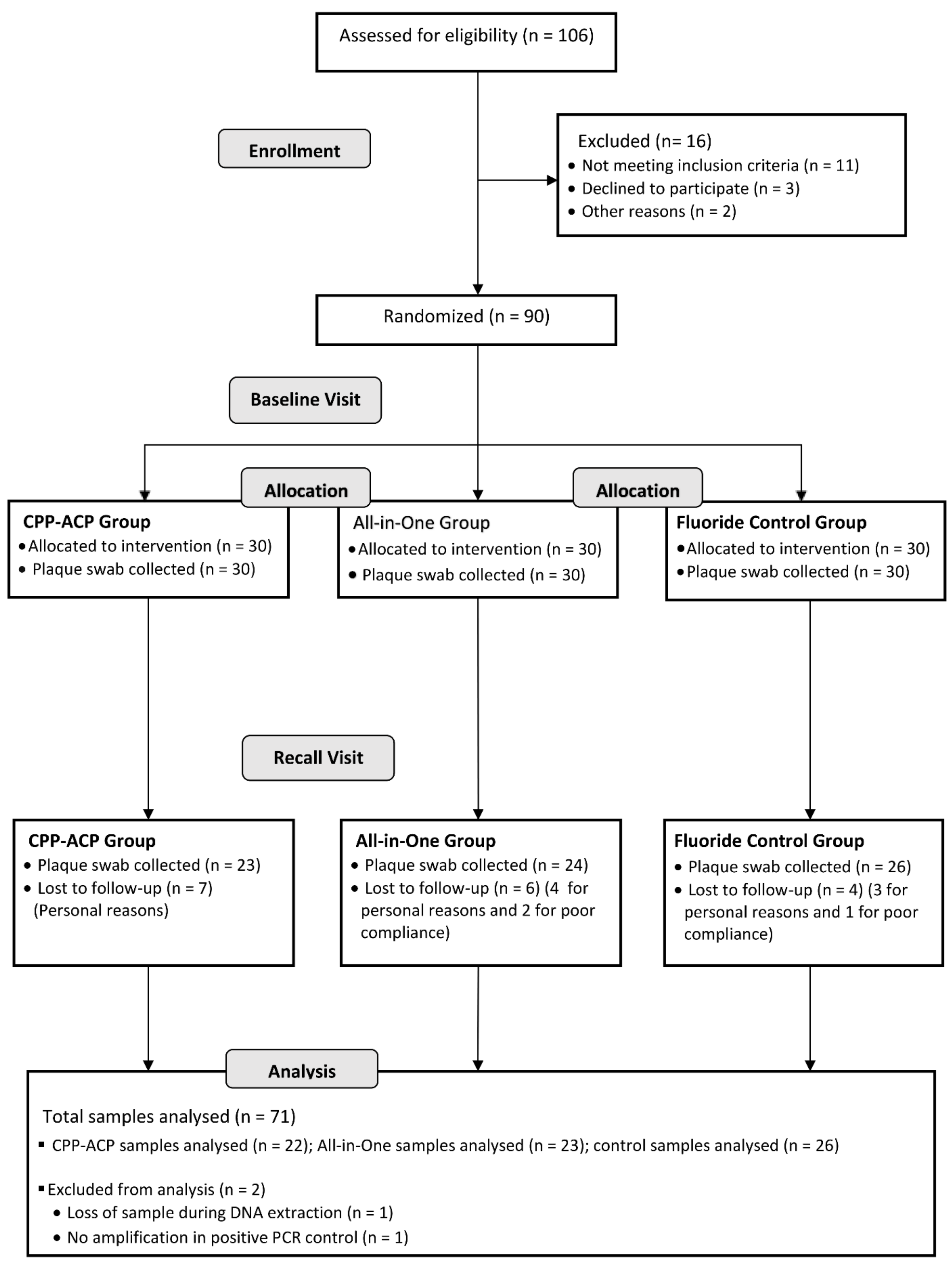


Figure 17. Study flow diagram

Trial Products

Except for the active agents (CPP-ACP and cranberry extract) in the interventional toothpastes, all the trial toothpastes had same ingredients and a standard 1100 ppm fluoride

concentration. The interventional toothpastes were compared with each other and with an active control fluoride dentifrice. Details of the three treatment groups are as follows:

- CPP-ACP group: dentifrice containing 10% (w/v) CPP-ACP (MI Paste® ONE, GC Corporation, Tokyo, Japan).
- All-in-One group: 0.25% (w/w) cranberry extract (Diana Food, Champlain, QC, Canada) was incorporated into the CPP-ACP dentifrice. The polyphenol-rich cranberry extract was a highly purified, organic, sugar-free natural extract that contained more than 80% A-PACs.
- Active control group: standard fluoride dentifrice (GC Corporation), which had the same vehicle and flavour as the interventional dentifrices.

Blinding and Randomisation

The trial dentifrices were coded and had the same external packaging, and their content was not known to the participants or the enrolling investigator. The dentifrice product codes were known only to the chief supervisor of the study (L.J.W) who was not involved in participant enrolment, product allocation or data analysis. The product codes were retained by the supervisor and broken only after final analysis of the study results. The randomisation allocation list was made by a single investigator (N.P) in SPSS version 25 (IBM, New York, NY, USA) using the random number generation function that equally distributed a third of the 90 trial subjects to each of the three coded trial groups. The same investigator enrolled participants and allocated the trial interventions to the study participants, without being aware of which dentifrice the individual trial participants had received.

Participants and Eligibility Criteria

The study protocol was approved by the institutional human research ethics committee (HREC/18/QPCH/7). The participants were child/teenage patients recruited from the orthodontic clinics of the University of Queensland School of Dentistry from July 2018 to October 2018.

The inclusion criteria for study participants were: (i) minimum of 10 years of age with at least 4 fully erupted permanent maxillary teeth; (ii) undergoing fixed orthodontic treatment in both arches with treatment having been underway for at least 1 month; (iii) not currently using antibiotics/antimicrobial mouth rinses; and (iv) available to attend a recall appointment in 5-6 weeks. The exclusion criteria were: (i) any medical condition or disability preventing self-tooth brushing; (ii) allergy to milk casein proteins or benzoate preservatives present in the CPP-ACP toothpaste; (iii) unwillingness to use a fluoridated toothpaste; and (iv) untreated periodontal disease or clinical evidence of active caries. All the study participants were given standardized oral

hygiene instructions and encouraged to use the allocated toothpaste for routine twice-daily tooth brushing throughout the trial period. Participant compliance with using the dentifrice was assessed based on the weight of the toothpaste tube at the end of the trial.

Dental Plaque Sample Collection

Dental plaque samples were collected from the study subjects at two time points - at a baseline visit just before the participants started using the trial toothpastes, and then at a recall visit scheduled after 5-6 weeks of using the allocated toothpastes. The plaque samples were collected by using a sterile microbrush to swab the labial and gingival third of the upper and lower anterior teeth. Sample were collected without removing any orthodontic modules, chain or ligature-ties. The microbrush tips were placed in 0.01% thiomersal (Sigma-Aldrich, St. Louis, MO, USA) solution and stored at -80°C until microbial analysis.

Microbial Analysis

The bacterial load of 14 selected bacterial species was determined using real-time quantitative PCR (qPCR) analysis. The bacterial panel included 8 caries-associated bacterial species (*Actinomyces gerensceriae*, *Lactobacillus gasseri*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus parasanguinis*, *Scardovia wiggsiae*, *Veillonella parvula* and *Prevotella denticola*), and 6 health-associated commensal bacterial species (*Streptococcus sanguinis*, *Streptococcus mitis*/*oralis*, *Steptococcus salivarius*/*thermophilus*, *Corynebacterium durum*, *Rothia aeria*/*dentocariosa* and *Neisseria flavescens*)

DNA extraction

DNA was extracted from the plaque samples using the MO BIO Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to manufacturer's instructions. RNA was removed using RNAse A (Thermo Fischer Scientific, Scoresby, Australia) and the amount of isolated DNA quantified spectrophotometrically.

16S rRNA sequencing

The bacterial load of the 14 selected bacterial species was determined using a custom-made qPCR array (16 x 24 format; Qiagen, Hilden, Germany). The assay used the 16S rRNA gene of the relevant bacterium, with the proprietary probes designed using the GreenGene version 13.8 database [DeSantis et al. 2006] for 16S sequences. Each DNA sample was mixed with a proprietary master mix and robotically dispensed into a 384-well plate (10µL, 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 (QuantStudio v1.3).

Statistical Analysis

The fold change of each bacterial amplicon was calculated using the comparative cycle threshold method (ΔΔCt). Briefly, for each sample, the Ct value of each bacterial target 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 trial group ΔΔCt for each bacterial species was then calculated as follows: $\Delta\Delta Ct^{\text{trial group}} = \Delta Ct^{\text{bacterial species (recall visit)}} - \Delta Ct^{\text{bacterial species (baseline visit)}}$. Fold increase, or decrease, in bacterial abundance from the baseline to recall visit for each trial group was calculated based on the formula: $2^{-\Delta\Delta Ct}$. For each bacterial species, one-way ANOVA was used to test for differences among the three trial groups using the ΔΔCt values [Yuan et al. 2006]. To adjust for the multiple bacterial comparisons, *P*-values were considered significant only if they were less than the Simes critical *P*-value [Simes 1986]. For bacterial species that showed significant ANOVA results, paired t-tests were performed, and the Bonferroni correction was used to assess for any significant differences between the treatment group pairs.

RESULTS

Age and gender distribution were comparable among the three trial groups (Table 14). At the baseline visit, there was no significant differences in the prevalence of any of the bacterial species across the treatment groups ($P > 0.05$). Except for 4 bacterial species (*L. gasseri*, *S. sobrinus*, *S. parasanguinis* and *S. wiggsiae*), all the other 10 bacterial species were highly prevalent in all groups at the baseline visit (Table 15).

Table 14. Demographic characteristics of the study population

	CPP-ACP Group	**All-in-One Group**	**Control Group**	***P*-values**
Mean Age ± S.D	14.33 ± 1.64	14.0 ± 1.55	14.03 ± 1.79	0.696
Female n (%)	19 (63%)	16 (53%)	19 (63%)	0.458

Table 15. Bacterial prevalence at the baseline visit across the three treatment groups

Bacterial Species	All Samples (n = 71)	CPP-ACP (n = 22)	All-in-One (n = 23)	Control (n = 26)	*P*-value
Actinomyces gerensceriae	70 (99%)	21 (96)	23 (100%)	26 (100%)	0.32
Corynebacterium durum	70 (99%)	21 (96)	23 (100%)	26 (100%)	0.32
Lactobacillus gasseri	13 (18%)	2 (9%)	4 (17%)	7 (27%)	0.28
Neisseria flavescens	63 (89%)	19 (86%)	22 (96%)	22 (85%)	0.44
Prevotella denticola	56 (79%)	17 (77%)	18 (78%)	21 (81%)	0.95
Rothia aeria/dentocariosa	71 (100%)	22 (100%)	23 (100%)	26 (100%)	δ
Streptococcus mitis/oralis	71 (100%)	22 (100%)	23 (100%)	26 (100%)	δ
Streptococcus mutans	54 (76%)	16 (73%)	18 (78%)	20 (77%)	0.90
Streptococcus parasanguinis	5 (7%)	0 (0%)	1 (4%)	4 (15%)	0.09
Streptococcus salivarius/ thermophilus	71 (100%)	22 (100%)	23 (100%)	26 (100%)	δ
Streptococcus sanguinis	71 (100%)	22 (100%)	23 (100%)	26 (100%)	δ
Streptococcus sobrinus	2 (2.8%)	0 (0%)	0 (0%)	2 (8%)	0.17
Scardovia wiggsiae	42 (59%)	13 (59%)	13 (57%)	16 (62%)	0.94
Veillonella parvula	71 (100%)	22 (100%)	23 (100%)	26 (100%)	δ

Baseline prevalence data showing number of samples in which the bacterial species was detected and values in brackets representing % prevalence. No significant differences between the treatment groups at the baseline visit ($P > 0.05$; chi-square test). δ: no statistics computed because bacterial prevalence was a constant.

The relative abundance of cariogenic and health-associated bacterial species at the baseline and recall visit, and the subsequent fold change in bacterial loads over time, are presented in the Tables 16 and 17 respectively. From the baseline to the recall visit, the treatment groups differed significantly in the mean changes in bacterial loads of two caries-associated species *V. parvula* ($P < 0.001$) and *S. mutans* ($P < 0.001$) (Table 18, Fig. 18A) and three health-associated bacterial species *C. durum* ($P = 0.008$), *N. flavescens* ($P = 0.005$), and *S. sanguinis* ($P < 0.001$) (Table 18, Fig. 18B). Bacterial loads of *V. parvula* and *S. mutans* decreased over time, both in the CPP-ACP group (mean fold decreases of 0.31 and 0.52 respectively) and in the All-in-One group (mean fold decreases of 0.11 and 0.23 respectively), and increased over time in the control group (mean fold increases of 1.10 and 2.51 respectively) (Table 18, Fig. 18A). Conversely, bacterial loads of *N. flavescens* and *S. sanguinis* increased over time in the CPP-ACP group (mean fold increases of 2.05 and 2.92

respectively) and in the All-in-One group (mean fold increases of 12.27 and 3.44 respectively), and decreased over time in the control group (mean fold decreases of 0.64 and 0.46 respectively). For *C. durum,* there were fold increases of 14.45, 6.11, and 1.92 in the CPP-ACP, All-in-One, and control groups respectively (Table 18, Fig. 18B). Mean changes in the loads of the other 9 bacterial species assessed did not differ between the treatment groups ($P > 0.05$) (Table 18).

Table 16. Relative abundance of each *caries-associated* bacterial species at the baseline and recall visits and the subsequent fold change in bacterial load over time

	ΔCt Baseline Visit			ΔCt Recall Visit			ΔΔCt			Fold change		
Bacterial Species	CPP-ACP	All-in-One	Control	CPP-ACP	All-in-One	Control	CPP-ACP	All-in-One	Control	CPP-ACP	All-in-One	Control
Veillonella parvula	-9.75	-9.50	-8.32	-8.06	-6.37	-8.46	1.68	3.13	-0.14	0.31	0.11	1.10
Streptococcus mutans	3.51	1.85	2.55	4.45	3.99	1.23	0.94	2.14	-1.33	0.52	0.23	2.51
Actinomyces gerensceriae	-4.33	-5.56	-4.60	-2.75	-4.46	-4.36	1.58	1.10	0.24	0.33	0.47	0.85
Streptococcus parasanguinis	9.55	9.27	7.97	10.40	10.59	9.42	0.85	1.32	1.45	0.56	0.40	0.37
Streptococcus sobrinus	9.55	9.86	9.44	10.26	10.59	9.92	0.71	0.73	0.48	0.61	0.60	0.72
Scardovia wiggsiae	5.37	4.94	5.09	6.18	4.92	4.49	0.81	-0.01	-0.60	0.57	1.01	1.52
Lactobacillus gasseri	9.01	8.63	9.13	9.10	9.04	9.21	0.09	0.41	0.08	0.94	0.75	0.95
Provotella denticola	-0.32	0.72	1.28	-1.45	-0.19	-0.96	-1.12	-0.92	-2.25	2.18	1.89	4.74

The Ct value of each bacterial target was normalized to the mean Ct value of all bacterial species ($\Delta Ct = Ct^{\text{bacteria species}} - Ct^{\text{mean of all bacterial species}}$). The ΔΔCt for each bacterial species was then calculated as follows: $\Delta\Delta Ct = \Delta Ct^{\text{bacterial species (recall visit)}} - \Delta Ct^{\text{bacterial species (baseline visit)}}$. Fold increase, or decrease, in bacterial abundance from the baseline to recall visit for each trial group was calculated based on the formula: $2^{-\Delta\Delta Ct}$. Ratio below 1 indicates lower relative abundance; a ratio above 1 indicates higher relative abundance

Table 17. Relative abundance of each *health-associated* bacterial species at the baseline and recall visits and the subsequent fold change in bacterial load over time

	ΔCt Baseline Visit			ΔCt Recall Visit			ΔΔCt			Fold change		
Bacterial Species	CPP-ACP	All-in-One	Control	CPP-ACP	All-in-One	Control	CPP-ACP	All-in-One	Control	CPP-ACP	All-in-One	Control
Corynebacterium durum	0.77	-0.81	-0.86	-3.09	-3.43	-1.80	-3.85	-2.61	-0.94	14.45	6.11	1.92
Neisseria flavescens	-0.06	1.14	1.58	-1.09	-2.48	2.23	-1.03	-3.62	0.65	2.05	12.27	0.64
Streptococcus sanguinis	-7.92	-7.27	-8.31	-9.47	-9.05	-7.19	-1.55	-1.78	1.12	2.92	3.44	0.46
Streptococcus mitis/oralis	-6.60	-6.13	-6.64	-6.34	-6.11	-6.03	0.26	0.02	0.62	0.84	0.98	0.65
Rothia aeria/dentocariosa	-5.63	-4.39	-6.00	-5.59	-5.12	-5.10	0.04	-0.73	0.89	0.97	1.65	0.54
Streptococcus salivarius/ thermophilus	-3.14	-2.74	-2.31	-2.56	-1.93	-2.59	0.59	0.81	-0.27	0.67	0.57	1.21

The Ct value of each bacterial target was normalized to the mean Ct value of all bacterial species ($\Delta Ct = Ct^{\text{bacteria species}} - Ct^{\text{mean of all bacterial species}}$). The ΔΔCt for each bacterial species was then calculated as follows: $\Delta\Delta Ct = \Delta Ct^{\text{bacterial species (recall visit)}} - \Delta Ct^{\text{bacterial species (baseline visit)}}$. Fold increase, or decrease, in bacterial abundance from the baseline to recall visit for each trial group was calculated based on the formula: $2^{-\Delta\Delta Ct}$. Ratio below 1 indicates lower relative abundance; a ratio above 1 indicates higher relative abundance.

Table 18. Comparison of treatment groups with regard to mean fold changes ($2^{-\Delta\Delta Ct}$) in bacterial loads of the caries-associated and health-associated bacterial species from baseline to recall visit

Bacterial Species	Treatment Groups			One-way ANOVA
	CPP-ACP	All-in-One	Control	*P*-values
Caries-associated				
Veillonella parvula	0.31 (0.14, 0.68)	0.11 (0.04, 0.32)	1.10 (0.68, 1.78)	< 0.001*
Streptococcus mutans	0.52 (0.23, 1.20)	0.23 (0.07, 0.71)	2.51 (1.30, 4.83)	< 0.001*
Actinomyces gerensceriae	0.33 (0.13, 0.85)	0.47 (0.22, 0.99)	0.85 (0.41, 1.75)	0.23
Streptococcus parasanguinis	0.56 (0.40, 0.77)	0.40 (0.15, 1.05)	0.37 (0.12, 1.15)	0.79
Streptococcus sobrinus	0.61 (0.45, 0.83)	0.60 (0.36, 1.01)	0.72 (0.42, 1.22)	0.83
Scardovia wiggsiae	0.57 (0.18, 1.81)	1.01 (0.36, 2.86)	1.52 (0.52, 4.43)	0.42
Lactobacillus gasseri	0.94 (0.46, 1.90)	0.75 (0.26, 2.20)	0.95 (0.56, 1.60)	0.89
Prevotella denticola	2.18 (0.42, 11.34)	1.89 (0.44, 8.01)	4.74 (1.46, 15.40)	0.58
Health-associated				
Corynebacterium durum	14.45 (6.59, 31.71)	6.11 (2.55, 14.64)	1.92 (0.66, 5.52)	0.008*
Neisseria flavescens	2.05 (0.71, 5.87)	12.27 (2.16, 69.68)	0.64 (0.23, 1.75)	0.005*
Streptococcus sanguinis	2.92 (1.61, 5.32)	3.44 (1.86, 6.37)	0.46 (0.28, 0.76)	< 0.001*
Streptococcus mitis/oralis	0.84 (0.36, 1.96)	0.98 (0.35, 2.77)	0.65 (0.34, 1.25)	0.77
Rothia aeria/dentocariosa	0.97 (0.51, 1.83)	1.65 (0.60, 4.53)	0.54 (0.37, 0.79)	0.07
Streptococcus salivarius/ thermophilus	0.67 (0.28, 1.60)	0.57 (0.27, 1.20)	1.21 (0.63, 2.33)	0.29

Mean fold change ($2^{-\Delta\Delta Ct}$) values (95% confidence intervals) for each bacterial species in the CPP-ACP group (n = 22), All-in-One group (n = 23) and control group (n = 26). Ratio below 1 indicates lower relative abundance; a ratio above 1 indicates higher relative abundance. * indicates where significant differences were found among the groups.

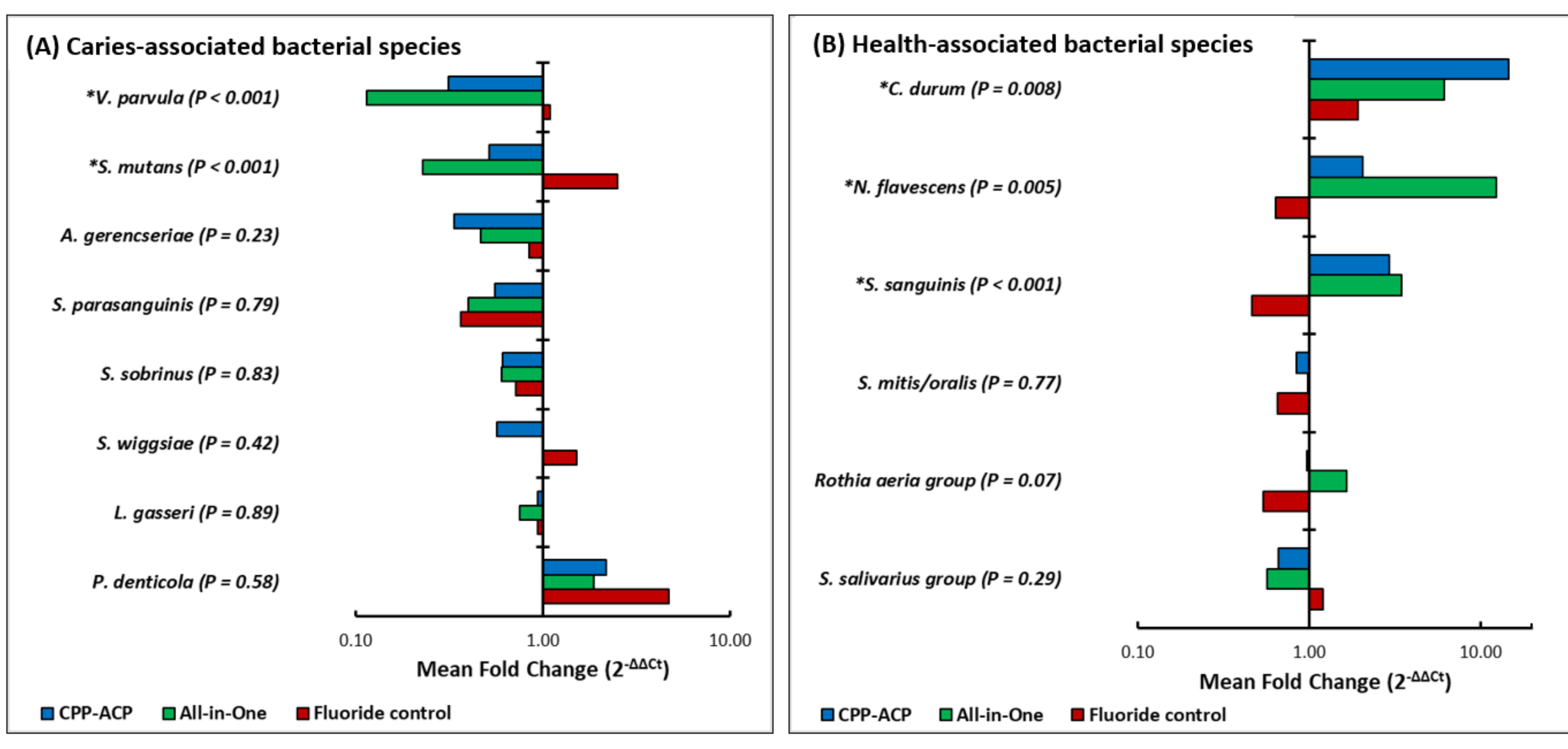


Figure 18 (A and B)**.** Fold change in relative bacterial abundance from baseline to recall visit

* indicates significant differences using the one-way ANOVA and Simes adjustment for the multiple bacterial comparisons

Post-hoc analysis revealed that the CPP-ACP group differed significantly from the control group with respect to mean changes in loads of *S. mutans* ($P = 0.032$), *C. durum* ($P = 0.007$) and *S. sanguinis* ($P < 0.001$), while the All-in-One group differed from the control group with respect to mean changes in loads of *S. mutans* ($P < 0.001$), *V. parvula* ($P < 0.001$), *N. flavescens* ($P = 0.003$) and *S. sanguinis* ($P < 0.001$) (Table 19). Compared to the CPP-ACP group, the All-in-One group generally showed greater mean fold decreases over time in the loads of the caries-associated bacteria, and higher mean fold increases over time in the loads of the health-associated bacteria (except for *C. durum*) (Fig. 18). However, these differences were not statistically significant ($P > 0.05$) (Table 19).

Table 19. Post-hoc pairwise comparisons between treatment groups

	P-values		
Bacterial species	**CPP-ACP versus Control**	**All-in-One versus Control**	**CPP-ACP versus All-in-One**
Caries-associated			
Veillonella parvula	0.061	< 0.001*	0.213
Streptococcus mutans	0.032*	< 0.001*	0.555
Health-associated			
Corynebacterium durum	0.007*	0.207	0.579
Neisseria flavescens	0.571	0.003*	0.158
Streptococcus sanguinis	< 0.001*	< 0.001*	1.00

Bonferroni correction was used to assess significance. * indicates where significant differences were found between the groups.

DISCUSSION

This clinical trial provides evidence that a CPP-ACP dentifrice can beneficially influence dental plaque microbial composition in a high caries-risk group. Specifically, we demonstrate species level changes in dental plaque after using dentifrices containing CPP-ACP with regards to two caries-associated bacterial species and three health-associated bacterial species. The addition of a highly purified polyphenol-rich cranberry extract to the CPP-ACP dentifrice gave a small enhancement to the microbial ecological effects of CPP-ACP, with the CPP-ACP – cranberry dentifrice significantly reducing *V. parvula* bacterial loads and significantly increasing *N. flavescens* bacterial loads compared to the fluoride control, effects that were not seen with the dentifrice containing CPP-ACP alone. This observation, combined with the fact the CPP-ACP – cranberry dentifrice effected a greater fold decrease in *S. mutans* levels and a higher fold increase in *S. sanguinis* levels than the CPP-ACP dentifrice, suggests the possibility that greater beneficial effects from the combination dentifrice could be seen if it was used for longer period, or a higher concentration of cranberry extract was used.

The proposed mechanisms for the microbial ecological effects of CPP-ACP observed in this study could be related to its buffering and anti-adhesion effects [Philip and Walsh, 2019]. Various clinical studies have reported that regular use of CPP-ACP topical crèmes had pronounced buffering influences on salivary and plaque pH [Caruana *et al.,* 2009; Heshmat *et al.,* 2014a; Heshmat *et al.,* 2014b; Marchisio *et al.,* 2010; Ozdas *et al.,* 2015; Peric *et al.,* 2015]. The buffering effects of CPP-ACP are due to its ability to act as a reservoir of peptides and phosphate ions. The catabolism of peptides by plaque peptidases would buffer against a pH fall through the production of ammonia (Reynolds and Riley 1989). CPP phosphoseryl residues that are more resistant to hydrolysis can readily accept protons and neutralize plaque acids (Reynolds 1987). Furthermore, the inorganic phosphate (PO_4^{3-}) and organic phosphate ($-O-PO_3^{2-}$) ions in the CPP-ACP nanocomplex also contribute to elevating salivary/plaque pH (Dashper et al. 2018). The buffering actions of CPP-ACP can result in a homeostatic oral environment that favours the growth of health-associated commensals. Besides its buffering effects, CPP-ACP is also known to inhibit bacterial co-aggregation and accumulation in dental plaque by binding to bacterial surfaces (with a strong affinity for *S. mutans*) and pellicle macromolecules (Reynolds et al. 2003; Rose 2000). In the present study, the CPP-ACP dentifrice effected a decrease in the bacterial load of *S. mutans* and an increase in bacterial loads of *C. durum* and *S. sanguinis.* These results can possibly be attributed to the anti-adhesion and buffering influences of CPP-ACP on the plaque biofilm.

Polyphenol-rich cranberry extracts and purified cranberry A-PACs have demonstrated potent inhibitory effects against *S. mutans* glycolytic, F_1F_0-ATPase, and glucosyltransferase (Gtf) enzymes, while also disrupting the structural architecture of *in vitro* biofilms [Duarte *et al.,* 2006; Gregoire *et al.,* 2007; Koo *et al.,* 2006; Philip *et al.,* 2019a, b]. The ability of cranberry polyphenols to influence key cariogenic virulence properties without affecting microbial viability make them potentially ideal agents to lower cariogenic virulence and improve the microbial ecological balance of dental plaque. The fact that the combination dentifrice showed the highest fold decreases in the cariogenic *S. mutans* and *V. parvula* bacterial species, suggests that the virulence-inhibitory effects of the polyphenol-rich cranberry extract extended to a clinical environment too.

The two bacterial species (*S. mutans* and *V. parvula*) that showed significant decreases in their bacterial loads have both been strongly associated with dental caries. *S. mutans*, even when present in low numbers, is still considered a keystone pathogen in the disease process as it is largely responsible for the initial assembly of the cariogenic glucan-rich biofilm matrix (Bowen et al. 2018).

Other acidogenic and aciduric bacteria lack the specific Gtfs to synthesize insoluble glucans that are considered the foundational building blocks of cariogenic biofilms (Hajishengallis et al. 2017). The *S. mutans*-derived Gtf exoenzymes orchestrate the virulent glucan-rich exopolysaccharide matrix scaffold, paving the way for other resident aciduric bacteria (e.g. lactobacilli, bifidobacteria, *Scardovia* spp. etc.) to dominate the microbiome as the plaque biofilm matures (Bowen et al. 2018; Burne 2018). *Veillonella*, an obligate anaerobic Gram-negative bacterium, is considered a bridge organism in the biofilm [Knapp *et al.,* 2017], and is commonly isolated from initial lesions [Aas *et al.,* 2005; Becker *et al.,* 2002; Gross *et al.,* 2012]. It was earlier perceived that presence of this bacterium in plaque may be beneficial as it neutralizes acidic pH by utilizing lactic acid as its nutritional source of energy. However, evidence from recent microbiome studies confirm that *Veillonella* is strongly associated with caries lesions [Jiang *et al.,* 2016; Richards *et al.,* 2017; Tanner *et al.,* 2016; Zhou *et al.,* 2016]. *Veillonella* was seen to promote *S. mutans* growth even in the presence of antagonistic *Streptococcus gordonii*, while the acetic acid produced from *Veillonella* lactate catabolism can also demineralize enamel (Bowen et al. 2018). Metatranscriptomic studies reveal that *Veillonella* is positively stimulated by low-pH conditions and exhibits intracellular pH control mechanisms (Do et al. 2015; Edlund et al. 2015). Furthermore, even aciduric organisms show reduced growth when pH drops below 4.5, and *Veillonella* can help the acidogenic microbial community by maintaining a relatively more neutral pH (Tanner et al. 2016).

In the present study, three health-associated bacterial species (*S. sanguinis, C. durum* and *N. flavescens*) showed significant increases in their bacterial loads from the baseline to the recall visit. These commensals have various strategies to foster an oral biofilm environment that discourages the emergence and dominance of opportunistic cariogenic pathogens. *S. sanguinis* belongs to a group of oral commensals known to possess the arginine deaminase system (ADS) that can neutralize acids produced from carbohydrate metabolism by generating ammonia from prebiotic dietary and salivary substrates [Nascimento, 2018]. The ammonia produced not only alkalizes the bacterial cytoplasm but also gets released outside the cell promoting an overall elevation in plaque pH. This creates an environment that supports the growth of other acid-sensitive commensals. Moreover, endogenous ammonia production promotes stability of health-associated biofilms by affording bioenergetic advantages to the commensal bacteria [Burne, 2018]. The bacterial species *C. durum* has an established nitrate (NO_3^-) reductase system which rapidly reduces salivary NO_3^- to nitrite (NO_2^-) as part of its respiration. In a low pH environment, NO_2^- dissociates to form a range of nitrogen oxides, most notably nitric oxide (NO). The NO_3^--NO_2^--NO reduction pathway can limit the

growth of cariogenic bacteria because of the antimicrobial effects of NO [Doel *et al.,* 2004]. *Neisseria* is a catalase-positive aerobic species that has been identified as part of the healthy "core microbiome" of the human oral cavity [Zaura *et al.,* 2009]. *Neisseria flavescens* is known to be asaccharolytic and has been associated with caries-free status in next-generation sequencing studies of saliva and plaque samples [Gross *et al.,* 2012; Richards *et al.,* 2017].

Prudency is required when interpreting the results of this study. Further studies are required to know whether the beneficial plaque microbial changes shown here can translate into lowering the caries increment in a high-risk population group, which should be the only criteria to judge the efficacy of new anti-caries agents. While the microarray and taxon-specific qPCR techniques employed in this study allowed quantification of multiple high-interest bacterial species from dental plaque, it does not make an evaluation of the numerous other microorganisms present in dental plaque. Moreover, the taxonomic composition of plaque samples alone does not give complete information about the functional output of plaque microbial communities or their role in health and disease [Takahashi, 2015]. Future research using metagenomic and metatranscriptomic analysis tools can provide genetic information of the whole microbial community and provide a better understanding of the metabolic output and functional profile of oral microbial communities.

There is now evidence that routine provision of interventions that deliver small but relevant benefits can support the maintenance of a symbiotic oral microbiome [Marsh, 2018]. A recent RCT demonstrated that that a toothpaste containing enzymes and proteins promoted an overall community shift by increasing the relative abundance of bacteria associated with gingival health and decreasing those associated with periodontal disease [Adams *et al.,* 2017]. The rationale for applying similar ecological approaches to dental caries prevention is apparent, and various strategies are currently under investigation [Philip *et al.,* 2018]. This study suggests the possibility of using milk-derived CPP-ACP and polyphenol-rich cranberry extracts in daily use oral care products to ecologically modulate plaque microbial communities.

CONCLUSIONS

Overall, the results of this clinical study indicate CPP-ACP delivered in a dentifrice exerts useful microbial ecological effects, and this is enhanced somewhat by including a polyphenol-rich cranberry extract as well. In the past two decades, CPP-ACP topical crèmes and chewing gums have been in use globally for its remineralizing actions. The present study shows that twice-daily brushing

with a CPP-ACP dentifrice can provide an additional ecological benefit that could potentially translate into lowering caries-risk. Further studies will be needed to resolve whether polyphenol-rich cranberry extracts or other additives can enhance the ecological effects of CPP-ACP. Finally, it should be emphasized that microbial ecology-based therapeutics are only one component within a holistic caries preventive strategy that should primarily aim to reverse the environmental stresses responsible for the dental plaque dysbiosis.

RAW DATA

Study 6: qPCR Cycle threshold (Ct) Data

Trial Group A Ct data (Baseline and Recall Visit)

Visit	Treatment_Grp	Sample_ID	Ct *A. gerencseriae*	Ct *C. durum*	Ct *L. Gasseri*	Ct *N. flavescens*	Ct *P. denticola*	Ct *Rothia spp*	Ct *S. wiggsiae*	Ct *Streptococcus spp 3*	Ct *S. mitis*	Ct *S. mutans*	Ct *S. parasanguinis*	Ct *S. sanguinis*	Ct *S. sobrinus*	Ct *V. parvula*
Baseline	Group A	103	22.90	29.25	40.00	40.00	40.00	23.11	27.74	25.26	26.31	30.43	40.00	24.72	40.00	24.57
Baseline	Group A	105	25.06	32.74	35.24	32.61	29.81	24.63	31.12	26.70	26.52	27.76	40.00	23.30	40.00	24.62
Baseline	Group A	109	24.95	25.83	40.00	23.30	26.35	22.84	33.16	25.39	24.83	26.68	27.68	20.49	40.00	22.49
Baseline	Group A	114	22.12	30.90	40.00	29.97	33.09	29.75	40.00	28.34	23.50	35.26	40.00	22.31	40.00	22.41
Baseline	Group A	115	26.23	29.81	40.00	34.13	40.00	27.60	40.00	27.68	23.19	40.00	29.69	23.67	40.00	21.21
Baseline	Group A	119	22.67	26.80	40.00	27.89	31.20	25.98	40.00	27.45	24.16	30.05	40.00	22.20	40.00	21.15
Baseline	Group A	129	22.61	35.90	37.50	33.24	40.00	20.40	25.18	27.50	23.69	26.26	40.00	21.30	40.00	19.71
Baseline	Group A	130	23.91	24.98	40.00	27.18	22.37	24.20	33.25	23.65	20.15	23.33	22.84	19.12	40.00	19.00
Baseline	Group A	131	25.94	34.97	40.00	19.07	32.24	23.04	40.00	33.49	20.31	34.79	40.00	22.14	40.00	19.99
Baseline	Group A	134	23.15	24.68	40.00	29.02	37.85	22.60	35.93	33.34	24.08	40.00	40.00	22.21	40.00	25.67
Baseline	Group A	135	34.15	25.48	40.00	25.81	33.98	24.37	40.00	29.55	22.66	32.23	40.00	23.83	40.00	25.13
Baseline	Group A	137	28.14	25.46	40.00	28.34	23.83	23.41	31.21	28.58	21.81	40.00	40.00	22.16	40.00	20.06
Baseline	Group A	139	24.21	25.97	40.00	27.80	22.96	25.04	35.40	28.23	23.80	40.00	40.00	20.49	40.00	24.10
Baseline	Group A	144	27.86	35.98	35.15	36.83	20.53	25.46	27.24	29.75	23.48	34.22	40.00	23.59	40.00	18.18
Baseline	Group A	145	22.12	25.74	40.00	28.26	25.75	23.20	36.81	26.87	23.51	33.30	40.00	20.70	40.00	21.89
Baseline	Group A	149	22.72	26.46	40.00	36.61	34.38	22.53	40.00	28.24	22.11	29.08	40.00	22.25	40.00	22.47
Baseline	Group A	150	27.00	35.98	35.72	34.79	28.01	22.55	33.82	24.70	21.97	29.90	40.00	19.44	30.09	19.48
Baseline	Group A	152	23.01	28.47	40.00	22.47	34.24	25.36	40.00	27.35	23.55	40.00	40.00	21.63	40.00	19.47
Baseline	Group A	154	29.70	24.09	40.00	32.01	33.59	21.73	27.91	26.91	29.14	24.98	40.00	20.76	40.00	21.82
Baseline	Group A	158	30.55	27.76	40.00	36.08	29.38	25.92	40.00	27.76	20.68	40.00	40.00	20.89	40.00	21.05
Baseline	Group A	161	26.39	34.96	40.00	25.30	28.86	24.64	40.00	27.72	20.61	30.35	40.00	21.23	40.00	21.16
Baseline	Group A	166	22.76	29.78	38.34	40.00	31.32	24.54	29.72	26.20	28.39	31.81	40.00	23.08	40.00	28.88
Baseline	Group A	179	24.97	31.09	36.19	40.00	24.21	24.15	33.68	27.67	23.82	26.34	26.31	20.96	40.00	19.22
Baseline	Group A	186	22.68	24.81	38.49	40.00	28.61	20.40	35.81	27.72	21.09	37.66	40.00	21.42	40.00	19.99
Baseline	Group A	189	31.28	34.89	40.00	30.13	40.00	24.07	33.57	25.42	21.21	30.06	40.00	18.34	34.52	19.65
Baseline	Group A	190	22.42	24.01	40.00	39.46	40.00	21.73	40.00	27.64	21.95	31.07	40.00	20.95	40.00	19.43
Recall	Group A	103	21.71	29.97	40.00	40.00	40.00	24.44	28.66	26.40	23.54	27.59	40.00	19.87	40.00	22.91
Recall	Group A	105	21.72	32.49	33.13	40.00	28.39	25.69	27.92	25.72	24.18	24.16	40.00	22.20	40.00	21.13
Recall	Group A	109	23.97	27.41	40.00	23.51	21.54	23.48	30.64	23.47	22.54	24.01	25.65	22.11	40.00	20.61
Recall	Group A	114	22.26	29.14	40.00	23.87	28.13	27.56	40.00	27.04	21.78	33.96	40.00	21.83	40.00	20.88
Recall	Group A	115	23.34	30.93	40.00	31.90	38.86	26.59	40.00	31.67	22.33	40.00	32.33	23.59	40.00	20.60
Recall	Group A	119	21.25	26.78	40.00	28.62	26.66	24.68	40.00	27.48	25.51	28.19	40.00	24.47	40.00	22.26
Recall	Group A	129	24.84	25.34	40.00	39.05	40.00	21.27	25.59	23.15	21.40	27.01	40.00	18.54	40.00	20.28
Recall	Group A	130	21.69	30.33	40.00	33.96	17.80	26.41	26.36	21.32	23.94	23.76	40.00	23.70	40.00	20.00
Recall	Group A	131	28.15	29.92	40.00	24.29	30.97	28.29	40.00	29.89	25.88	35.64	40.00	25.26	40.00	22.61
Recall	Group A	134	25.89	23.76	40.00	27.55	34.87	22.00	40.00	33.09	24.60	40.00	40.00	25.37	40.00	27.67
Recall	Group A	135	34.03	24.09	40.00	25.44	29.22	22.10	40.00	24.98	25.04	32.64	40.00	24.11	40.00	24.24
Recall	Group A	137	27.54	25.88	40.00	26.20	30.24	24.57	35.80	25.68	21.43	37.72	40.00	23.51	40.00	20.11
Recall	Group A	139	24.91	26.22	40.00	26.33	24.19	24.99	40.00	28.73	24.39	37.72	40.00	22.09	40.00	24.53
Recall	Group A	144	25.55	30.97	40.00	38.20	20.93	26.04	26.65	32.42	24.45	33.77	40.00	26.26	40.00	20.83
Recall	Group A	145	22.63	29.49	40.00	25.98	24.65	26.31	32.19	28.50	28.64	32.11	40.00	25.48	40.00	24.42
Recall	Group A	149	26.30	26.30	37.23	37.72	33.43	23.96	35.88	31.00	24.94	30.36	40.00	23.76	40.00	23.38
Recall	Group A	150	28.61	25.95	37.84	35.60	23.78	23.64	33.12	28.60	21.18	29.24	40.00	18.18	33.12	18.02
Recall	Group A	152	24.70	30.99	40.00	24.06	27.23	26.30	35.50	26.01	23.86	31.30	40.00	22.43	40.00	22.71
Recall	Group A	154	33.56	23.66	40.00	40.00	26.50	23.76	30.04	28.31	23.75	28.73	40.00	22.45	40.00	20.65
Recall	Group A	158	30.03	28.70	40.00	31.39	34.63	28.01	36.30	28.57	23.70	37.66	40.00	23.01	40.00	21.40
Recall	Group A	161	27.28	26.97	40.00	24.48	25.87	24.97	33.96	25.11	23.03	29.80	40.00	20.32	40.00	19.19
Recall	Group A	166	24.16	27.13	36.19	38.74	27.74	22.92	29.79	24.13	25.34	28.58	40.00	22.44	40.00	22.18
Recall	Group A	179	24.85	29.03	31.07	40.00	21.76	23.24	25.46	26.71	24.94	22.37	40.00	20.92	40.00	17.86
Recall	Group A	186	27.04	28.59	37.04	40.00	28.77	21.56	35.78	25.96	23.40	38.53	40.00	23.73	37.72	19.26
Recall	Group A	189	22.23	30.03	40.00	31.27	21.80	24.41	40.00	23.97	21.62	21.47	40.00	20.57	40.00	18.37
Recall	Group A	190	21.31	26.13	40.00	32.76	40.00	23.08	40.00	27.87	20.93	28.53	40.00	19.80	40.00	16.94

Study 6: qPCR Cycle threshold (Ct) Data

Trial Group B Ct data (Baseline and Recall Visit)

Visit	Treatment_Grp	Sample_ID	Ct *A. gerencseriae*	Ct *C. durum*	Ct *L. Gasseri*	Ct *N. flavescens*	Ct *P. denticola*	Ct *Rothia spp*	Ct *S. wiggsiae*	Ct *Streptococcus spp 3*	Ct *S. mitis*	Ct *S. mutans*	Ct *S. parasanguinis*	Ct *S. sanguinis*	Ct *S. sobrinus*	Ct *V. parvula*
Baseline	Group B	102	22.90	31.09	40.00	40.00	24.52	26.61	34.41	29.16	25.36	24.30	40.00	22.20	40.00	21.10
Baseline	Group B	108	24.42	31.14	40.00	34.82	32.95	28.81	34.23	27.81	25.79	30.05	40.00	22.86	40.00	24.44
Baseline	Group B	117	25.60	33.85	40.00	27.62	40.00	24.18	40.00	26.24	23.89	40.00	40.00	21.57	40.00	21.19
Baseline	Group B	118	24.85	29.14	40.00	27.15	24.21	22.07	33.50	23.93	23.00	30.22	40.00	20.69	40.00	20.94
Baseline	Group B	127	40.00	25.94	37.55	26.51	29.90	23.24	40.00	28.36	22.74	26.19	40.00	21.26	40.00	18.36
Baseline	Group B	128	27.31	40.00	40.00	36.89	36.15	21.13	40.00	34.21	22.50	40.00	40.00	23.99	40.00	20.95
Baseline	Group B	136	25.43	32.76	40.00	27.40	25.13	28.38	33.35	31.05	27.25	32.85	40.00	25.26	40.00	22.06
Baseline	Group B	138	23.46	29.92	40.00	22.50	29.04	23.27	36.15	30.38	23.96	40.00	40.00	23.87	40.00	23.71
Baseline	Group B	148	24.52	31.29	40.00	35.19	25.96	27.95	40.00	30.25	21.46	32.90	40.00	22.72	40.00	18.57
Baseline	Group B	151	24.72	30.29	40.00	22.65	21.22	26.77	33.87	25.87	22.80	32.83	40.00	20.56	40.00	19.92
Baseline	Group B	153	25.67	28.37	40.00	28.85	25.14	22.89	32.46	26.68	24.79	32.82	40.00	19.98	40.00	21.99
Baseline	Group B	155	32.81	27.51	40.00	28.62	40.00	23.44	40.00	27.28	21.59	35.66	40.00	23.91	40.00	18.82
Baseline	Group B	156	23.40	32.02	40.00	23.82	24.67	24.79	35.69	25.63	23.85	35.46	40.00	23.15	40.00	23.14
Baseline	Group B	168	26.91	29.98	30.55	40.00	40.00	22.30	40.00	24.95	24.90	40.00	40.00	22.57	40.00	20.03
Baseline	Group B	172	24.58	33.87	40.00	31.29	28.79	24.75	35.32	21.54	27.53	33.16	40.00	24.93	40.00	18.18
Baseline	Group B	173	31.22	34.96	40.00	35.16	29.75	20.92	40.00	21.98	25.82	30.18	40.00	22.81	40.00	19.46
Baseline	Group B	174	25.14	29.12	40.00	24.83	40.00	27.18	31.15	27.10	18.45	40.00	40.00	18.50	40.00	16.70
Baseline	Group B	180	26.38	27.73	40.00	27.40	32.03	23.66	40.00	32.01	21.59	38.13	40.00	19.95	40.00	21.92
Baseline	Group B	181	24.09	32.67	40.00	22.25	26.51	26.65	40.00	25.05	22.15	33.57	40.00	23.77	40.00	20.33
Baseline	Group B	184	21.44	29.98	40.00	37.15	40.00	24.90	30.68	26.17	23.81	40.00	40.00	22.91	40.00	18.96
Baseline	Group B	185	24.11	35.56	40.00	40.00	23.04	29.23	27.20	28.72	28.67	37.09	40.00	24.95	40.00	25.52
Baseline	Group B	188	25.79	29.63	40.00	28.54	23.83	22.88	29.96	26.43	22.95	21.77	40.00	23.28	40.00	19.25
Recall	Group B	102	23.92	27.04	36.81	36.54	26.55	23.55	30.39	28.26	26.30	24.20	40.00	22.81	40.00	21.61
Recall	Group B	108	25.24	25.02	40.00	27.44	27.23	25.93	34.78	22.85	22.23	30.95	40.00	20.18	40.00	21.75
Recall	Group B	117	24.58	28.89	40.00	25.71	40.00	27.51	40.00	28.85	25.48	40.00	40.00	19.31	40.00	24.35
Recall	Group B	118	23.87	26.73	40.00	25.32	24.48	24.18	35.99	27.49	21.40	31.13	40.00	22.67	40.00	21.76
Recall	Group B	127	40.00	23.35	36.30	25.30	29.81	22.42	40.00	26.86	20.54	28.39	40.00	20.17	40.00	19.13
Recall	Group B	128	28.74	30.42	40.00	31.12	33.78	20.29	40.00	28.75	23.60	40.00	40.00	20.75	40.00	24.17
Recall	Group B	136	36.85	32.73	40.00	24.12	20.63	27.96	40.00	27.67	22.77	40.00	40.00	18.79	40.00	22.94
Recall	Group B	138	25.80	24.48	40.00	18.46	26.21	22.24	40.00	30.17	22.81	34.95	40.00	21.13	40.00	23.27
Recall	Group B	148	22.97	26.96	35.98	40.00	25.32	23.85	40.00	27.66	20.07	35.29	40.00	20.24	40.00	17.91
Recall	Group B	151	23.25	27.15	40.00	21.73	22.26	24.48	32.11	25.82	26.24	32.46	40.00	19.02	40.00	22.15
Recall	Group B	153	23.11	23.40	40.00	22.47	33.89	23.19	40.00	28.79	24.45	40.00	40.00	18.34	40.00	23.25
Recall	Group B	155	30.80	22.39	40.00	34.45	27.76	20.78	40.00	27.51	20.86	32.20	40.00	19.81	40.00	19.77
Recall	Group B	156	23.59	25.57	40.00	22.68	28.75	24.09	35.63	27.65	21.69	33.44	40.00	21.92	40.00	20.87
Recall	Group B	168	32.21	26.52	35.96	40.00	40.00	22.32	40.00	26.95	27.14	39.07	40.00	20.01	40.00	24.28
Recall	Group B	172	23.04	25.65	40.00	27.32	23.44	23.51	40.00	22.28	19.84	28.92	40.00	19.02	40.00	17.30
Recall	Group B	173	33.37	24.21	36.68	32.33	30.60	21.57	30.73	26.84	24.68	29.12	40.00	18.72	40.00	21.61
Recall	Group B	174	25.93	25.46	40.00	23.01	40.00	22.98	32.00	26.01	20.08	39.68	40.00	18.75	40.00	17.77
Recall	Group B	180	26.73	27.67	35.74	22.71	26.72	25.50	35.19	25.70	21.26	34.04	40.00	19.00	40.00	21.05
Recall	Group B	181	26.46	26.66	33.96	25.76	26.18	27.05	34.48	27.98	28.19	37.18	40.00	20.02	40.00	25.90
Recall	Group B	184	23.26	22.69	40.00	31.57	19.03	26.96	27.84	29.48	25.27	37.81	40.00	19.15	40.00	24.84
Recall	Group B	185	23.76	30.43	40.00	37.00	21.69	26.36	28.63	25.24	22.35	38.35	40.00	20.40	40.00	18.68
Recall	Group B	188	23.35	29.95	40.00	32.18	25.13	21.58	29.45	26.26	24.57	22.06	40.00	22.77	40.00	19.51

Study 6: qPCR Cycle threshold (Ct) Data

Trial Group C Ct data (Baseline and Recall Visit)

Treatment_Grp	Sample_ID	Ct *A. gerencseriae*	Ct *C. durum*	Ct *L. Gasseri*	Ct *N. flavescens*	Ct *P. denticola*	Ct *Rothia spp*	Ct *S. wiggsiae*	Ct *Streptococcus spp 3*	Ct *S. mitis*	Ct *S. mutans*	Ct *S. parasanguinis*	Ct *S. sanguinis*	Ct *S. sobrinus*	Ct *V. parvula*
Group C	101	22.51	32.55	29.49	40.00	27.65	26.85	29.44	22.77	24.72	21.00	40.00	23.80	40.00	19.49
Group C	104	29.29	34.20	40.00	27.31	32.48	28.31	32.09	29.68	25.97	40.00	40.00	25.15	40.00	21.22
Group C	110	27.34	31.18	40.00	32.46	31.31	34.75	29.84	28.99	34.04	34.56	40.00	26.58	40.00	26.98
Group C	112	30.60	30.98	40.00	30.22	30.66	26.96	35.37	34.78	31.46	28.78	40.00	24.45	40.00	20.14
Group C	113	27.45	31.14	40.00	27.49	27.78	27.74	40.00	28.93	26.11	40.00	40.00	26.52	40.00	20.91
Group C	120	23.81	27.14	30.96	28.53	40.00	24.58	19.91	24.40	25.67	23.95	40.00	23.09	40.00	19.09
Group C	123	22.45	32.10	40.00	37.92	40.00	25.06	33.36	25.69	22.75	26.36	26.54	21.37	40.00	18.86
Group C	124	23.26	27.68	40.00	35.17	31.27	27.33	35.31	27.04	18.58	40.00	40.00	22.03	40.00	23.56
Group C	126	20.90	31.75	40.00	27.67	40.00	25.37	31.65	23.99	21.92	24.13	40.00	20.01	40.00	17.20
Group C	133	22.60	29.83	40.00	31.55	22.23	22.81	31.22	23.36	22.19	28.07	40.00	21.62	40.00	20.48
Group C	140	26.72	26.33	40.00	26.03	38.23	25.00	40.00	30.58	20.52	40.00	40.00	20.06	40.00	18.93
Group C	142	23.15	22.97	40.00	25.33	24.11	20.83	29.78	24.93	20.92	39.59	40.00	20.81	40.00	20.52
Group C	143	35.14	28.76	40.00	26.88	22.61	29.63	40.00	27.40	25.76	26.74	40.00	23.88	40.00	21.25
Group C	146	20.52	25.20	40.00	31.96	24.19	25.62	32.40	27.75	21.98	31.29	40.00	20.08	40.00	19.64
Group C	160	20.92	35.24	40.00	32.90	40.00	31.26	40.00	29.19	23.12	40.00	40.00	23.87	40.00	22.64
Group C	163	19.85	24.04	40.00	33.83	30.50	18.81	40.00	25.60	21.45	25.22	40.00	22.89	40.00	18.43
Group C	164	24.51	23.91	40.00	32.77	34.08	20.87	40.00	25.14	24.43	28.00	40.00	19.86	40.00	22.16
Group C	165	22.48	27.63	40.00	35.24	27.77	23.40	36.76	29.31	24.63	37.32	40.00	24.42	40.00	22.42
Group C	170	27.44	34.95	40.00	35.89	29.75	29.28	40.00	30.97	22.78	38.21	40.00	21.79	40.00	20.48
Group C	177	24.34	30.43	40.00	30.08	25.19	26.65	40.00	27.39	22.26	34.02	40.00	23.32	40.00	19.20
Group C	178	22.23	31.40	33.97	34.01	28.15	21.43	29.70	24.88	23.88	24.80	40.00	23.91	40.00	20.42
Group C	182	23.56	26.70	40.00	29.74	40.00	26.13	40.00	31.27	23.44	37.88	40.00	24.29	40.00	22.10
Group C	183	24.33	28.44	37.34	26.46	21.96	23.56	40.00	26.11	23.61	25.99	40.00	22.35	40.00	18.75
Group C	101	24.46	30.12	40.00	29.37	26.47	24.39	31.78	27.34	22.48	30.98	40.00	19.60	40.00	18.94
Group C	104	22.74	31.49	40.00	27.80	24.21	25.35	34.46	25.67	21.04	37.35	40.00	21.90	40.00	20.16
Group C	110	25.14	23.02	40.00	26.01	27.83	23.75	28.09	29.54	23.88	37.32	40.00	22.09	40.00	40.00
Group C	112	27.41	23.09	40.00	20.38	29.45	22.94	31.82	30.27	22.89	30.88	40.00	20.93	40.00	22.14
Group C	113	25.43	29.85	40.00	24.34	26.80	25.83	40.00	27.77	24.81	40.00	40.00	20.03	40.00	24.28
Group C	120	23.01	24.01	31.41	20.02	40.00	22.43	21.89	23.99	21.35	23.48	40.00	19.24	40.00	22.58
Group C	123	23.12	24.16	32.01	40.00	29.61	19.50	32.78	26.13	24.11	40.00	40.00	18.40	40.00	19.26
Group C	124	22.59	27.87	40.00	21.78	40.00	25.14	40.00	34.08	25.88	40.00	40.00	19.43	40.00	23.63
Group C	126	23.96	25.66	40.00	34.75	40.00	22.52	27.70	24.49	22.14	24.34	40.00	20.21	40.00	23.47
Group C	133	24.12	25.37	40.00	18.56	27.94	20.61	25.20	27.53	19.43	30.08	40.00	19.48	40.00	22.57
Group C	140	23.11	26.04	40.00	24.85	32.74	27.85	40.00	28.94	25.98	39.03	40.00	24.20	40.00	24.45
Group C	142	28.44	22.69	40.00	28.47	28.94	20.21	35.33	24.33	22.54	40.00	40.00	20.08	40.00	21.52
Group C	143	40.00	26.65	40.00	22.12	23.63	25.16	40.00	23.35	23.56	30.34	40.00	21.30	40.00	23.32
Group C	146	27.32	23.92	40.00	40.00	30.94	36.93	40.00	34.16	29.76	37.70	40.00	19.68	40.00	26.82
Group C	160	19.81	28.37	40.00	31.73	40.00	26.73	40.00	26.88	22.43	38.85	40.00	20.29	40.00	22.95
Group C	163	21.89	23.17	40.00	22.35	26.33	20.10	33.72	25.14	21.14	24.14	40.00	18.55	40.00	23.81
Group C	164	22.17	23.53	40.00	32.56	33.64	22.88	36.41	27.43	24.39	27.67	40.00	19.13	40.00	20.74
Group C	165	22.72	25.65	40.00	26.63	27.52	24.94	40.00	29.91	19.27	35.88	40.00	22.51	40.00	22.52
Group C	170	27.48	26.44	40.00	22.31	26.92	24.40	40.00	27.86	24.92	36.22	40.00	20.32	40.00	21.68
Group C	177	23.71	27.95	40.00	24.55	21.76	24.88	32.71	30.25	24.10	32.58	40.00	21.05	40.00	21.00
Group C	178	26.36	26.02	28.66	36.78	21.93	25.22	30.69	23.48	24.71	25.10	40.00	19.07	40.00	20.74
Group C	182	23.87	27.28	40.00	18.91	22.90	22.72	33.40	26.67	21.97	33.69	40.00	19.41	40.00	20.34
Group C	183	25.01	25.24	32.23	25.08	22.42	24.14	33.67	26.78	23.05	32.50	40.00	21.37	40.00	22.98

5. GENERAL DISCUSSION

The purpose of this thesis was to develop natural approaches to attenuate cariogenic biofilm virulence and maintain a symbiotic oral microbiome. The previous chapters have reviewed the literature and presented results from the *in vitro* and clinical studies investigating the effects of polyphenol-rich natural extracts and milk-derived CPP-ACP on microbial biofilms. The work in this thesis has addressed several gaps in the literature and provided insights about the potential of using natural products and CPP-ACP to ecologically modulate dental biofilms and thereby lower caries-risk. This chapter presents an overview of the study in relation to current knowledge, discusses how the research gaps identified have been addressed, and proposes recommendations for future research. Figure 19 broadly summarizes the gaps in knowledge, results of the thesis and directions for future work.

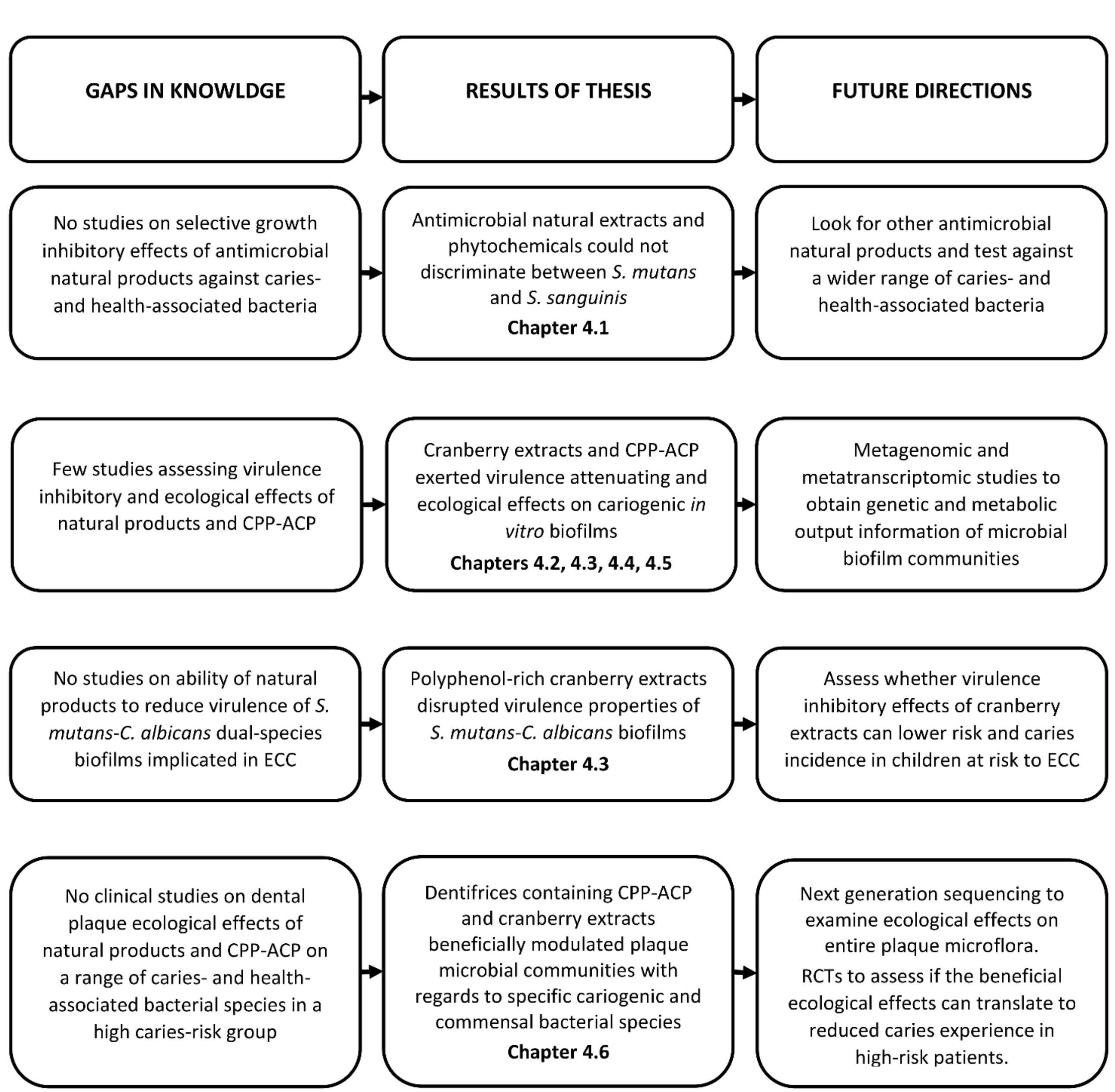


Figure 19. Summary of research gaps, thesis results and future directions

RESEARCH OVERVIEW

Prevention and management of dental caries is remarkably different from other infectious diseases. While dental caries is essentially a microbially induced disease caused by bacterial metabolites, it cannot be considered an infectious disease in classical terms since its aetiology is polymicrobial and the opportunistic pathogens involved are found even in healthy individuals [Lopez-Lopez *et al.,* 2017]. Insights from the Human Microbiome Project have revealed that maintaining microbial equilibrium is vital for health and preventing disease [Turnbaugh *et al.,* 2007]. This is evident in oral diseases like dental caries where plaque dysbiosis, rather than any specific microorganism, is the trigger for lesion initiation. Recent paradigms have also emphasised the role that health-associated commensals can play in abating disease pathogenesis. This evolving view of ecological battles (health- vs. caries-associated bacteria) and polymicrobial synergies within the structured heterogeneous biofilm environment has direct implications for developing effective modern-day therapeutics against dental caries [Bowen *et al.,* 2018].

Fluoride-based preventive strategies have the strongest and most consistent level of evidence supporting their use for caries control and are singularly responsible for the dramatic decline in caries prevalence rates over the past 60 years [Fontana *et al.,* 2018]. Yet, the recent Global Burden of Diseases report identified dental caries as the most common human disease condition worldwide [Kassebaum *et al.,* 2017]. In Australia itself, the 2018 Australian Institute of Health and Welfare report highlighted that 42% of Australian children aged between 5 and 10 years of age experience dental caries, while 30% of the adults in the 25-40 years age bracket have untreated caries lesions [AIHW, 2018]. Recent epidemiological reports from United States and Scandinavian nations show similar worrying trends [Agustsdottir *et al.,* 2010; Dye *et al.,* 2017]. This suggests that dental caries remains a “silent epidemic”, even in developed countries where there is widespread use of fluoride products and good access to dental care [Bowen, 2002]. The persistence of dental caries in many high-risk groups even with regular exposure to fluoride could be due to the inability of the remineralizing agent to significantly alter microbial composition and reverse the plaque dysbiosis. Thus, while fluoride will continue to be the cornerstone of any caries management protocol, additional antimicrobial or ecological strategies are needed to supplement the physicochemical cariostatic effects of fluoride.

The current antimicrobial approach to preventing dental plaque-related diseases like caries is however non-specific, mostly centred on removal of plaque by mechanical and/or chemical means

[Zhang *et al.,* 2018]. Eradication of dental plaque is a completely logical approach in a healthy mouth, but may not be as effective in caries conducive environments [Liu *et al.,* 2018]. Conventional mechanical/chemical plaque control measures do not reverse the microbiome dysbiosis seen in high-risk patients, and susceptible tooth surfaces are often repopulated with a microbiome similar in composition to the one that was removed [Burne, 2018]. Re-establishing a health oral ecosystem and maintaining a symbiotic oral microbiome is thus crucial, not only for long-term control over the disease, but also has important metabolic, physiological and immunologic benefits [Marsh, 2018]. This has resulted in increasing research interest in therapeutic interventions that modulate and restore ecological balance in dental biofilms [Nascimento, 2018]. This thesis has attempted to close some of the research gaps identified in the use of natural products and CPP-ACP as ecological agents to maintain a healthy plaque microbiome.

RESEARCH GAPS ADDRESSED

The paradigm shifts in caries aetiology and their implications for the development of therapeutic agents against the disease have been the most significant change in cariology research over the past couple of decades. As summarized in the introduction to the literature review (Chapters 2.1.1 and 2.1.2), the rationale for adopting ecological approaches to caries prevention is now evident, from aetiological conceptual perspectives to the important clinical implications it has for preventing lesion recurrence in patients. Various iterations of the ecological plaque hypothesis have emphasised that dental caries results from a shift in the composition of biofilm microbiota driven by local environmental stress [Filoche *et al.,* 2010; Kleinberg, 2002; Marsh, 1994, 2003; Takahashi and Nyvad, 2008, 2011]. It is thus vital that preventive measures address the underlying ecological basis of the disease in order to achieve long-term control over dental caries. Clinicians need to be aware that restorative interventions alone will have no significant effect on the microbiome dysbiosis or avoiding the vicious cycle of treatment and retreatment of CARS [Domejean *et al.,* 2017; Featherstone and Chaffee, 2018].

The literature review Chapters 2.2.2 and 2.3.2 discussed the role that cranberry polyphenols and CPP-ACP can potentially play as therapeutic ecological agents to reverse dental plaque dysbiosis. However, there were significant gaps in the literature on whether natural agents and CPP-ACP can be used to disrupt cariogenic virulence properties and modify the oral microbiome (Chapter 2.4). Research on cariostatic natural products has mostly focussed on their growth inhibitory effects against cariogenic bacteria, with only few studies examining their virulence inhibitory activity, and

no research on any potential biofilm ecological effects. For CPP-ACP, while there have been multiple reports on its ability to inhibit bacterial adhesion and buffer biofilm pH, the vast majority of the CPP-ACP research has been rightly directed towards its remineralizing effects (Chapter 2.3.1). Evidence of the dental plaque ecological effects of CPP-ACP has just started emerging, with results from this study providing among the earliest confirmation for this.

This research project has attempted to close the aforementioned gaps in the literature on virulence inhibitory and ecological effects of natural products and CPP-ACP. A commercially available polyphenol-rich cranberry extract was shown to disrupt critical cariogenic virulence factors of monospecies *S. mutans* (Chapter 4.2), dual-species *S. mutans-C. albicans* biofilms (Chapter 4.3) and saliva-derived polymicrobial biofilms (Chapter 4.4). The virulence inhibitory effects resulted in beneficial microbial ecological changes in polymicrobial biofilms growing in a cariogenic environment (Chapters 4.4). The present study further demonstrated that treating polymicrobial biofilms twice-daily with 2% CPP-ACP lowered biofilm acid production and microbial counts, with the buffering and anti-adhesion influences of CPP-ACP the likely mechanisms for these effects. The CPP-ACP treatment of polymicrobial biofilms growing in a cariogenic environment was also able to effect a beneficial microbial ecological change compared to control-treated biofilms (Chapter 4.5). This *in vitro* study on the ecological effects of CPP-ACP on saliva-derived polymicrobial biofilms extended and confirmed the results from a recent study on microbial modulatory effects of CPP-ACP in a 6-species biofilm model [Dashper *et al.,* 2018].

The randomised controlled trial conducted as part of this project (Chapter 4.6) closed another important gap in natural product and CPP-ACP clinical research. The evidence cited in previous studies on the cariostatic effects of natural products (Chapter 2.2.1) are almost entirely from *in vitro* studies. A single *in vivo* study in an animal caries model showed that treatment with cranberry A-PACs was able to reduce the incidence and severity of caries lesions [Koo *et al.,* 2010], indicating the potential of using natural products for caries prevention. However, there are no studies on whether any of the natural products with proposed cariostatic effects could lower caries-risk in a clinical environment. With regards to CPP-ACP, multiple clinical studies have shown that regular use of CPP-ACP lowered salivary counts of *S. mutans* (Chapter 2.3.2). However, new data strongly suggests that saliva may not be an appropriate sample for caries aetiological studies or for caries-risk tests [Mira, 2018]. What is more relevant from the therapeutic point of view would the effects of CPP-ACP on a range of disease- and health-associated bacteria in dental plaque. The RCT

in the current study showed that twice-daily brushing with dentifrices containing CPP-ACP and a polyphenol-rich cranberry extract was able to effect significant improvements in the dental plaque microbial ecology. The clinical translation of the *in vitro* virulence inhibitory and ecological effects could contribute towards lowered risk of developing new caries lesions, an important goal sought by patients, clinicians and policy makers.

FUTURE RESEARCH DIRECTIONS

Preventive approaches to dental caries must in future be more logical, focussing not just on the remineralizing early hard tissue lesions, but also on reversing the microbiome dysbiosis causing the lesions [Zhan, 2018]. Rather than attempting to eliminate entire plaque microflora with short-term antimicrobial treatment, regular provision of interventions that promote symbiosis and prevent dysbiosis could deliver relevant benefits to support the maintenance of a symbiotic oral microbiome [Marsh, 2018].

The science of cariology with regards to microbiome dysbiosis is well-advanced, but the challenge is translating mechanistic research into practical solutions for patients and clinicians. Based on the results of this study, it is recommended that future research on natural products and ecological agents be driven in the following areas:

1. Bacterial growth inhibitory treatments will generally be unfruitful in preventing oral polymicrobial diseases like dental caries. Rather, strategies directed towards modulating microbial interactions and their metabolic output should be developed further.

2. Enormous intraspecies gene diversity and plasticity of oral microbes are exhibited by commensal and cariogenic bacteria manifesting as phenotypic heterogeneity. Thus, function-based, rather than taxonomy-only studies, are needed in the future to understand what the plaque microflora are doing regardless of its taxonomic composition.

3. Specific flavonoids in the cranberry extracts like the A-PACs are known to exert the most potent virulence disruptive effects. Future *in vitro* and clinical studies using the isolated bioactive phytochemicals in different combinations and concentrations are needed to assess whether they can cause greater inhibition of cariogenic virulence properties and result in better ecological effects on the dental plaque microbiome.

4. The caries preventive efficacy of cranberry extracts and CPP-ACP in low concentration fluoride dentifrices (250-500 ppm) requires further investigation. If successful, these combination toothpastes could safely be used by infants and children below 6 years for enhanced protection against ECC.

5. Further research is also required to evaluate the microbial ecological effects and caries preventive efficacy of fluoride-cranberry combinations (without CPP-ACP), in daily use mouthwashes and dentifrices. Such combinations may be more cost-effective for low to moderate caries-risk patients, while the CPP-ACP-fluoride-cranberry combination could be used in patients assessed to be at high risk to dental caries.

6. The recent *in vitro* demonstration of the enhanced remineralizing and ecological effects of CPP-ACP - SnF_2 combination [Dashper *et al.,* 2018] suggests the possibility that incorporation of the cranberry extract to this combination may further enhance the beneficial effects. Additional research needs to be directed towards such CPP-ACP combinations.

7. Besides caries prevention, there is a need to investigate whether CPP-ACP or natural products can be used against other oral infections and diseases. For example, periodontal disease is also known to be triggered by microbiome dysbiosis. It will be of interest to evaluate whether the prebiotic effects of CPP-ACP or virulence inhibitory effects of cranberry extracts can promote growth of bacteria associated with periodontal health, while concomitantly inhibiting those associated with periodontal disease. Furthermore, in conditions like oral candidiasis, natural products with antimicrobial effects would be preferred over synthetic biocides or antifungals.

8. The ultimate success of any caries preventive agent should be judged only on its proficiency to lower caries experience and prevent new lesions from developing. Hence, well-designed RCTs, in high-risk population groups, are required to assess the preventive effectiveness of the CPP-ACP - cranberry dentifrices suggested in this study.

CONCLUDING REMARKS

The disease of dental caries can have long-term and broad dental, medical, societal, economic and oral health-related quality of life consequences [Fontana *et al.,* 2018a]. Despite

significant improvements in dental caries prevalence rates, the disease remains a major public health problem, affecting all age groups, and with multiple vulnerable population groups at heightened risk. Concerted clinical and research efforts are needed to reduce the scourge of dental caries around the world and throughout an individual's lifespan.

Ecological approaches to dental caries prevention could prove to be the missing link in eradicating this ubiquitous and age-old disease. While reducing dietary sugar exposures is the most effective way to prevent dental plaque dysbiosis, modifying dietary behaviour is often difficult to sustain in the present-day consumerist culture. Clearly, additional ecological measures may be required in many high caries-risk individuals and population groups. The work presented in this thesis provides evidence to support the possibility of using polyphenol-rich cranberry extracts and CPP-ACP as ecological agents to lessen caries-risk and prevent dental caries.

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7. APPENDIX

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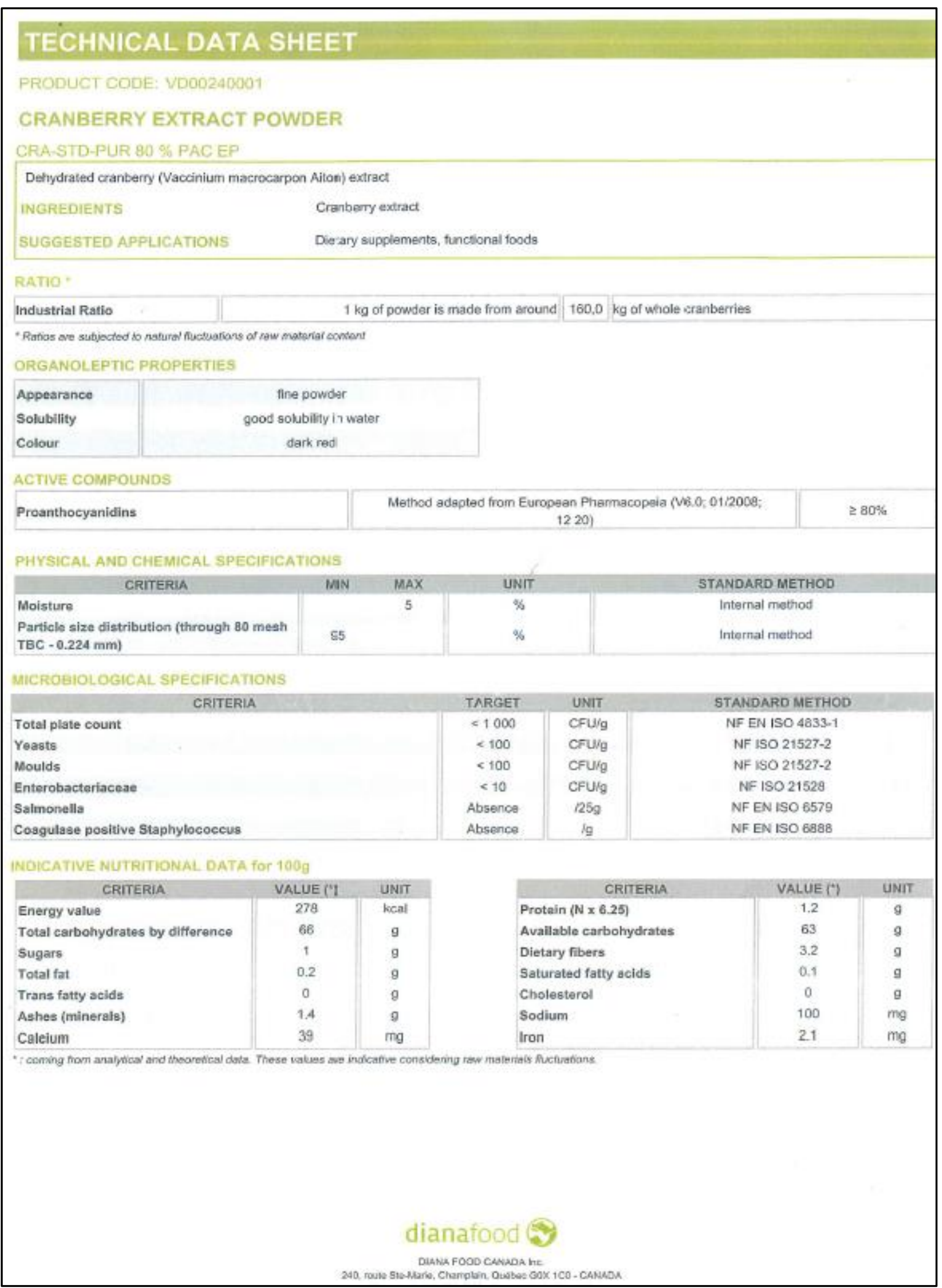
Appendix 7.1: Technical Data Sheet and Certificate of Analysis of the Cranberry Extract



TECHNICAL DATA SHEET

PRODUCT CODE: VD00240001

CRANBERRY EXTRACT POWDER

CRA-STD-PUR 80 % PAC EP

Dehydrated cranberry (Vaccinium macrocarpon Aiton) extract

INGREDIENTS	Cranberry extract
SUGGESTED APPLICATIONS	Dietary supplements, functional foods

RATIO *

Industrial Ratio	1 kg of powder is made from around	160,0	kg of whole cranberries

* Ratios are subjected to natural fluctuations of raw material content

ORGANOLEPTIC PROPERTIES

Appearance	fine powder
Solubility	good solubility in water
Colour	dark red

ACTIVE COMPOUNDS

Proanthocyanidins	Method adapted from European Pharmacopeia (V6.0; 01/2008; 12 20)	≥ 80%

PHYSICAL AND CHEMICAL SPECIFICATIONS

CRITERIA	MIN	MAX	UNIT	STANDARD METHOD
Moisture		5	%	Internal method
Particle size distribution (through 80 mesh TBC - 0.224 mm)	55		%	Internal method

MICROBIOLOGICAL SPECIFICATIONS

CRITERIA	TARGET	UNIT	STANDARD METHOD
Total plate count	< 1 000	CFU/g	NF EN ISO 4833-1
Yeasts	< 100	CFU/g	NF ISO 21527-2
Moulds	< 100	CFU/g	NF ISO 21527-2
Enterobacteriaceae	< 10	CFU/g	NF ISO 21528
Salmonella	Absence	/25g	NF EN ISO 6579
Coagulase positive Staphylococcus	Absence	/g	NF EN ISO 6888

INDICATIVE NUTRITIONAL DATA for 100g

CRITERIA	VALUE (*)	UNIT	CRITERIA	VALUE (*)	UNIT
Energy value	278	kcal	Protein (N x 6.25)	1.2	g
Total carbohydrates by difference	66	g	Available carbohydrates	63	g
Sugars	1	g	Dietary fibers	3.2	g
Total fat	0.2	g	Saturated fatty acids	0.1	g
Trans fatty acids	0	g	Cholesterol	0	g
Ashes (minerals)	1.4	g	Sodium	100	mg
Calcium	39	mg	Iron	2.1	mg

* : coming from analytical and theoretical data. These values are indicative considering raw materials fluctuations.

dianafood

DIANA FOOD CANADA Inc.
240, route Ste-Marie, Champlain, Québec G0X 1C0 - CANADA

TECHNICAL DATA SHEET

PRODUCT CODE: VD00240001

CRANBERRY EXTRACT POWDER

CRA-STD-PUR 80 % PAC EP

PACKAGING, SHELF-LIFE AND STORAGE

	Packaging 1
Shelf life (from production date)	36 months
Packaging	in a double polyethylene bag in a cardboard box
Net weight	10 kg (22.05 lbs)
Storage conditions	at ambiant temperature (recommended <20°C, <68°F) away from direct sunlight and hot humid place

FOOD SAFETY AND LEGISLATION

GMO	This product is non GMO in accordance with Regulations (EC) No 1829/2003 and (EC) No 1830/2003 and their amendments.
Allergen	This product does not contain allergens in accordance with Regulation (EU) No 1169/2011 and its amendments.
Ionization	This product and its ingredients are not irradiated / treated with ionising radiation in accordance with Directives 1999/2/EC and 1999/3/EC and their amendments.
Contaminants maximum levels	This product complies with Regulation (EC) No 1881/2006 and its amendments.
Pesticides maximum residue levels	This product complies with the European Pharmacopoeia.
Pharmacologically active substances residue limits	Not applicable
Sanitary agreement	Not applicable
Safety	This product does not meet any of the criteria as defined in article 31 of Regulation (EC) No 1907/2006 (REACH) and its amendments – No mandatory material safety data sheet. Specific precaution: the wearing of dust / aerosol filter type P3 (in accordance with standars EN 143) is recommended because of the pulverulent powder.
Packaging	The packaging in direct contact with the product complies with the provisions of Regulation (EC) No 1935/2004 and its amendments.
Specific legislation	This product complies with Directive 2009/32/EC and its amendments regarding extraction solvents.

DIETARY INFORMATION

Kosher	Yes certified
Halal	Yes certified

Ovo-lacto-vegetarians	Yes suitable
Vegans	Yes suitable

The above information is given with regard to in-force European legislation and to the best of our knowledge.

It is the customer's responsibility to make sure use and conditions of use comply with the legislation in force in their own markets and countries.

DIANA FOOD CANADA Inc.
240, route Ste-Marie, Champlain, Québec G0X 1C0 - CANADA

well-being
by nature

CERTIFICATE OF ANALYSIS

PO Number		Delivery Number	
Customer Product Name		Customer Number	
Product reference	*Cranberry extract powder (VD00240001)*		
Batch	*764*	DN Order Number	
Best before (3ANS)	*26/10/2019*	Your Reference	
Date of production	*26/10/2016*		

ORGANOLEPTIC ANALYSIS

Criterion	Method	Target	Unit	Result
Appearance	Internal	Fine powder 95-100% through 80 mesh TBC		Conform
Colour	Internal	Dark red		Conform
Solubility	Internal	Good solubility in water		Conform

ACTIVE COUMPOUNDS

Criterion	Method	Target	Unit	Result
Proanthocyanidins	Method adapted from European Pharmacopeia	≥ 80	%	99.67

PHYSICO-CHEMICAL ANALYSIS

Criterion	Method	Target	Unit	Result
Moisture	Internal	< 5	%	3.8

MICROBIOLOGICAL ANALYSIS

Criterion	Method	Target	Unit	Result
Total Plate Count	USP 61	< 1000	CFU /G	< 10
Salmonella	USP 62	Negative	CFU /G	Negative
Yeasts	USP 61	< 100	CFU /G	< 10
Moulds	USP 61	< 100	CFU /G	< 10
Coagulase positive Staphylococcus	USP 62	Negative	CFU /G	Negative

Germs present in our technical data sheet but not in the COA are analyzed according to a monitoring program.

For other product information, please refer to the technical data sheet.

Conclusion: Result conform

Plant director	*R GAGNON*	Sales contact
Date	*18/04/2017*	

Appendix 7.2: The University of Queensland Human Research Ethics Approval for Saliva Collection from Single Adult Donor

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Ecological Approaches to Dental Caries Prevention: Alteration of Oral Plaque Biofilms by Casein Phosphopeptide-Amorphous Calcium Phosphate and Natural Products
Chief Investigator:	Mr Nebu Philip
Supervisor:	Prof Laurence Walsh, Dr Shaneen Leishman, Dr Nihal Bandara
Co-Investigator(s):	Prof Laurence Walsh, Dr Shaneen Leishman, Dr Nihal Bandara
School(s):	School of Dentistry
Approval Number:	2017000519
Granting Agency/Degree:	PhD
Duration:	31st December 2017

Comments/Conditions:

Expedited Review - Low Risk

- HREA Application Form, 13/04/2017
- Project Description, 13/04/2017
- Participant Information and Consent Form, 13/04/2017

Note: If this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
University of Queensland Human Research Ethics Committee B
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Dr. Frederick Khafagi
Chairperson
University of Queensland Human Research Ethics Committee B
Registration: EC00457

Signature ______________________ Date **29/05/2017**

Appendix 7.3: The University of Queensland Human Research Ethics Committee Approval for Saliva Collection from Multiple Adult Donors

THE UNIVERSITY OF QUEENSLAND

Sub-Committee Human Research Ethics Approval

Project Title:	Formation of multi-species oral biofilm for in vitro antimicrobial assessment
Chief Investigator:	Dr Yan He, Prof Laurence Walsh, Dr Chun Xu
Supervisor:	None
Co-Investigator(s):	Dr Nebu Philip, Dr Patricia Wright, Mr Zhihao Li, Miss Yuxue Cao
School(s):	School of Dentistry, The University of Queensland
Approval Number:	2017001492
Granting Agency/Degree:	None
Duration:	31st December 2020

Comments/Conditions:

- HREA Form, 04/10/2017
- Advertisement, 04/10/2017
- Participant Consent Form v1.0, 03/10/2017
- Participant Information Sheet 12/10/2017
- Participants' information record v1.0, 03/10/2017
- Project Description 13/10/2017
- Frequently Asked Questions v1.0, 03/10/2017

Note: If this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Sub-Committee:
University of Queensland Health and Behavioural Sciences, Low & Negligible Risk Ethics Sub-Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Sub-Committee representative:
Associate Professor Guy Wallis
Chairperson
University of Queensland Health and Behavioural Sciences, Low & Negligible Risk Ethics Sub-Committee

Signature ______________________ Date 13/10/2017

Appendix 7.4: The Prince Charles Hospital Human Research Ethics Committee Approval for Randomised Controlled Clinical Trial

Enquiries to: ResearchTPCH@health.qld.gov.au
Office Ph: (07) 3139 4198
(07) 3139 4500
Our Ref: HREC Final Approval

14 February 2018

The Prince Charles Hospital
Human Research Ethics Committee
The Prince Charles Hospital
Building 14
Rode Road, Chermside QLD 4032

Professor David Healey
C/- Professor Laurence J Walsh
The University of Queensland
School of Dentistry
UQ Oral Health Centre
288 Herston Road
HERSTON QLD 4006

Dear Professor Healey,

Re: HREC/18/QPCH/7: Ecological approaches to dental caries prevention: A controlled trial to evaluate the influence of CPP-ACP – Cranberry toothpastes in effecting an ecological change in the oral plaque microbiome

Thank you for submitting the requested documents for the above project for further review which was received on 10 January 2018. This project was considered by The Prince Charles Hospital Human Research Ethics Committee (HREC) (EC00168).

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007), NHMRC and Universities Australia Australian Code for the Responsible Conduct of Research (2007)* and the *CPMP/ICH Note for Guidance on Good Clinical Practice.*

I am pleased to advise that this proposal meets the requirements of the National Statement and that The Prince Charles Hospital Human Research Ethics Committee has granted final approval of this research project. The documents reviewed and approved for the above mentioned project include:

Document	Version	Date
Application	AU/1/AF43310	
Protocol	1	5 January 2018
Participant Information Sheet and Consent Form – Parent/ Guardian	1.1	31 January 2018
Participant Information Sheet and Consent Form – Adult Participant	1.1	31 January 2018
Documents acknowledged:	**Version**	**Date**
eBS Submission Document for a Clinical Trial Application Application No.: CT-2017-CTN-05069-1 Submission No.: CT-2018-00034-1	-	-
eCTN application CT-2017-CTN-05069-1	-	8 January 2018
Australian Register of Therapeutic Goods Certificate ARTG Identifier: 271415	-	26 February 2016

Investigator CV: A/Prof David Healey	-	-
Investigator CV: Professor Laurence Walsh	-	-

This information will be tabled at the next meeting on 22 March 2018 for noting.

Please note the following conditions of approval:

1. The Principal Investigator will immediately report anything which might warrant review of ethical approval of the project in the specified format, including:

    a. Unforeseen events that might affect continued ethical acceptability of the project.

    b. Serious Adverse Events that materially impact on the continued ethical acceptability of the project. In addition the Investigator must provide, at least six monthly, a summary of serious adverse events, in the specified format, including a comment as to suspected causality.

2. Amendments to the research project which may affect the ongoing ethical acceptability of a project must be submitted to the TPCH HREC for review. Major amendments should be reflected in a cover letter from the principal investigator, providing a description of the changes, the rationale for the changes, and their implications for the ongoing conduct of the study. Hard copies of the revised amendments, the cover letter and all relevant updated documents with tracked changes must also be submitted to the TPCH HREC coordinator as per standard HREC SOP. Further advice on submitting amendments is available from http://www.health.qld.gov.au/ohmr/documents/researcher_userguide.pdf

3. Proposed amendments to the research project which may affect both the ethical acceptability and site suitability of the project must be submitted firstly the TPCH HREC for review and, once TPCH HREC approval has been granted, submitted to the RGO.

4. Amendments which do not affect either the ethical acceptability or site acceptability of the project (e.g. typographical errors) should be submitted in hard copy to the TPCH HREC coordinator. These should include a cover letter from the principal investigator providing a brief description of the changes and the rationale for the changes, and accompanied by all relevant updated documents with tracked changes.

5. In accordance with Section 3.3.22 (b) of the National Statement the Principal Investigator will report to the TPCH HREC annually in the specified format and a final report is to be submitted on completion of the study. https://www.health.qld.gov.au/metronorth/research/ethics-governance/post-approval-reporting/default.asp

6. The Principal Investigator will notify the TPCH HREC if the project is discontinued at the participating site before the expected completion date, with reasons provided. Any plan to extend the duration of the project past the approved period, the Principal Investigator will submit any associated required documentation for TPCH HREC approval ***before*** expiry of the project, listed below.

7. The Hospital & Health Service Administration and the Human Research Ethics Committee may inquire into the conduct of any research or purported research, whether approved or not and regardless of the source of funding, being conducted on hospital premises or claiming any association with the Hospital; or which the Committee has approved if conducted outside The Prince Charles Hospital & Health Services.

HREC approval is valid until **14 February 2021**.

Please advise The Prince Charles Hospital Human Research Ethics Committee of the date you commence the research project for the approved site(s) using the Notification of Commencement Form: **https://www.health.qld.gov.au/metronorth/research/ethics-governance/post-approval-reporting/default.asp**

If the research does not commence within 3 months of this letter, please inform the committee in formal correspondence of any delays occurring with your project.

Should you have any queries about the TPCH HREC's consideration of your project please contact the Manager of Research, Ethics & Governance Unit on the above phone numbers or email addresses. The HREC terms of Reference, Standard Operating Procedures, membership and standard forms are available from http://www.health.qld.gov.au/ohmr/html/regu/regu_home.asp

You are reminded that this letter constitutes ethical approval only. You must not commence this research project at a site until separate authorisation from the Hospital & Health Services CEO or Delegate of that site has been obtained.

A copy of this approval must be submitted to the relevant Hospital & Health Services Research Governance Officer/s or Delegated Personnel with a completed Site Specific Assessment (SSA) Form for authorisation from the CEO or Delegate to conduct this research at the site/s.

The Prince Charles Hospital Human Research & Ethics Committee wishes you every success in your research.

Yours faithfully

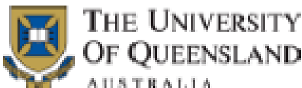
Dr Russell Denman
Chair
The Prince Charles Hospital
Human Research Ethics Committee

List of approved Sites:

No.	Site	Investigator
1.	UQ Oral Health Centre	Associate Professor David Healey

Note: If additional sites are engaged prior to the commencement of, or during the research project, the Coordinating Principal Investigator is required to notify the TPCH HREC. Notification of the withdrawn sites should also be provided to the TPCH HREC in a timely fashion accompanying a close out report for that site.

Appendix 7.5: The University of Queensland Governance Approval for the Clinical Trial



Human Ethics Research Office

Cumbrae-Stewart Building #72
The University of Queensland
St Lucia, QLD 4072

CRICOS PROVIDER NUMBER 00025B

20 March 2018

Mr Nebu Philip
School of Dentistry

Dear Nebu,

Clearance Number: 2018000564
Project Title: "(HREC/18/QPCH/7) Ecological approaches to dental caries prevention: A controlled trial to evaluate the influence of CPP-ACP - Cranberry toothpastes in effecting an ecological change in the oral plaque microbiome"

Following administrative review of the human research ethics approval from The Prince Charles Hospital Human Research Ethics Committee, I am pleased to advise that, as the University of Queensland's authorised delegate for the University of Queensland's Human Research Ethics Committees A & B, approval is granted for this project.

Approval has been based on the already approved documents in TPCH HREC Approval Letter dated 14/02/2018.

This project has been approved to 14th February 2021.

We would like to take this opportunity to remind you that, should any modifications be made to this project, they will need to be approved by the lead human research ethics committee prior to being forwarded to the University of Queensland's Human Research Ethics Office for administrative review and approval.

Furthermore, conditions of the University of Queensland HREC Approval, require the researcher to provide an annual report and a final report on completion of the study (copy of lead HREC report will suffice). On commencement of this research, the researcher provides an undertaking to notify the University of Queensland's Human Research Ethics Office of all complaints or adverse events that may arise from this research.

Please keep a copy of this document for your records.

Kind regards,

Chris Rose'Meyer
Governance Officer
Office of Research Ethics
The University of Queensland

Address. Human Research Ethics Office | Cumbrae-Stewart Building #72 The University of Queensland St Lucia, QLD 4072 | E humanethics@research.uq.edu.au W www.uq.edu.au/research/integrity-compliance/human-ethics

Appendix 7.6: Trial Participant Information Sheet

Oral Health Services
Metro North Hospital and Health Services

Participant Information Sheet – Parent/Guardian

Metro North Oral Health Services

Project Title: A controlled trial to evaluate the influence of CPP- ACP – Cranberry toothpastes in effecting an ecological change in the oral plaque microbiome

Principal Investigators: Prof. Laurence Walsh; Assoc. Prof. David Healey; Nebu Philip; Dr. Shaneen Leishman.

Part A: What does the child's participation involve?

1. Introduction

This is an invitation for the child/teenager in your care to take part a research project that is testing new preventive toothpastes for tooth decay in orthodontic patients. This Participant Information Sheet/Consent Form gives you details about the research project. Knowing what is involved will help you decide if you want the child/teenager to take part in the research.

Please read this information carefully. Ask questions about anything that you don't understand or want to know more about. Before deciding whether or not the child/teenager can take part, you might want to talk about it with a relative, friend or the child/teenager's local doctor.

Participation in this research is entirely voluntary. If you do not wish for the child/teenager to take part, they do not have to. He/she will continue to receive the best possible dental care whether or not they take part.

If you decide you want the child/teenager to take part in the research project, you will be asked to sign the Consent Form. By signing it you are telling us that you:

- Understand what you have read
- Consent to the child/teenager taking part in the research project
- Consent for the child/teenager to have the treatments that are described
- Consent to use of the child/teenager's personal information as described.

You will be given a copy of this Participant Information and Consent Form to keep.

2. What is the purpose of this research?

This research is being conducted by The University of Queensland School of Dentistry. The project aims to find out whether brushing with new preventive toothpastes can change bacterial composition of dental plaque deposits on teeth and make them less likely to induce tooth decay.

Two new toothpastes are being investigated, one containing casein phosphate-amorphous calcium phosphate (CPP-ACP), and the other containing a combination of CPP-ACP and highly-purified organic cranberry extracts. CPP-ACP is a complex of proteins and minerals derived from cow's milk that is scientifically proven to prevent tooth decay, erosion, and sensitivity. Cranberry extracts, long used in foods and juices, have been shown to inhibit the oral bacteria responsible for causing tooth decay in laboratory studies.

CPP-ACP has been successfully used in oral care products in Australia to prevent tooth decay when used as a topical crème (applied on teeth after brushing with a fluoridated toothpaste) or in chewing gums. The two CPP-ACP oral care products to be used in this project are meant to be used as regular toothpastes for routine twice-daily toothbrushing. Both these new toothpastes contain fluoride at concentrations consistent with regular commercially available toothpastes and meet all guidelines required to maintain oral hygiene when used as instructed.

3. What does participation in this research involve?

If your child/teenager is eligible and you consent to their participation, he/she will be randomly allocated to one of three toothpaste groups: a CPP-ACP toothpaste group; a CPP-ACP – Cranberry toothpaste group; and a fluoridated toothpaste group without either CPP-ACP or cranberry that will serve as the active control group. It will not be known which of the toothpaste treatments your child/teenager is receiving. The study clinician will also not know (a double-blind study). However, in certain circumstances the study clinician can find out which treatment the participant is receiving. This research project has been designed to make sure the researchers interpret the results in a fair and appropriate way and avoids study clinicians/researchers or participants jumping to conclusions. The toothpaste group your child/teenager is in will only be revealed after the completion of all the analysis of the study.

Your child/teenager will be asked to routinely brush their teeth twice daily (morning and night) with their allocated toothpaste. Both the test toothpastes and the placebo toothpaste will contain an amount of fluoride which is consistent with commercially available toothpastes. All toothpastes will be supplied free of charge to participants for the duration of their involvement in the study.

To determine the effects of the toothpastes on dental plaque deposits, plaque swab samples will be collected from the participant's teeth on two separate occasions. The first dental plaque sample will be collected at baseline just before the child/teenager starts using his/her allocated toothpaste. The second dental plaque sample will be collected after 5-6 weeks of using the toothpaste. On both occasions, plaque samples will be collected by swabbing the upper and lower teeth with a microbrush. Prior to the

plaque sample collection, your child/teenager will be instructed not to brush in the morning and to refrain from food/drink for two hours before the plaque collection. Both plaque sample collections can be done to coincide with your child/teenager's scheduled orthodontic appointment 5-6 weeks apart.

4. What will happen to the collected plaque samples?

The plaque samples collected will be sent to The University of Queensland research laboratory at the Herston Oral Health Centre. The samples will be tested for the presence of decay-causing bacteria and general oral bacteria and how these levels change over the 5-6 weeks of the study. At the end of the analysis, any remaining oral plaque samples will be destroyed in compliance with biological safety requirements. You will not be advised of the individual results of your child's plaque sample.

5. Does the child have to take part in this research project?

Participation in any research project is voluntary. If you do not wish for the child/teenager to take part, they do not have to. If you decide that they can take part and later change your mind, you are free to withdraw the child/teenager from the project at any stage. If you do decide that the child can take part, you will be given this Participant Information and Consent Form to sign and you will be given a copy to keep. Your decision that the child/teenager can or cannot take part, or that they can take part and then be withdrawn, will not affect their routine orthodontic/dental treatment, relationship with those treating them, or their relationship with *Metro North Oral Health Services*.

6. Other relevant information about the research project

In total, a little over 60 orthodontic patients will be recruited to participate in this project. This project is a collaborative project between The University of Queensland School of Dentistry and Metro North Oral Health Services.

7. What are the possible benefits of taking part?

CPP-ACP topical crèmes have been proven to be very effective in preventing white spot lesions (first stage of tooth decay) that often develop during the course of orthodontic treatment. Toothpastes containing CPP-ACP are expected to offer the same benefits and will thus be particularly helpful for patients undergoing orthodontic treatment.

No monetary benefits will be provided. However, resources such as toothbrushes and toothpastes will be issued free of charge during the trial period.

8. What are the possible risks and precautions of taking part?

CPP-ACP toothpaste is a milk-based product which has been shown to be very safe and effective against dental decay. It works by protecting the teeth against bacterial acids. However, children/teenagers who are allergic to cow's milk should not use the CPP-ACP toothpaste. If your child/teenager is identified as having an allergy to cow's milk during

the study, immediately stop using the toothpaste supplied and notify us. Cranberry extracts added to one of the test toothpastes is a commonly used natural product and is not expected to have any safety issues. Like for regular toothpastes, excessive swallowing of the test toothpastes should be avoided. All dental products issued should be kept out of reach of children.

9. What if I withdraw the child/teenager from this research project?

If you decide to withdraw the participant from the project at any stage, please notify a member of the research team. You should be aware that data collected up to the time of withdrawal will form part of the research project. If you do not want this, you must tell them before the participant joins the research project.

10. What happens when the research project ends?

After the research finishes, the trial toothpaste will no longer be available. You will be encouraged to have your child/teenager brush twice daily with commercially available fluoridated toothpaste.

At the end of the research study, all participants who completed the study will be emailed flyers on the overall results of the study.

Part B: How is the research project being conducted?

1. What will happen to information about the child?

By signing the consent form you consent to the relevant research staff collecting and using personal information about the child/teenager for the research project. The personal information collected for this research project is restricted to name, age, gender and contact information of the participant. Any information obtained in connection with this research project that can identify the child/teenager will remain confidential and securely stored. The child's information will only be used for the purpose of this research project and it will only be disclosed with your permission, except as required by law.

It is anticipated that the results of this research project will be published and or presented in a variety of forums. In any publication and/or presentation, information will be provided in such a way that the child/teenager cannot be identified.

In accordance with relevant Australian privacy and other relevant laws, you have the right to request access to the participant's information collected and stored by the study team. You also have the right to request that any information with which you disagree be corrected. Please contact a study team member if you would like to access the participant's information.

2. Complaints and Compensation

If the participant suffers any injuries or complications as a result of this research project, you should contact the study team as soon as possible and you will be assisted with arranging appropriate medical/dental treatment for the participant. If the participant is eligible for Medicare, they can receive any medical treatment required to treat the injury or complication free of charge, as a public patient in any Australian public hospital.

3. Who is organising and funding the research?

This research project is being conducted and supervised by Prof. Laurence Walsh at The University of Queensland School of Dentistry. The project is being funded by Prof. Walsh internal research grants. The test toothpastes being trialed in this study are being provided by GC Corporation based in Japan. This company has licensed the CPP-ACP technology for dental products and is the sole global manufacturer of CPP-ACP dental products. GC Australia role in this project is restricted to providing the trial toothpastes and they have placed no constraints on publishing the results obtained from the clinical trial.

A positive outcome would be advantageous for GC Corporation (through potential future product sales). You will not benefit financially from the child's involvement in this research project even if, for example, the child's samples (or knowledge acquired from analysis of the samples) prove to be of commercial value. No member of the research team will receive a personal financial benefit from the child's involvement in this research project (other than their ordinary wages).

4. Who has reviewed the research project?

All research in Australia involving humans is reviewed by an independent group of people called a Human Research Ethics Committee (HREC). This study adheres to the Guidelines of the ethical review process of the HREC of Metro North Health and The University of Queensland and the *National Statement on Ethical Conduct in Human Research (2007)*. This statement has been developed to protect the interests of people who agree to participate in human research studies. Whilst you are free to discuss your participation in this study with project staff (please see below), if you would like to speak to an officer of the University not involved in the study, you may contact the UQ Ethics Coordinator on 3365 3924.

5. Further information and who to contact

The person you may need to contact will depend on the nature of your query. If you want any further information concerning this project, you can contact the following people:

For further details about this research project or to report any adverse effects on using the trial toothpastes, you may contact:

Research project contact person

Name	*Laurence Walsh*
Position	*Professor of Dental Science*
Telephone	*0401 990 555*
Email	*l.walsh@uq.edu.au*

For matters relating to research at the site at which the child/teenager is participating, the details of the local site person are:

Site complaints contact person

Name	***Jacqueline Robinson***
Position	*Research Governance Officer, Metro North Hospital and Health Service HREC, Royal Brisbane and Women's Hospital*
Telephone	*3646 8579*
Email	RBWH-RGO@health.qld.gov.au

If you have any complaints about any aspect of the project, the way it is being conducted or any questions about being a research participant in general, then you may contact:

Reviewing HREC Office contact

Name	*Anne Carle*
Position	*HREC Coordinator and Manager, Metro North Hospital and Health Service HREC, The Prince Charles Hospital*
Telephone	*3139 4500*
Email	ResearchTPCH@health.qld.gov.au

Appendix 7.7: Trial Participant Consent Form

Consent Form – Parent/Guardian

Project Title: A controlled trial to evaluate the influence of CPP- ACP – Cranberry toothpastes in effecting an ecological change in the oral plaque microbiome

Principal Investigators: Prof. Laurence Walsh, Assoc. Prof David Healey, Nebu Philip, Dr. Shaneen Leishman

Declaration by Parent/Guardian

I have read the Participant Information Sheet or someone has read it to me in a language that I understand.

I understand the purposes, procedures and risks of the research described in the project.

I give permission for the child's oral health professionals to release information to The University of Queensland concerning the child's oral health and associated treatment for the purposes of this project. I understand that such information will remain confidential.

I consent to the storage and use of plaque samples taken from the child for use, as described in the relevant section of the Participant Information Sheet, for this specific research project.

I have had an opportunity to ask questions and I am satisfied with the answers I have received.

I freely agree to the child/teenager participating in this research project as described and understand that I am free to withdraw them at any time during the research project without affecting their future health care.

I understand that I will be given a signed copy of this document to keep.

Name of Child/Teenager (please print)

Name of Parent/Guardian (please print) ______________________________

Signature of Parent/Guardian ______________________ Date ____________

Declaration by Study Doctor/Senior Researcher†

I have given a verbal explanation of the research project, its procedures and risks and I believe that the parent/guardian has understood that explanation.

Name of Senior Researcher† (please print) ______________________________

Signature ______________________ Date ______________________

† A senior member of the research team must provide the explanation of, and information concerning the research project.

Natural Products Trial – PICF Version 1.1 Last updated 05/01/2018 Page | 1
Master Version 1.0 approved XX/XX/XXXX by TPCH HREC
MNHHS Oral Health Site Specific Version X dated XX/XX/XXXX

Appendix 7.8: ANZCTR Clinical Trial Registry Approval

ANZCTR Admin <actr@ctc.usyd.edu.au>

Wed 28/02/2018 8:34 AM

Deleted Items

To:Nebu Ivan Philip <n.philip@uq.edu.au>;

Cc:actr@ctc.usyd.edu.au <actr@ctc.usyd.edu.au>;

Updated Successfully

Dear: Nebu Philip,
Re: A clinical trial to evaluate the influence of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) – Cranberry toothpastes in changing the bacterial composition of dental plaque deposits on teeth of orthodontic patients.

Thank you for updating the trial information of the above trial registered with the Australian New Zealand Clinical Trials Registry (ANZCTR): 12618000095268

All your updated trial information has now been approved and successfully updated on the website.

Please be reminded that the quality and accuracy of the trial information submitted for registration is the responsibility of the trial's Primary Sponsor
or their representative (the Registrant). The ANZCTR allows you to update trial data, but please note that the original data lodged at the time of trial
registration and the tracked history of any changes made will remain publicly available.

The ANZCTR is recognised as an ICMJE acceptable registry (http://www.icmje.org/faq.pdf) and a Primary Registry in the WHO registry
network (http://www.who.int/ictrp/network/primary/en/index.html).

If you have any enquiries or have received this email by mistake please contact the ANZCTR Admin.

Thank you,
ANZCTR Staff

Tel: +61 2 9562 5333
Fax: +61 2 9565 1863
Email: info@actr.org.au
Website: [www.ANZCTR.org.au]www.ANZCTR.org.au

Appendix 7.9: TGA Clinical Trial Notification

Clinical Trial CT-2017-CTN-05069-1 v1 Repository

Submission Date: 08/01/2018
Acknowledged by TGA

Application

Sponsor Name School of Dentistry, The University of Queensland
Sponsor Address UQ Oral Health Centre corner Bramston Terrace and Herston Road Herston QLD 4006
Notification Fee $350

Trial Details

Contact Name	Nebu Philip
Contact Phone Number	0452355711
Contact Email	n.philip@uq.edu.au
Protocol Number	05069
Expected Trial Start Date	01/06/2018
Expected Completion Date	31/12/2018
Potential Use of Restricted Goods	No
Title of Study	Ecological approaches to dental caries prevention: A controlled trial to evaluate the influence of CPP-ACP - Cranberry toothpastes in effecting an ecological change in the oral plaque microbiome
Trial Type	Phase 1,Device
Trial Type Description (if necessary)	This research project aims to evaluate whether the microbial ecology of dental plaque biofilms can be altered by toothpastes containing either Casein Phosphopetide-Amorphous Calcium Phosphate (CPP-ACP) or a combination of CPP-ACP and cranberry.
Total Number of Patients to be Enrolled in Trial	51-200
Therapeutic Area	Other
This Trial	Involves the use of a Medical Device Is comparator controlled

Devices

Product Name	Is this a kit?	Manufacturer	GMDN	Description	Intended Purpose	Other Description
CPP-ACP toothpaste (MI Paste� ONE)	Single Device	GC Australasia Dental Pty Ltd	Dental material, desensitizing resin (45232)		Investigational Product	
CPP-ACP - Cranberry toothpaste ('All-in-one')	Single Device	GC Australasia Dental Pty Ltd		A CPP-ACP toothpaste enriched with a cranberry additive. Highly purified organic cranberry extract (250-500�g/ml) is added to the MI Paste One CPP-ACP toothpaste to enhance its antimicrobial effects against cariogenic bacteria responsible for tooth decay. Cranberry has been shown to inhibit bacterial synthesis of insoluble glucans responsible for establishing a cariogenic oral plaque biofilm. The 'All-in-one' CPP-ACP - Cranberry toothpaste is intended to be used for routine twice-daily tooth brushing. Product category: Medical Device Class 1	Investigational Product	
Standard fluoride toothpaste	Single Device	GC Australasia Dental Pty Ltd		A toothpaste that is similar to the two investigational toothpastes in all aspects (including its 1100ppm fluoride concentration), except it does not contain either CPP-ACP or cranberry, is proposed to be used as the comparator representing standard oral care therapy.	Comparator	

Sites

Site	Site Address, Location	Expected Site Start Date	Principal Investigator Name	Principal Investigator Contact Phone Number	Principal Investigator Contact Email	HREC Name, Code	HREC Contact Officer, Position	HREC Contact Phone	HREC Contact Email	Approving Authority Name	Approving Authority Contact Officer, Position	Contact Phone	Contact Email
UQ Oral Health Centre	Herston, Brisbane, QLD	01/06/2018	Nebu Philip	0452355711	n.philip@uq.edu.au	The Prince Charles Hospital Human Research Ethics, EC00168	Anne Carle, HREC Coordinator and Manager	0731394500	ResearchTPCH@health.qld.gov.au	Metro North Hospital and Health Service	Jacqueline Robinson, Research Governance Officer	0736468579	RBWH-RGO@health.qld.gov.au

Change to Trial Details

Completion

Appendix 7.10: The University of Queensland Biosafety Approval for Plaque Sample Microbial Analysis

BIOSAFETY
Potentially Hazardous Biological Material – FORM v1.3

THE UNIVERSITY OF QUEENSLAND AUSTRALIA

For high risk biological work (other than GM) – tick appropriate box:

- ☐ Risk Group 2 microorganisms* cultured in large volumes (10L or greater)
- ☐ Risk Group 2 microorganisms which require special precautions*
- ☐ Risk Group 3 or 4 microorganisms*
- ☐ Infectious / potentially infectious animals, tissues or fluids *(involving microorganisms of the categories mentioned above)*
- ☑ Unscreened Specimens *(i.e. Human tissue or body fluids that are known to contain microorganisms listed above, or have not been screened for infectious disease; animal tissue or body fluids that could contain zoonoses or have not been screened for such).*
- ☐ Poisonous or venomous animals *(e.g. snakes, spiders, cone-shells)*
- ☐ Biological toxins *(excluding toxoids)*
- ☐ Biological material on the Defence Strategic Goods List (DSGL)
- ☐ Security sensitive biological material (SSBA's)
- ☐ Other; please give full details below

* As listed in AS2243.3 2010, Section 3 or any microorganism categorized as Dangerous Goods Class 6.2 (Infectious Substances) or those falling under UN2814 & UN2900 in the Dangerous Goods Regulations (IATA).

Refer to Webpage http://www.uq.edu.au/ohs/biosafety-approvals-for-potentially-hazardous-biologicals

Guideline: Work Involving Potentially Hazardous Biologicals http://www.uq.edu.au/ohs/biosafety-forms

It is the researcher's responsibility to contact the UQ Biosafety Advisors for clarification if unsure of categorization.

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1. **Title of Project:** A controlled trial to evaluate the influence of CPP-ACP – Cranberry toothpastes in effecting an ecological change in the oral plaque microbiome

2. **Details of Project Supervisor submitting proposal:**

   Name: Prof. Laurence Walsh
   Position within the organization: Group Head of Research in Advanced Materials and Therapeutics
   School/Institute: School of Dentistry
   Relevant qualifications: BDSc, PhD (Qld), DDSc (Qld), QCEd, FFOP(RCPA), FADI, FPFA, FIADFE
   Relevant experience: Active in research since 1982, with extensive experience in cell, tissue, and organ culture of human cells and bacteria, and analysis of plaque and saliva. Registered specialist in special needs dentistry and expert in infection control (including member of chair of national committees in the area). Over 25 years experience as a WHSR or WHSO.
   Phone: 336 581061/ 336 58130
   Email: l.walsh@uq.edu.au
   Date of UQ Biosafety Training: 25/11/2014 — Biosafety training ✓ 11/11/2018

3. **Details of other staff involved in the work (attachment may be included if insufficient space):**

   Name: Nebu Philip
   Qualifications: BDS, MDS
   Experience: Two years' experience working in a PC2 laboratory environment with relevant experience of the microbiological techniques required for the current research project.
   Role in project: Doctoral Research Student
   Date of UQ Biosafety Training: 11/04/2016

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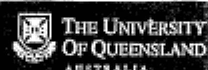
Approved by: Biosafety Advisor ☑ / IBSC ☐ OHNA referral? ☐

BIOSAFETY
Potentially Hazardous Biological Material – FORM v1.3



4. **Aim/s of Project:** Determine any microbiological changes in the dental plaque of high caries-risk individuals using either an 'One-step' CPP-ACP toothpaste or an 'All-in-one' CPP-ACP - Cranberry toothpaste. The outcome measure is the bacterial load of 14 selected bacterial species indicative of health or dental caries, using real-time PCR analysis.

   This application will specifically address the experimental procedures around the extraction of microbial DNA from human oral plaque swab samples.

5. **Main experimental procedures of the work (including waste treatment and decontamination):**
   Note:- if dealing with biological toxins, venoms or poisons, please detail the concentration of stocks and dilutions. Please include any known information on lethal doses in vertebrates (LDL), and if possible provide a safety data sheet (SDS).

   Use of human bodily fluids (oral plaque samples) is needed for this research project. Oral plaque samples may contain bacteria from Risk Group 2, hence all unscreened plaque samples will be treated as potentially infectious and all precautions following AS/NS S2243.3 will be followed in handling these samples. All personnel prior to commencement of work will check that their vaccinations are up to date (in particular HepB and tetanus). Personnel will be advised to contact the UQ OHS Occupational Nurse to check their vaccination status if unsure.

   Sample collection will be carried out in the Metro North Oral Health Clinics (Division of Orthodontics) located at the UQ Oral Health Centre, Herston. The plaque samples will be collected using sterile microbrushes from upper and lower teeth of orthodontic patients by trained orthodontists/dental assistants following AS/NS S4815 for infection control. The oral swabs will be placed in a sample tube containing 1ml sterile saline and held temporarily in a specified refrigerator.

   For final storage, the samples will be transferred to a -80 °C freezer in the UQ Oral Health Centre PC2 Research Laboratory (Rm 6412) until their microbial analysis. Transportation of the samples from the Level 3 Orthodontic Clinics to the Level 6 PC2 Research Laboratory, will be done on a daily basis by UQ personnel who have undergone the required transportation of biological hazards training. Samples will be packed in a rigid eski with icepacks and absorbent packaging between primary and secondary receptacle. Labelling will include UN numbering, destination, and itemized list of contents of the package.

   DNA extraction experiments and other molecular biology procedures will be carried out in Rooms 6410 and 6413. Plaque samples will be centrifuged to concentrated the bacterial cells. Sealed tubes and centrifuges with aerosol containment lids will be used to contain the production of aerosols. Following centrifugation, sample tubes will be opened in a Class II biosafety cabinet and the supernatant will be discarded leaving any pellet and 0.1ml of supernatant. The supernatant will be discarded into a waste container containing sodium hypochlorite and subsequently sewered. The remaining sample will be transferred to PowerBead tubes for cell lysis. Cells are lysed by a combination of chemical agents from steps 1-4 of the isolation protocol and mechanical shaking in step 5 of the protocol. All mixing, vortexing, heating, and sample transfer will be carried out in a Class II biosafety cabinet. All items will be decontaminated before removal from the biosafety cabinet. Bacterial Cell Lysis will be complete following step 5 of the isolation protocol and the samples, therefore, will be rendered non-infectious from this point forward.

   The volumes of chemicals handled and the waste generated in this protocol are small and, consequently, the risk from chemical incompatibility are relatively low. One of the chemicals used in the isolation kit are incompatible with strong oxidizers such as bleach. If liquids containing these chemicals are split, the split will be contained with absorbent material and will be overlaid with paper towels and the area cleaned with neutral detergent that does not react with solutions contained within the kit. If the split liquid also contains the potentially infectious agent (plaque sample), the spill will be contained with absorbent material and will be overlaid with paper towels (which will be disposed of in the clinical waste stream) and the area cleaned with neutral detergent before being disinfected with a fresh solution of at least 1 % available chlorine allowing 10 minutes contact time. All waste, excluding chemical waste, (i.e.

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Approved by: Biosafety Advisor ☑ IBSC ☐ OHNA referral? ☐

pipettes tips, sample tubes, wipes) will be disposed in the clinical waste stream. With the exception of solutions C2 and C6, all chemical waste generated will be disposed through the UQ chemical waste program. As stipulated in the respective MSDS, solutions C2 (containing ammonium acetate) and C6 (Tris buffer) will be sewered with running water.

6. **Details of facilities where work will be conducted:**
Sample Collection
Building/s Name and Number: Orthodontic Clinics at UQ Oral Health Centre (#883)
Room numbers: Level 3 Clinic 3.4a
Do you have appropriate approval to use this facility? (Provide name): Yes, Assoc. Prof. David Healy, Discipline Lead of Orthodontics

Sample Storage
Building/s Name and Number: PC2 Research Laboratory at UQ Oral Health Centre (#883)
Room numbers: 6412
Do you have appropriate approval to use this facility? (Provide name): Yes, Prof. Pauline Ford, Head, School of Dentistry

DNA extraction from samples
Building/s Name and Number: PC2 Research Laboratory at UQ Oral Health Centre (#883)
Room numbers: 6410 and 6413
Do you have appropriate approval to use this facility? (Provide name): Yes, Prof. Pauline Ford, Head, School of Dentistry

7. RISK ASSESSMENT: **Provide an approved risk assessment of possible hazards associated with the work (e.g. biological, chemical, physical, field work), including emergency procedures, health surveillance & vaccination considerations if applicable.**

Provide the UQ risk assessment ID number/s here (or provide complete copies if RAs reside on an alternative database):

ID 55149: DNA extraction of oral plaque samples using MoBio PowerSoil isolation kit....................

8. **Work is not to commence until approval is received**

Expected completion date of work: December 2018

ETHICS: If your research project involves animals or humans or their products, ensure you have the appropriate approvals in place from either the Animal Ethics Committee or the Human Ethics Committee: http://www.uq.edu.au/research/rid/ethical-clearances: Human ethics approval: **Pending**

HEALTH SURVEILLANCE: if your research project involves infectious material or animals, you may benefit from Health Surveillance. The Occupational Health Nurse Advisor will provide advice: (http://www.uq.edu.au/ohs/index.html?page=45274)

Signature of Chief Investigator: [signature] **Date:** 5/1/18

Signature IBSC Chair/Biosafety Advisor [signature] **Date:** 11/1/2018

Office use only: BIOSAFETY NUMBER: IBC/2238/ Dent / 2018 Review: 10/1/2023
Approved by: Biosafety Advisor ☑ / IBSC ☐ OHNA referral? ☐

Appendix 7.11: CONSORT Checklist for Reporting a Randomised Trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	238
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	239
Introduction			
Background and	2a	Scientific background and explanation of rationale	240-241
objectives	2b	Specific objectives or hypotheses	241-242
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	242
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	None
Participants	4a	Eligibility criteria for participants	244
	4b	Settings and locations where the data were collected	244
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	243-244
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	245-246
	6b	Any changes to trial outcomes after the trial commenced, with reasons	None
Sample size	7a	How sample size was determined	242
	7b	When applicable, explanation of any interim analyses and stopping guidelines	-
Randomisation:			
Sequence	8a	Method used to generate the random allocation sequence	244
generation	8b	Type of randomisation; details of any restriction (such as blocking and block size)	244

Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	244
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	244
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	244
	11b	If relevant, description of the similarity of interventions	-
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	246
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	-
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	Figure 1
	13b	For each group, losses and exclusions after randomisation, together with reasons	Figure 1
Recruitment	14a	Dates defining the periods of recruitment and follow-up	244
	14b	Why the trial ended or was stopped	-
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Figure 1
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	246-249
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	-
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	-

Harms	19	All-important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	None
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	253
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	253
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	250-253
Other information			
Registration	23	Registration number and name of trial registry	242
Protocol	24	Where the full trial protocol can be accessed, if available	-
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	None

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.