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# The prebiotic effect of CPP-ACP sugar-free chewing gum

James R. Fernando, Catherine A. Butler, Geoffrey G. Adams, Helen L. Mitchell, Stuart G. Dashper, Karen Escobar, Brigitte Hoffmann, Peiyan Shen, Glenn D. Walker, Yi Yuan, Coralie Reynolds, Eric C. Reynolds*

*Oral Health Cooperative Research Centre, Melbourne Dental School, Bio21 Institute, The University of Melbourne, Victoria, Australia*

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ABSTRACT

*Objectives:* To determine if chewing gum containing casein phosphopeptide stabilised amorphous calcium phosphate (CPP-ACP) promoted an increase in the abundance of *Streptococcus sanguinis* and other species associated with dental health in supragingival plaque in a clinical study.

*Materials and methods:* Nineteen participants were recruited for a three-leg cross-over, randomised, controlled clinical trial. Participants chewed a sugar-free gum with or without CPP-ACP six times daily for 20 min over two weeks. The study also involved no gum chewing (no gum) for the same two week period. Participants were randomly assigned to one of the test gums or no gum for each intervention period. Participants abstained from oral hygiene and had washout periods of two weeks between intervention periods. After each intervention period, supragingival plaque was collected and analysed for bacterial composition by sequencing the V4 variable region of the 16S rRNA gene. Data were analysed using a linear mixed model.

*Results:* The CPP-ACP gum intervention produced a significant ($p < 0.01$) increase in the proportions of *S. sanguinis* (112%), as well as the commensal species *Rothia dentocariosa* (127%), *Corynebacterium durum* (80%) and *Streptococcus mitis* (55%) when compared with the no gum intervention. All the species that were promoted by the CPP-ACP gum are known to possess one or both of the alkali-producing enzymes arginine deiminase and nitrate reductase.

*Conclusion:* This clinical study demonstrated that chewing a sugar-free gum containing CPP-ACP promoted prebiosis by significantly increasing the proportion of *S. sanguinis* and other health-associated bacterial species in supragingival plaque.

*Clinical significance:* Regular chewing of CPP-ACP sugar-free gum increases the proportions of health-associated commensal species in supragingival plaque to promote prebiosis and oral homeostasis.

## 1. Introduction

Dental caries progresses when there is an imbalance between demineralisation and remineralisation of the tooth resulting in a net loss of ions and an increase in tooth porosity. A clinical goal of caries management is to modify the oral environment to favour remineralisation during or after acid challenges. This not only involves increasing bioavailable calcium, phosphate and fluoride to the tooth surface, but also the promotion of commensal microorganisms that colonise and maintain plaque with a neutral pH. These favourable commensal microorganisms include the non-mutans streptococci such as *Streptococcus sanguinis* [1], which is a species known to be a biomarker of dental health possessing an inverse relationship with *Streptococcus mutans* correlating to caries status [2–4].

The primary anticariogenic effect of casein phosphopeptide stabilised amorphous calcium phosphate (CPP-ACP) is the ability to deliver bioavailable calcium, phosphate and fluoride ions to mineral deficient carious lesions to promote remineralisation [5–7]. Recent clinical evidence suggests casein phosphopeptide (CPP)-containing products can also reduce the number of mutans streptococci in dental biofilms thereby limiting acid production and demineralisation potential [8,9]. An increase in plaque pH and buffering capacity following the use of CPP-containing products has also been observed [10–12].

Due to the evidence suggesting therapeutic use of CPP-containing products reduces mutans streptococci levels and maintains a neutral pH, it was hypothesised that a CPP-containing product would accordingly favour an increase in abundance of *S. sanguinis* in dental plaque. To test this hypothesis, a randomised double-blind crossover clinical

* Corresponding author at: Melbourne Dental School, The University of Melbourne, 720 Swanston Street, Carlton, Melbourne, Victoria, 3010, Australia.
*E-mail address:* e.reynolds@unimelb.edu.au (E.C. Reynolds).

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study was conducted assessing the change in plaque composition based on chewing gum activity. The objective was to compare the abundance of *S. sanguinis* and accompanying microorganisms in supragingival dental plaque obtained from healthy participants following regular use of CPP-ACP sugar-free chewing gum, sugar-free chewing gum or no gum chewing. The null hypothesis was that chewing CPP-ACP sugar-free chewing gum would not significantly increase *S. sanguinis* abundance in dental plaque.

## 2. Materials & methods

### 2.1. Participants

Nineteen healthy participants living in Melbourne, Australia were recruited for this clinical study according to selection criteria and a clinical protocol approved by the University of Melbourne Human Research Ethics Committee (Application number 1441865, approved 20th February 2015). Australian and New Zealand Clinical Trials registration number is ACTRN 12617000148370. The study was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) [13]. The participants provided informed, written approval to participate in the study. The number of participants required for the study was based on power calculations using the results of a previous study [6]. The required sample size was calculated using the G*Power Version 3.1 sample size package [14]. With a 5% significance level and a power of 90% at least 18 participants were required. To allow for 5% participant attrition 19 participants were recruited for the study. Inclusion criteria for participants required having at least 20 natural teeth, a stimulated whole salivary flow rate above or equal to 1.0 ml/min, and an unstimulated whole salivary flow rate above or equal to 0.2 ml/min. Participants were screened clinically and excluded from the study for any of the following reasons: Allergy to milk protein or any ingredient in the experimental chewing gums; Pregnancy or lactation; Treatment with antibiotics or anti-inflammatory medications in the previous month prior to starting the study; Medical conditions requiring antibiotic prophylaxis prior to invasive dental procedures such as extractions or periodontal probing; Wearing dentures or orthodontic appliances; Dental veneers or more than one incisor with a crown; Oral pathology including periodontitis, untreated dental caries or tumours of the soft or hard dental tissues; Participation in another clinical study.

During clinical screening of participants, the presence of periodontal disease was determined by assessing 10 preselected teeth according to the Community Periodontal Index of Treatment Needs (CPITN) utilising a World Health Organisation periodontal probe [15]. Any individual with a CPITN score of 3 or above were deemed to have periodontal disease and unable to participate in the study.

### 2.2. Clinical protocol

The study was conducted at the Royal Dental Hospital of Melbourne in 2017. The study had a randomised, double-blind, three-period, crossover design. Participants were randomly assigned to one of the three 14-day interventions. A block randomized schedule was generated with block size six. This ensured that each of the treatment sequences (ABC, ACB, BCA, BAC, CAB, CBA) were assigned proportionately, and that the study was balanced for treatment and carry-over effects. With each block, the order of the six sequences was determined randomly. One week prior to commencing the first intervention, participants were instructed to use a specific toothbrush with a sodium fluoride toothpaste supplied to them (Colgate Sparkling Mint Gel (1000 ppm F); Colgate-Palmolive, New York, USA) and advised not to use any interdental cleaning aids such as floss during the entirety of the study. Between interventions, participants had a 14-day washout period after which they commenced another intervention in randomised order until they had completed all three interventions. Participants were instructed not to use any oral hygiene measures (including mouthrinse) during the intervention periods, however during the 14-day washout periods participants were permitted to resume their normal oral hygiene routine with the supplied toothpaste and toothbrush. The three interventions (A, B and C) were as follows: Intervention A: Abstain from oral hygiene and chew two pellets of gum for 20 min six times a day each day (sugar-free gum containing 18.8 mg CPP-ACP per gum pellet); Intervention B: Abstain from oral hygiene and chew two pellets of gum for 20 min six times a day each day (control sugar-free gum); and Intervention C: Abstain from oral hygiene. The chewing gums were manufactured by Mondelez International (Deerfield, USA).

**Table 1**
Ingredients of the sugar-free chewing gums used in the interventions. Ingredients in the right column were less than 2% of final composition. The chewing gums in different interventions differed only by the addition of CPP-ACP.

| Ingredients | |
|---|---|
| Sorbitol | Acesulfame potassium |
| Gum base | Aspartame |
| Glycerin | Butylated Hydroxyanisole (BHA) |
| Mannitol | Butylated Hydroxytoluene (BHT) |
| Natural and artificial flavouring | Green colour |
| Xylitol | Soy lecithin |
| | Acetylated monoglycerides |
| | Monoglycerides |
| | CPP-ACP (one intervention only) |

For periods requiring participants to chew gum, a log-sheet was given to record time and duration of chewing; participants were advised to leave no less than 50 min between chewing times. Participants were blinded as to which gum they were chewing during interventions A and B, and the dental examiner and laboratory staff were blinded as to which intervention the participants were completing. The ingredients of both gums are outlined in Table 1.

At the commencement of each intervention period participants received a scale and clean using an ultrasonic scaler and a polish with pumice paste. At the end of each intervention period participants attended a similar appointment however prior to the scale and clean supragingival plaque was collected from the upper right first and second molars using a sterile sickle scaler; these plaque collections were pooled for both teeth, labelled and frozen until DNA processing. Participants received a sodium fluoride mouthrinse at the conclusion of the final appointment. Before each clinical session participants were screened for any adverse events or change in medical history. All clinical appointments were conducted by the same dental examiner.

### 2.3. Microbiomic analysis

Genomic DNA was extracted from 57 plaque samples using methodology based on the NIH Human Microbiome Project Manual of Procedures [16,17]. Total DNA was extracted from plaque using PowerLyzer PowerSoil DNA Isolation Kits according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA quantitation was attained using a Qubit dsDNA High Sensitivity Assay kit (Thermo Fisher Scientific, Scoresby, Australia). Targeted amplification of the V4 region of the 16S rRNA phylogenetic marker gene was performed using PCR with custom barcoded primers. Amplicon libraries were purified using Agencourt AMPure XP Reagent according to the manufacturer's instructions (Beckman Coulter, Brea, USA). Purified barcoded amplicon libraries were quantified with a LabChip GX Touch 24 Nucleic Acid Analyzer using DNA high sensitivity kits (Perkin Elmer, Waltham, USA). Emulsion PCR of pooled amplicon libraries was performed using an Ion OneTouch 2 System with 400 bp protocols (Thermo Fisher Scientific, Scoresby, Australia) and multiplex sequencing was subsequently performed on an Ion Torrent Personal Genome Machine utilising Hi-Q chemistry with 525 reagent flows per run (Thermo Fisher Scientific,

Scoresby, Australia). Torrent Suite Software (Thermo Fisher Scientific, Scoresby, Australia) was used to demultiplex samples and trim sequencing adapters and barcodes. The Galaxy / GVL 4.1.0 workbench and the 'Find, Rapidly, OTUs with Galaxy Solution' (FROGS) pipeline [18] was used for filtering and clustering of sequences. Primer sequences were trimmed and sequences were removed that did not contain both forward and reverse primers, were less than 220 bp in length or greater than 350 bp, contained ambiguities, contained homopolymers with size > 7 nt, or contained two poor qualities scores (< 10) that were < = 10 nt apart. Clustering was performed using the Swarm method [19], chimeric DNA sequences were detected and removed as were any clusters containing only a single sequence. The resulting 16S ribosomal RNA gene sequences were then analysed against the MicroSEQ ID 16S rRNA Full Gene Library (Thermo Fisher Scientific, Scoresby, Australia), Greengenes 16S rRNA gene database [20], and the Human Oral Microbiome Database (HOMD [21];) to identify the bacteria to the genus or species level.

### 2.4. Statistical analysis

Descriptive statistics (mean and standard deviation) were calculated for the relative abundance of bacteria data, and then analysed using an analysis of variance (ANOVA) for a cross-over study approach. Intervention group (A, B or C) and intervention period (1, 2, or 3) were included in the models as within-participant effects. Intervention sequence (ABC, ACB, BAC, BCA, CAB and CBA) was included in the model as a between-participant effect, with participants nested beneath Intervention sequence. *Post hoc* comparisons of intervention differences were performed on the marginal means using the Sidak adjustment for multiple comparisons. Modelling assumptions were checked using residual and normal probability plots. Complementary log-log transformations of the relative abundance data were used prior to analysis. P values less than 0.05 were regarded as being statistically significant. All analyses were conducted using statistical software (Stata version 14.2; StataCorp LLC., College Station, USA).

## 3. Results

The mean age of the 19 participants recruited for the trial was 33.3 ± 9.2 years of age (20 to 50 years) with 6 males and 13 females. All participants completed the study with no adverse events. Their unstimulated saliva flow rate was 1.02 ± 0.72 ml/min and stimulated flow rate 2.98 ± 1.33 ml/min. The supragingival plaque microbiome data for the three groups (no gum, CPP-ACP gum and control gum) are presented in Fig. 1. The proportion of plaque bacteria identified as *S. sanguinis* following the no gum intervention was 2.6%. This was increased to 3.6% after chewing the control sugar-free gum, and 5.5% after chewing the CPP-ACP gum. While there was a trend for an increase in *S. sanguinis* abundance following periods of chewing the control sugar-free gum, only the CPP-ACP gum intervention showed a significant difference in *S. sanguinis* abundance ($p = 0.004$) when compared to the no gum intervention (Fig. 2), and hence the null hypothesis was rejected. This significant difference was more than a two-fold increase in *S. sanguinis* abundance that resulted from chewing the CPP-ACP gum. The *S. sanguinis* abundance after chewing the CPP-ACP sugar-free gum was also higher than that produced by the control sugar-free gum but the difference did not reach statistical significance with the limited power of the study (Table 2, Fig. 2).

In addition to *S. sanguinis*, over 300 different bacterial taxa were identified from the 57 samples. The 40 most abundant taxa accounted for approximately 77% of the total bacteria in the plaque (Fig. 1). The most abundant taxa were members of the *Corynebacterium*, *Streptococcus* and *Actinomyces* genera. Major pathogenic species associated with dental caries such as *Streptococcus mutans*, *Lactobacillus casei* and *Bifidobacteria* spp. were not commonly detected in the plaque samples of the dentally healthy participants.

Comparison of the supragingival plaque composition across interventions revealed significant differences between the interventions with chewing gum (either CPP-ACP gum or the sugar-free gum) and no chewing gum (see Table 2). Significant differences were found in the abundances of *Actinomyces massiliensis*, *Corynebacterium durum, Lautropia* genus, *Leptotrichia* sp./*wadei, Leptotrichia shahii, Rothia dentocariosa, Streptococcus gordonii/mitis* as well as *S. sanguinis*. Bacteria of the *Actinomyces* genus decreased in abundance during the sugar-free gum period compared with the no gum period. *Lautropia mirabilis*/sp., *Leptotrichia buccalis* and the *Propionibacterium* genus significantly decreased in the CPP-ACP chewing gum period compared with the no gum period. Supplementary Table S1 summarizes the relative abundances of the 40 most prevalent taxa.

## 4. Discussion

Chewing gum is known to increase saliva flow for up to 2 h after chewing [22], increasing the clearance rate of nutrients and microorganisms as well as the buffering capacity, calcium phosphate level and antimicrobial activity [23]. Consequently, commensal species that tolerate or thrive in biofilms with neutral pH and relatively high calcium phosphate concentrations are more favoured to dominate after chewing gum, which was demonstrated in the current study. Among a low caries risk population these species would be expected to be naturally high in abundance in supragingival plaque. In accordance, only a few species were detected to significantly change in abundance between intervention periods, and the overall supragingival plaque bacterial composition among all individuals belonged to taxa typical in oral health [24], with no mutans streptococci or lactobacilli among the 40 most abundant species detected in any individuals.

The CPP-ACP chewing gum significantly increased the proportion of *S. sanguinis* compared with the non-chewing group, a species that has been consistently associated with dental health and low caries incidence [2,25]. *S. sanguinis* is a well characterised commensal microorganism that is an early coloniser of the dental hard tissues [4]. Multiple studies have suggested *S. sanguinis* possesses an antagonistic relationship with *S. mutans*, promoting a delay or inhibition of *S. mutans* colonisation largely due to its production of $H_2O_2$ [2,4,26–28]. Previous investigators who have demonstrated a decrease in plaque *S. mutans* following use of CPP-ACP-containing products have suggested that CPP-ACP's strong buffering effect and interference with bacterial adherence impeded *S. mutans* colonisation [9,29]. However, the CPP-ACP associated increase in *S. sanguinis* in the current study suggests increased *S. sanguinis* antagonism may have also contributed to decreased *S. mutans* levels observed in those previous studies.

*S. sanguinis* is also known to metabolise arginine through expression of the enzyme arginine deiminase (AD) which produces alkali to inhibit drops in plaque pH [30]. There has been cumulative evidence that bacterial alkali production through AD is an important mechanism maintaining oral biofilm homeostasis in healthy individuals, translating to a lower risk of developing caries [31–36]. Arginine occurs naturally in saliva in its free form at a concentration of approximately 50 μM and is additionally present within numerous salivary proteins/peptides [31,37]. AD expression occurs when bacteria are exposed to arginine and low pH in order to generate adenosine triphosphate, which additionally produces citrulline and ammonia as by-products. Citrulline and ammonia are released into the surrounding plaque fluid, the latter having a buffering effect [33]. One of the major CPPs, β-casein(1-25), contains N- and C- terminal arginine which can be released from the peptide through enzymatic hydrolysis (peptidase) and catabolised through the AD pathway by oral bacteria to produce ammonia [38,39]. It has been shown that chewing CPP-ACP gum results in the incorporation of CPP-ACP into supragingival plaque and that the CPP-ACP could still be detected in plaque three hours after chewing one piece of gum [40]. Therefore, it would be expected that chewing the CPP-ACP gum in the current study would have loaded the plaque with

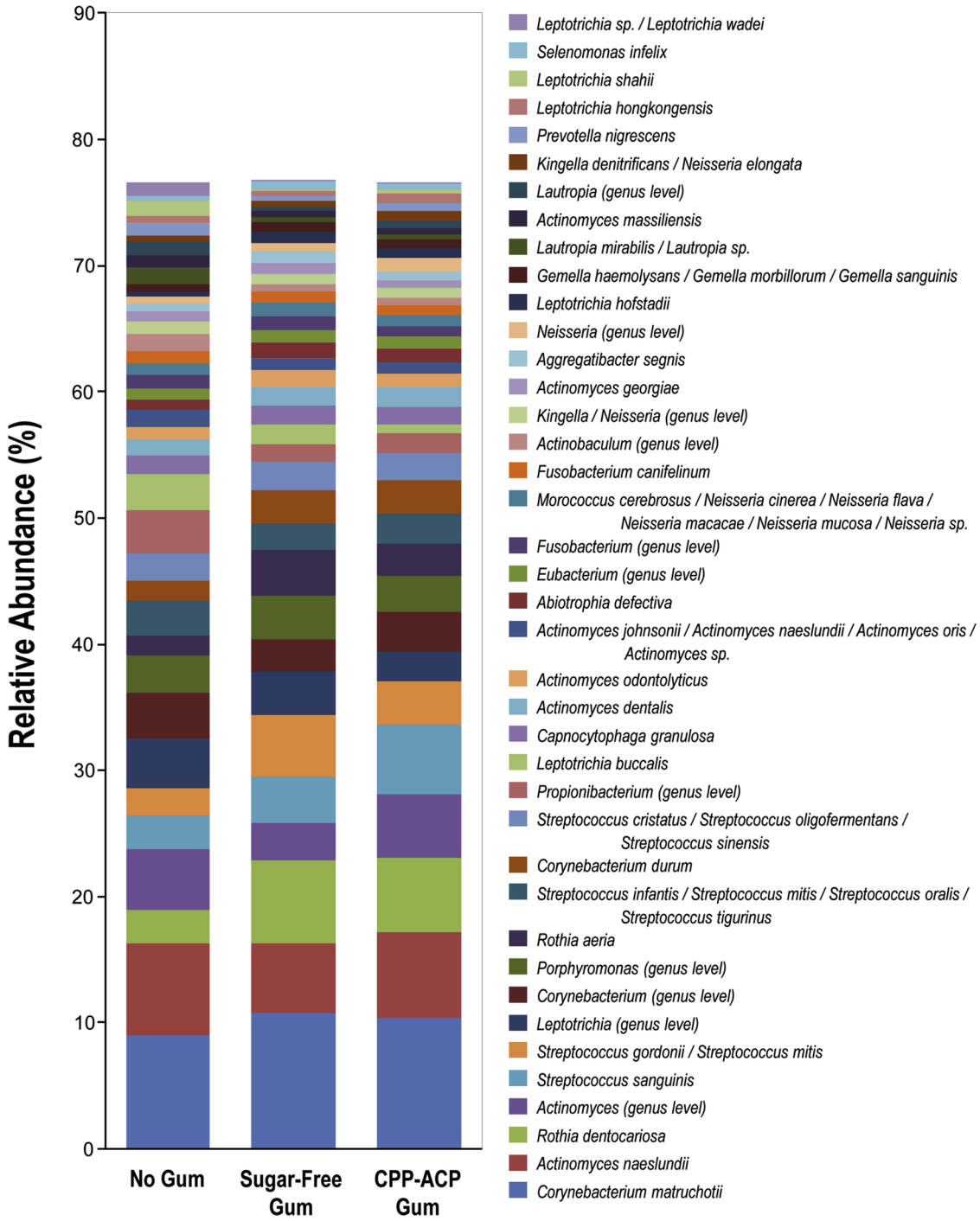


**Fig. 1.** Supragingival plaque microbiome data for the three interventions (No gum, CPP-ACP gum and Control Sugar-Free gum). The 40 most abundant species are shown, sorted from top to bottom by increasing average relative abundance.

CPP-ACP. This may have promoted AD expression and production of ammonia, and consequently favoured the colonisation and metabolic activity of microorganisms such as *S. sanguinis* [39]. As individuals with a history of dental caries have previously been shown to have lower free salivary arginine than caries-free adults [37], chewing CPP-ACP gum may provide these individuals with a supplementary form of arginine and other ammonia-generating amino acids, thereby promoting colonisation/emergence of ammonia-producing bacteria in plaque.

Chewing the sugar-free chewing gums also significantly increased the abundance of *S. gordonii/mitis* compared with the no gum intervention (these two species were unable to be differentiated with the current analysis). Both *S. mitis* and *S. gordonii* are AD positive, $H_2O_2$ producing bacteria, though they are believed to play a less significant role in caries inhibition than *S. sanguinis* [28,41].

Compared with the no gum period, both the CPP-ACP sugar-free gum and control sugar-free gum interventions also significantly increased the proportion of *C. durum* and *R. dentocariosa*. In a recent study assessing the plaque microbiome in caries-free and caries-active children the *Rothia* and *Corynebacterium* genera, in addition to *S. sanguinis* were significantly higher in caries-free children [25]. Both *C. durum* and *R. dentocariosa* are nitrate reducing bacteria and are now considered commensals [42]. Evidence of a caries preventive effect by nitrate reducing bacteria has been reported where individuals with high salivary nitrate levels and a high nitrate reducing capacity of resident oral

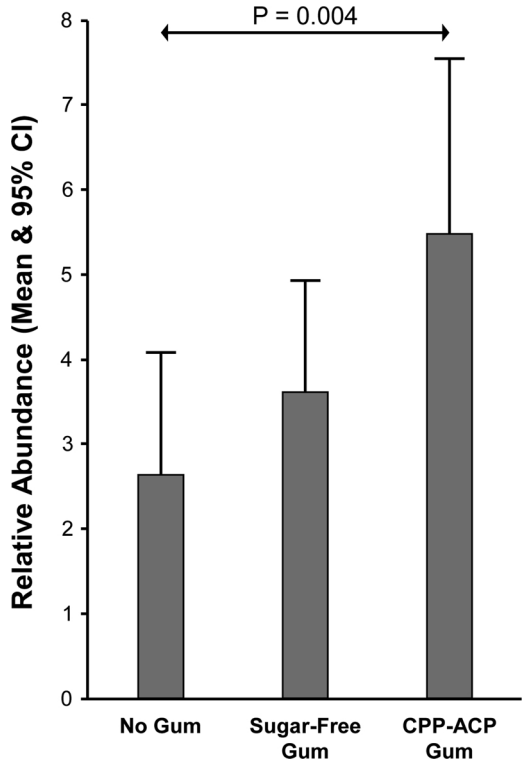


**Fig. 2.** Mean abundance and 95% confidence interval of *S. sanguinis* according to treatment period. A significant difference between the CPP-ACP gum and no gum interventions was observed (p = 0.004).

bacteria exhibited less dental caries than individuals with low salivary nitrate and low bacterial nitrate reducing capacity [43]. The bacterial enzyme nitrate reductase (NR) is active in certain bacteria to enable nitrate metabolism during anaerobic conditions when their preferred oxygen dependent metabolism is impeded. Intra-oral nitrate ($NO_3^-$) is mainly sourced from saliva and diet and is reduced to nitrite ($NO_2^-$) through bacterial NR. During acidic conditions $NO_2^-$ is protonated to nitrous acid ($HNO_2$) which is inherently unstable and forms dinitrogen trioxide ($N_2O_3$), nitrogen dioxide ($NO_2$) and nitric oxide (NO) [44]. Expression of NR inhibits drops in plaque pH through the acidification of nitrite and additionally inhibits *S. mutans* and *Lactobacillus casei* levels due to production of NO [45]. Intra-oral bacterial NR activity has also been proposed to have an important role in maintaining circulatory NO essential for vascular health as ingested nitrite is absorbed and converted to NO in the bloodstream [46]. Stimulated saliva has an increased buffering capacity and total intraoral nitrate output [23,47], while CPP-ACP additionally increases plaque pH and buffering capacity [10–12]. NR activity is promoted when the salivary pH increases from 6 to 8 and when salivary nitrate levels increase [48], hence the buffering effect of CPP-ACP may have indirectly promoted NR activity.

In addition to the significant increase in the proportion of *C. durum* and *R. dentocariosa*, chewing the control sugar-free or CPP-ACP sugar-free gum significantly decreased the proportion of *A. massiliensis*, *L. shahii, L.* sp./*wadei*, genus *Lautropia*, *L. mirabilis*/sp., L. *buccalis*, and genus *Propionibacterium* when compared with the no gum intervention (Table 2). No aetiological or protective role for these microorganisms has been established with regards to dental caries however as some are Gram negative LPS-producing species they may have a role in gingival inflammation such that a decrease in abundance may also have a protective role in periodontal disease.

The current study showed regular chewing of CPP-ACP gum resulted in a significant increase in *S. sanguinis* abundance compared to not chewing gum. This was postulated to occur through plaque CPP-ACP incorporation promoting a biofilm with a high buffering capacity to favour neutral pH and providing a source of arginine and other amine-rich amino acids to generate ammonia and maintain pH and oral homeostasis. The bacterial species detected were considered non-cariogenic which was consistent with the low caries risk individuals who participated in the study. Had the study included high caries risk participants perhaps a marked effect on acidogenic microorganisms may have resulted following the chewing gum interventions. The findings suggest that chewing CPP-ACP gum may have an additional anticariogenic effect apart from promoting remineralisation: and that is prebiosis of supragingival plaque promoting health-related commensal species over cariogenic (acidogenic and aciduric) species. This may help explain the significant reduction in caries experience observed in children chewing the CPP-ACP sugar-free gum when compared with a control sugar-free gum in a randomised, controlled clinical trial [7]. Hence, for prevention of dental caries, chewing CPP-ACP sugar-free gum may be recommended over chewing gum without CPP-ACP.

In conclusion, chewing CPP-ACP sugar-free gum significantly

**Table 2**
Significantly different changes in bacterial genus and species abundance in supragingival plaque for Day 14 by CPP-ACP gum, sugar-free gum or no gum interventions. The results are presented as averages of the percentage of the total bacteria in supragingival plaque.

| | Abundance (%)[c] | | | Significance (Overall comparison) |
|---|---|---|---|---|
| | CPP-ACP Gum | Sugar-free Gum | No Gum | |
| ***Genus Level*** | | | | |
| *Actinomyces* | 5.0 (4.2) | 3.0 (2.7) [b] | 4.8 (3.2) | 0.04 |
| *Lautropia* | 0.6 (1.2) [a] | 0.2 (0.4) [b] | 1.0 (1.4) | 0.0003 |
| *Propionibacterium* | 1.5 (2.8) [a] | 1.4 (1.9) [b] | 3.4 (4.6) | 0.02 |
| ***Species Level*** | | | | |
| *Actinomyces massiliensis* | 0.5 (0.5) [a] | 0.5 (0.5) [b] | 1.0 (0.9) | 0.002 |
| *Corynebacterium durum* | 2.7 (2.2) [a] | 2.7 (1.9) [b] | 1.5 (1.6) | 0.00005 |
| *Lautropia mirabilis / Lautropia sp.* | 0.4 (0.5) [a] | 0.4 (0.5) [b] | 1.3 (2.1) | 0.01 |
| *Leptotrichia buccalis* | 0.7 (0.8) [a] | 1.6 (2.4) | 2.9 (3.7) | 0.04 |
| *Leptotrichia shahii* | 0.3 (0.5) [a] | 0.2 (0.3) [b] | 1.1 (2.1) | 0.002 |
| *Leptotrichia sp./wadei* | 0.1 (0.1) [a] | 0.1 (0.1) [b] | 1.1 (2.8) | 0.0005 |
| *Rothia dentocariosa* | 5.9 (7.0) [a] | 6.6 (6.0) [b] | 2.6 (2.4) | 0.0001 |
| *Streptococcus gordonii/mitis* | 3.4 (3.4) [a] | 4.9 (6.5) [b] | 2.2 (4.0) | 0.04 |
| ***Streptococcus sanguinis*** | **5.5 (4.3)** [a] | **3.6 (2.7)** | **2.6 (2.0)** | **0.005** |

[a, b]Significantly different to the No Gum treatment.
[c]Data are displayed as mean (standard deviation). The abundance data were sorted first by the overall average abundance for each species and the 30 species with the highest average abundances were selected for further analysis. The abundance data were then sorted separately for each treatment group and the 30 species with the highest average abundances were identified for each treatment group. This resulted in a further 10 species being selected for further analysis. The 40 species identified accounted for approximately 77% of the bacterial species abundance in each treatment group. The unit of analysis was the participant.

increased the abundance of *S. sanguinis* and other alkali-producing species positive for arginine deiminase and nitrate reductase in supragingival plaque when compared with a no gum intervention.

## CRediT authorship contribution statement

**James R. Fernando:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Catherine A. Butler:** Methodology, Investigation, Writing - review & editing. **Geoffrey G. Adams:** Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing - review & editing. **Helen L. Mitchell:** Investigation, Formal analysis, Writing - review & editing. **Stuart G. Dashper:** Conceptualization, Supervision, Formal analysis, Writing - review & editing. **Karen Escobar:** Investigation, Project administration, Writing - review & editing. **Brigitte Hoffmann:** Methodology, Investigation, Writing - review & editing. **Peiyan Shen:** Methodology, Investigation, Supervision, Project administration, Writing - review & editing. **Glenn D. Walker:** Methodology, Investigation, Writing - review & editing. **Yi Yuan:** Methodology, Investigation, Writing - review & editing. **Coralie Reynolds:** Methodology, Investigation, Project administration, Writing - review & editing. **Eric C. Reynolds:** Conceptualization, Methodology, Resources, Supervision, Visualization, Writing - original draft, Writing - review & editing, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdent.2019.103225.

## References

[1] P.D. Marsh, Microbiology of dental plaque biofilms and their role in oral health and caries, Dent. Clin. North Am. 54 (2010) 441–454.
[2] R.A. Giacaman, S. Torres, Y. Gomez, C. Munoz-Sandoval, J. Kreth, Correlation of *Streptococcus mutans* and *Streptococcus sanguinis* colonization and *ex vivo* hydrogen peroxide production in carious lesion-free and high caries adults, Arch. Oral Biol. 60 (2015) 154–159.
[3] P.M. Corby, J. Lyons-Weiler, W.A. Bretz, T.C. Hart, J.A. Aas, T. Boumenna, J. Goss, A.L. Corby, H.M. Junior, R.J. Weyant, B.J. Paster, Microbial risk indicators of early childhood caries, J. Clin. Microbiol. 43 (2005) 5753–5759.
[4] P.W. Caufield, A.P. Dasanayake, Y. Li, Y. Pan, J. Hsu, J.M. Hardin, Natural history of *Streptococcus sanguinis* in the oral cavity of infants: evidence for a discrete window of infectivity, Infect. Immun. 68 (2000) 4018–4023.
[5] N.J. Cochrane, E.C. Reynolds, Calcium phosphopeptides—mechanisms of action and evidence for clinical efficacy, Adv. Dental Res. 24 (2012) 41–47.
[6] N.J. Cochrane, P. Shen, S.J. Byrne, G.D. Walker, G.G. Adams, Y. Yuan, C. Reynolds, B. Hoffmann, S.G. Dashper, E.C. Reynolds, Remineralisation by chewing sugar-free gums in a randomised, controlled in situ trial including dietary intake and gauze to promote plaque formation, Caries Res. 46 (2012) 147–155.
[7] M.V. Morgan, G.G. Adams, D.L. Bailey, C.E. Tsao, S.L. Fischman, E.C. Reynolds, The anticariogenic effect of sugar-free gum containing CPP-ACP nanocomplexes on approximal caries determined using digital bitewing radiography, Caries Res. 42 (2008) 171–184.
[8] S. Chandak, A. Bhondey, A. Bhardwaj, J. Pimpale, M. Chandwani, Comparative evaluation of the efficacy of fluoride varnish and casein phosphopeptide – amorphous calcium phosphate in reducing *Streptococcus mutans* counts in dental plaque of children: an *in vivo* study, J. Int. Soc. Prev. Community Dent. 6 (2016) 423–429.
[9] S. Emamieh, Y. Khaterizadeh, H. Goudarzi, A. Ghasemi, A.A. Baghban, H. Torabzadeh, The effect of two types chewing gum containing casein phosphopeptide-amorphous calcium phosphate and xylitol on salivary *Streptococcus mutans*, J. Conserv. Dent. 18 (2015) 192–195.
[10] P.C. Caruana, S.A. Mulaify, R. Moazzez, D. Bartlett, The effect of casein and calcium containing paste on plaque pH following a subsequent carbohydrate challenge, J. Dent. 37 (2009) 522–526.
[11] H. Heshmat, S. Banava, E. Mohammadi, M.J. Kharazifard, F. Mojtahedzadeh, The effect of recommending A CPP-ACPF product on salivary and plaque pH levels in orthodontic patients: a randomized cross-over clinical trial, Acta Odontol. Scand. 72 (2014) 903–907.
[12] T. Peric, D. Markovic, B. Petrovic, V. Radojevic, T. Todorovic, B.A. Radicevic, R.J. Heinemann, G. Susic, A.P. Popadic, V.T. Spiric, Efficacy of pastes containing CPP-ACP and CPP-ACFP in patients with Sjogren's syndrome, Clin. Oral Investig. 19 (2015) 2153–2165.
[13] Bulletin of the World Health Organization World Medical Association, World Medical Association Declaration of Helsinki, Ethical principles for medical research involving human subjects, Bull. World Health Organ 79 (2001) 373.
[14] F. Faul, E. Erdfelder, A.G. Lang, A. Buchner, G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences, Behav Res Methods 39 (2007) 175–191.
[15] T.W. Cutress, J. Ainamo, J. Sardo-Infirri, The community periodontal index of treatment needs (CPITN) procedure for population groups and individuals, Int. Dent. J. 37 (1987) 222–233.
[16] S. Yuan, D.B. Cohen, J. Ravel, Z. Abdo, L.J. Forney, Evaluation of methods for the extraction and purification of DNA from the human microbiome, PLoS One 7 (2012) e33865.
[17] P. McInnes, M. Cutting, Manual of Procedures for Human Microbiome project, Core Microbiome Sampling, Protocol A, HMP Protocol # 07-001, (2010).
[18] F. Escudie, L. Auer, M. Bernard, M. Mariadassou, L. Cauquil, K. Vidal, S. Maman, G. Hernandez-Raquet, S. Combes, G. Pascal, FROGS: find, rapidly, OTUs with galaxy solution, Bioinformatics 34 (2018) 1287–1294.
[19] F. Mahe, T. Rognes, C. Quince, C. de Vargas, M. Dunthorn, Swarm: robust and fast clustering method for amplicon-based studies, PeerJ 2 (2014) e593.
[20] T.Z. DeSantis, P. Hugenholtz, N. Larsen, M. Rojas, E.L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, G.L. Andersen, Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB, Appl. Environ. Microbiol. 72 (2006) 5069–5072.
[21] T. Chen, W.H. Yu, J. Izard, O.V. Baranova, A. Lakshmanan, F.E. Dewhirst, The Human Oral Microbiome Database: a Web Accessible Resource for Investigating Oral Microbe Taxonomic and Genomic Information, Database (Oxford) 2010, 2010, p. baq013.
[22] C. Dawes, K. Kubieniec, The effects of prolonged gum chewing on salivary flow rate and composition, Arch. Oral Biol. 49 (2004) 665–669.
[23] C. Dawes, Salivary flow patterns and the health of hard and soft oral tissues, J. Am. Dent. Assoc. 139 (Suppl) (2008) 18S–24S.
[24] E. Zaura, B.J.F. Keijser, S.M. Huse, W. Crielaard, Defining the healthy "core microbiome" of oral microbial communities, BMC Microbiol. 9 (2009) 259.
[25] M. Agnello, J. Marques, L. Cen, B. Mittermuller, A. Huang, N. Chaichanasakul Tran, W. Shi, X. He, R.J. Schroth, Microbiome associated with severe caries in Canadian first nations children, J. Dent. Res. 96 (2017) 1378–1385.
[26] Y. Ge, P.W. Caufield, G.S. Fisch, Y. Li, *Streptococcus mutans* and *Streptococcus sanguinis* colonization correlated with caries experience in children, Caries Res. 42 (2008) 444–448.
[27] J. Kreth, J. Merritt, W.Y. Shi, F.X. Qi, Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm, J. Bacteriol. 187 (2005) 7193–7203.
[28] J. Kreth, Y. Zhang, M.C. Herzberg, Streptococcal antagonism in oral biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus mutans*, J. Bacteriol. 190 (2008) 4632–4640.
[29] M.L. Pukallus, K.A. Plonka, T.F. Holcombe, A.G. Barnett, L.J. Walsh, W.K. Seow, A randomized controlled trial of a 10 percent CPP-ACP cream to reduce mutans streptococci colonization, Pediatr. Dent. 35 (2013) 550–555.
[30] K.J. Ferro, G.R. Bender, R.E. Marquis, Coordinately repressible arginine deiminase system in *Streptococcus sanguis*, Curr. Microbiol. 9 (1983) 145–149.
[31] R.A. Burne, R.E. Marquis, Alkali production by oral bacteria and protection against dental caries, FEMS Microbiol. Lett. 193 (2000) 1–6.
[32] V. Gordan, C. Garvan, M. Ottenga, R. Schulte, P. Harris, D. McEdward, I. Magnusson, Could alkali production be considered an approach for caries control? Caries Res. 44 (2010) 547–554.
[33] Y.L. Liu, M. Nascimento, R.A. Burne, Progress toward understanding the contribution of alkali generation in dental biofilms to inhibition of dental caries, Int. J. Mol. Sci. 4 (2012) 135–140.
[34] M.M. Nascimento, V.V. Gordan, C.W. Garvan, C.M. Browngardt, R.A. Burne, Correlations of oral bacterial arginine and urea catabolism with caries experience, Oral Microbiol. Immunol. 24 (2009) 89–95.
[35] M.M. Nascimento, Y. Liu, R. Kalra, S. Perry, A. Adewumi, X. Xu, R.E. Primosch, R.A. Burne, Oral arginine metabolism may decrease the risk for dental caries in children, J. Dent. Res. 92 (2013) 604–608.
[36] M.M. Nascimento, A.J. Alvarez, X. Huang, S. Hanway, S. Perry, A. Luce, V.P. Richards, R.A. Burne, Arginine metabolism in supragingival oral biofilms as a potential predictor of caries risk, JDR Clin Trans Res. (2019) 2380084419834234.
[37] B. Van Wuyckhuyse, H. Perinpanayagam, D. Bevacqua, R. Raubertas, R. Billings,

W. Bowen, L. Tabak, Association of free arginine and lysine concentrations in human parotid saliva with caries experience, J. Dent. Res. 74 (1995) 686–690.
[38] K.J. Cross, N.L. Huq, J.E. Palamara, J.W. Perich, E.C. Reynolds, Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes, J. Biol. Chem. 280 (2005) 15362–15369.
[39] E.C. Reynolds, P.F. Riley, Protein dissimilation by human salivary-sediment bacteria, J. Dent. Res. 68 (1989) 124–129.
[40] E.C. Reynolds, F. Cai, P. Shen, G.D. Walker, Retention in plaque and remineralization of enamel lesions by various forms of calcium in a mouthrinse or sugar-free chewing gum, J. Dent. Res. 82 (2003) 206–211.
[41] J. Carlsson, Y. Iwami, T. Yamada, Hydrogen peroxide excretion by oral streptococci and effect of lactoperoxidase-thiocyanate-hydrogen peroxide, Infect. Immun. 40 (1983) 70–80.
[42] J.J. Doel, N. Benjamin, M.P. Hector, M. Rogers, R.P. Allaker, Evaluation of bacterial nitrate reduction in the human oral cavity, Eur. J. Oral Sci. 113 (2005) 14–19.
[43] J.J. Doel, M.P. Hector, C.V. Amirtham, L.A. Al-Anzan, N. Benjamin, R.P. Allaker, Protective effect of salivary nitrate and microbial nitrate reductase activity against caries, Eur. J. Oral Sci. 112 (2004) 424–428.
[44] J.O. Lundberg, E. Weitzberg, J.A. Cole, N. Benjamin, Nitrate, bacteria and human health, Nat. Rev. Microbiol. 2 (2004) 593–602.
[45] L.S. Mendez, R. Allaker, J. Hardie, N. Benjamin, Antimicrobial effect of acidified nitrite on cariogenic bacteria, Oral Microbiol. Immunol. 14 (1999) 391–392.
[46] W.G. Wade, The oral microbiome in health and disease, Pharmacol. Res. 69 (2013) 137–143.
[47] T. Granli, R. Dahl, P. Brodin, O.C. Bockman, Nitrate and nitrite concentrations in human saliva: variations with salivary flow-rate, Food Chem. Toxicol. 27 (1989) 675–680.
[48] J. Xu, X. Xu, W. Verstraete, Quantitative measurement of the nitrate reductase activity in the human oral cavity, Food Chem. Toxicol. 39 (2001) 393–400.