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Effects of a Topical Crème Containing CPP-ACP with Fluoride (Tooth Mousse Plus™) on Dental Plaque during Orthodontic Treatment

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Recent studies have shown prebiotic effects of casein phosphopeptides on dental plaque microflora in orthodontic patients. The aim of this study was to assess dental plaque ecological changes in orthodontic patients that resulted from the application of a topical crème containing casein phosphopeptides and 900 ppm fluoride (GC Tooth Mousse Plus™) (TMP) over a 4-week period. In this study, orthodontic patients being treated with fixed appliances were randomized to 3 groups; 21 received TMP, 22 received a placebo, and 21 were untreated controls. Subjects applied TMP once daily at bedtime. Plaque was stained with GC TriPlaque ID Gel at baseline and after 28 days. Areas of thin (pink), mature (violet) and acid producing (light blue) plaque biofilm in the cervical half of the maxillary anterior teeth were analyzed in a blinded manner, tracking each site over time. The plaque composition at the site with the strongest acid production was assessed using molecular methods (RT-qPCR) for 14 bacterial species. TMP caused a significant reduction in total plaque area, mature plaque area, and acid producing plaque area (all p values < 0.0001). A dose response relationship was noted for these TMP effects, with low users (mean 0.11 g/day) having less changes in plaque acid production than high users (mean 0.30 g/day). On the other hand, more total, mature and acid-producing plaque was seen in the placebo and control groups. With TMP, samples of the most acidogenic plaque had increased levels of the health-related

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bacterial species *Streptococcus salivarius.* An opposite trend was seen in both the placebo and control groups. Thus, daily use of TMP exerts beneficial ecological effects on dental plaque composition and metabolism in orthodontic patients.

Keywords: CPP-ACP; orthodontics; dental plaque; disclosing; prebiotic.

1. INTRODUCTION

One of the common complications of orthodontic treatment is an increase in the risk of demineralization of teeth [1]. This occurs because the irregular surfaces of brackets, bands, wires, and other attachments are stagnation areas for dental plaque, and make tooth cleaning more difficult. Fixed and removable orthodontic appliances provide an increased surface area for retention of biofilm which may induce oral ecologic changes, leading to increased levels of the keystone caries pathogen *Streptococcus mutans* in saliva and dental plaque biofilms, with corresponding reductions in key health-associated bacteria such as *Streptococcus salivarius* and *Streptococcus sanguinis* [1,2].

Changes in dental plaque metabolic activity can be tracked by using pH responsive dyes. TriPlaque ID™ gel (GC Corporation, Tokyo, Japan) uses two acid-sensitive dyes (Rose Bengal, brilliant blue FCF) that change colour according to pH. The product contains sucrose as a substrate for biofilm acid production. In plaque that is less than 24 hours old, the red dye binds to the plaque, which in turn is stained pink/red. Mature deposits of dental plaque bind and trap both dyes, and are stained a dark blue/purple colour [3]. Areas of acid-producing dental plaque biofilm appear in a light blue color, as a consequence of a drop in pH that turns the red dye colour lighter, and the dark blue dye into light blue [4-6]. Areas of biofilm that are stained in light blue have been shown to correlate with high levels of *S. mutans* and other cariogenic bacteria, and to the occurrence of caries [5]. Light blue stained plaque is the most damaging in terms of risks of decalcification of enamel during orthodontic treatment [6].

The topical application of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) has been shown to have benefits for caries prevention [7-10], especially for preventing and reversing the development of enamel decalcification lesions during fixed orthodontic treatment [11-17]. Likewise, glass ionomer cement materials that release CPP-ACP and fluoride can help prevent the formation of subsurface lesions of enamel adjacent to orthodontic brackets [18]. A likely contributing reason for the caries preventive effects of CPP-ACP on dental caries in the orthodontic setting is its ability to alter the composition and metabolism of the dental plaque biofilm, exerting a prebiotic action which counters the dysbiosis responsible for dental caries [19,20]. Laboratory studies using polymicrobial biofilms have shown that CPP-ACP exerts prebiotic effects and causes an increase in the abundance of the health-associated species *Streptococcus salivarius* and *Streptococcus sanguinis*, and reduced levels of cariogenic species [21,22]. Physical disruption of single species biofilms of S. mutans by CPP-ACP has also been reported [23]. Such actions align with the most recent concepts of dental caries prevention, based on ecological shifts, prebiotic effects and disrupting cariogenic virulence factors [9, 24].

In clinical trials, once-daily application of a CPP-ACP crème in children aged 18 to 24 months of age reduced levels of *S. mutans* [25], and elevated dental plaque biofilm pH levels in orthodontic patients [26,27]. Likewise, recent clinical trials of a fluoride dentifrice that contained the same level of CPP-ACP (10% by weight) showed prebiotic effects (CPP-ACP) and was able to beneficially modulate the microbial ecology of dental plaque in orthodontic patients with fixed appliances [24].

Given these positive findings from previous investigations, the aim of this study was of interest to explore whether the application of a fluoride-containing CPP-ACP crème (GC Tooth Mousse Plus™) (TMP) to orthodontic patients could alter the plaque microflora and reduce acid production following a sucrose challenge, when assessed using TriPlaque ID gel, with a treatment period of 4 weeks. The null hypothesis was that daily application of TMP onto the teeth would not reduce the area of mature dental plaque or the extent of acid production in patients undergoing fixed orthodontic treatment.

2. MATERIALS AND METHODS

The study design was a parallel 3-group double-blinded randomized controlled trial, with an active arm, a placebo arm, and a no treatment arm. The study followed a repeated measures design, tracking each subject from baseline, so that changes could be compared within each individual subject. As described previously [28], a power analysis was used to determine the sample size needed for a 3-group, 1-way analysis of variance (ANOVA) test, assuming an alpha of 0.05, and a power of 80%. This analysis revealed that a total sample size of 60 was needed (20 per group). The study was conducted in a university orthodontic postgraduate clinic, with institutional ethics approval (No. 2013001324) and informed written consent. A total of 64 patients (aged between 10 and 49 years) who were currently undergoing fixed orthodontic treatment in both maxillary and mandibular arches were recruited. The purpose of the project was explained to the subjects, and to the subjects and their parents, for those aged under 18 years. Written informed consent was obtained.

The inclusion criteria were as follows: at least four fully erupted permanent maxillary anterior teeth, (2) good physical health and an absence of systemic disease, as determined by a review of the medical history, and (3) existing fixed orthodontic appliances present in both arches for at least 1 month. Exclusion criteria were (1) current or recent use of antibiotics or an antibacterial/anti-plaque mouthrinse, (2) any medical conditions or disability that impaired manual tooth brushing, (3) allergy to milk casein proteins or benzoate preservatives, or known intolerance to any of the components of Tooth Mousse Plus crème, (3) unwillingness to use a fluoridated dentifrice, (4) untreated periodontal disease, (5) clinical evidence of high caries activity with more than 1 untreated carious lesions, and (6) strict adherence to a Halal diet. The latter requirement relates to the use of porcine enzymes for one stage of the production of CPP-ACP from bovine milk. After enrolment, subjects were assigned randomly to one of the three experimental groups.

The tubes containing the active and the placebo crème were coded and had the same external packaging, and their content was not revealed to the participants or the enrolling investigator. The dentifrice product codes were known only to the supervisor of the study (LJW), who was not involved in participant enrolment or product allocation. The product codes were broken only after the final analysis of the study results.

2.1 Baseline Assessment

All subjects were seen at the same time of the day, i.e. in the early afternoon. This was intended to standardize factors such as changes in salivary flow and eating patterns between the two visits of the study. At the baseline visit, before removing orthodontic modules, chain or ligature-ties, existing plaque was stained using TriPlaque ID™ gel (GC Corporation, Tokyo, Japan), following the manufacturer's instructions. The gel was applied with a microbrush onto the labial surfaces of all maxillary anterior teeth and left in place for 4 minutes, then patients were instructed to rinse their mouth gently with water and expectorate.

A series of three standardized high resolution (14 megapixel) digital photographs were taken, using a professional clinical camera with a macro lens (Canon EOS 650D with EFS 100 mm macro lens and MR-14EX Macro Ring Lite flash, Canon, Tokyo, Japan). The first photograph was perpendicular to the contact point between teeth 13 and 12, the second was perpendicular to the contact point between 11 and 21, and the third was perpendicular to the contact point between 22 and 23. All photographs were taken at exactly the same distance of 40 cm, with the aid of a positioning cord running from the body of the camera and to the subject's face. Consistent camera settings were used (manual focus, aperture f32, shutter speed 1/200 sec, speed ISO 100, constant manual white balance settings, consistent flash exposure). Automatic white balance and automatic exposure settings were not used as these cause variations in colour balance and exposure. During photography, the oral cavity was lit with a daylight colour temperature LED operating light (Belmont AL902 Bel-Halo 900 series, Belmont, Somerset, NJ, USA), while ambient room lighting was from daylight colour temperature fluorescent lamps. There was no ambient sunlight in the clinical area. Images were coded to allow subsequent analysis in a fully blinded manner.

To provide a consistent platform of mechanical plaque control, once the baseline photographs had been taken, each subjects was given a manual toothbrush (TePe Select ™, Tepe, Malmo, Sweden) and a tube of a 1500 ppm fluoride dentifrice (Colgate® Maximum Cavity

Protection, Colgate-Palmolive, Villawood, Australia), together with standardized oral hygiene instruction. Subjects were instructed to use the supplied dentifrice with twice daily manual brushing throughout the 4 weeks of the trial.

2.2 Treatment Groups

There were three groups. Subjects in the experimental group (Group A) received the active product (GC Tooth Mousse Plus, GC Corporation, Tokyo, Japan), a topical crème containing 10% w/w CPP-ACP and 900 ppm fluoride (as sodium fluoride). Subjects in the placebo group (Group B) received the placebo product. This placebo was identical in composition and flavour, but lacked the CPP-ACP component. The placebo contained the same level of fluoride (900 ppm F) as the active paste. Patients were not aware of the identity of the crème, as all tubes were coded. As mentioned earlier, products were supplied by the manufacturer in unmarked tubes, to ensure blinding. All tubes were numbered individually, and were pre-weighed so that the extent of product use could be tracked.

Subjects in groups A and B were instructed to first brush their teeth with the supplied dentifrice, and then after rinsing to rub a pea size amount of the crème across the labial surfaces of 6 maxillary anterior teeth using a clean finger. This was to be done just before bed each evening, for 4 weeks. After applying the crème, they were instructed to expectorate gently, but not to rinse their mouth, eat, or drink. To encourage compliance, two text messages were sent to each subject in the first and second week, and they were also contacted by telephone once.

Subjects in Group C served as untreated controls. They were given the same fluoride dentifrice, but did not receive a treatment crème. They underwent the same plaque disclosing and oral hygiene procedures.

2.3 Post-treatment Review

All subjects were contacted the day before their scheduled 4 week appointment, and instructed to brush after breakfast, but to refrain from brushing after lunch before their usual afternoon appointment. Patients in Groups A and B were asked to bring in the product tubes for collection, so that those could be weighed. At the commencement of the review visit, all subjects were questioned regarding their compliance with instructions regarding oral hygiene on the day of the review, so that those patients who had brushed immediately before their usual orthodontic afternoon appointment could be identified and excluded from the final analysis.

At the 4-week review appointment, all subjects were questioned regarding adverse effects from the products, and their compliance with applying the products once daily at bedtime in line with the study protocol. Subjects who had acted against instructions and brushed their teeth at midday immediately before the review appointment were excluded from the study at this point, since this would invalidate assessments of their dental plaque. Likewise, subjects who failed to attend the 4 week review appointment on the assigned day were also excluded from further involvement in the study.

2.4 Plaque Sampling

This aspect of the study was designed to assess changes in the composition and metabolism of the dental plaque biofilm, choosing the part of the plaque biofilm showing the **greatest** acid production, and then assessing microbial composition using real-time quantitative polymerase chain reaction (RTqPCR) analysis to determine changes in the bacterial loads of 14 key bacterial species (8 caries associated and 6 health associated) in the dental plaque at that site. This method has been described in detail in previous publications from our group [19,24].

In each subject, at the baseline and at the 4 week review appointment, the area of dental plaque with the greatest acid production (i.e. light blue stained plaque) was chosen, and a sample taken using a microbrush with an area of 4 mm^2. The microbrush tips were cut off and placed into microcentrifuge tubes containing tris(hydroxymethyl) aminomethane buffer with ethylenediaminetetraacetic acid, and the samples stored at -80^oC until analyzed.

2.5 Image Analysis

Images were coded to allow blinded analysis, without knowledge of the treatment group or whether the images were from baseline or from the 4 week review. The allocation of images was undertaken by the lead investigator once the image analysis was completed. The identification of groups was revealed only after the statistical analysis was complete.

The region of interest for the study was gingival aspect of the maxillary anterior teeth, specifically the area between the gingival margin and the orthodontic bracket and arch wire. This region was selected because it has the greatest risk of caries developing during fixed orthodontic appliance treatment [3,27-29]. This area was defined and the number of pixels counted using Adobe Photoshop™ software.

Data were collected at the individual tooth level. Separate areas for pink (thin) plaque, dark blue/violet (mature) plaque, and light blue (acid producing) plaque were measured (Fig. 1).

The counting process involved combining all the relevant stained for that dye colour on the one tooth surface. Data for pixel counts were entered into a spreadsheet and coded with an identifier, to ensure blinding for the analysis. At the completion of the study, data sets for baseline and 4 weeks were combined into before/after pairs for repeated measures analysis of differences. Results for each group where then collected, and differences between groups analyzed. Only after this was done was the blinding code broken so that the allocation of subjects into one of the three arms of the study was revealed.

The total area of plaque on the tooth surface was determined by adding the areas of pink, violet/blue and light blue stained plaque together. The plaque free area was then determined by subtracting the total plaque area from the total area at that site. A quantitative percentage score was then calculated for the following: the total percentages of the area covered by dental plaque (of any colour), and the percentages of mature (violet/blue) plaque, young (pink/red) plaque, and acid producing (light blue) plaque.

2.6 Intra-examiner Reliability

To assess the intra-examiner reliability of the plaque scoring method, a total of 15 subjects (5 from each group) were selected randomly for repeat measurements. This was undertaken on the same image sets, for a total of 61 sites, in a blinded manner, 3 weeks after the initial assessment of the same images.

2.7 Statistical Analysis of Plaque Parameters

Normality of data sets was assessed using the Kolmogorov-Smirnov test. Information on subject age was collected, and the three groups compared using nonparametric analysis of variance (Kruskal-Wallis test), since these data sets did not show a normal distribution. The gender distribution between the three groups was assessed using the Chi-squares test for independence.

ANOVA was used to compared the three groups in terms of demographic characteristics (age and daily product use) and plaque percentages at baseline.

To assess the differences between treatment groups, the Kruskal-Wallis test (nonparametric ANOVA) and Wilcoxon matched-pairs signed-ranks test were used to compare effects between the three treatment groups, and to compare the baseline and one month results within each group.

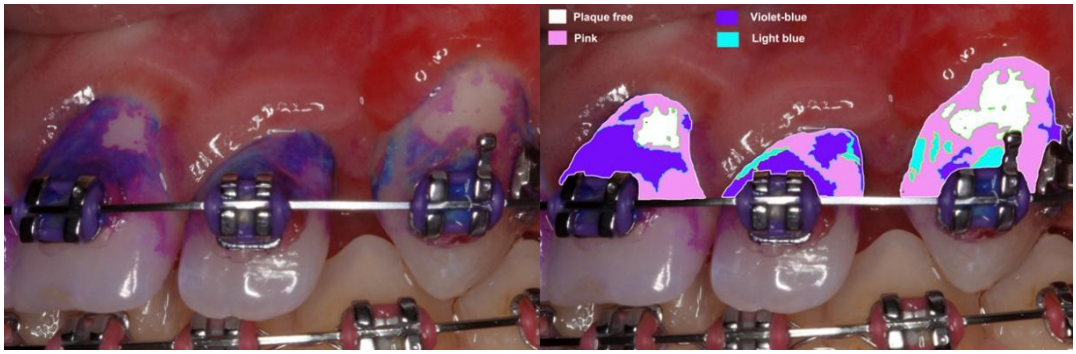

Fig. 1. Multi-colour disclosing of dental plaque. Left: clinical image showing plaque biofilm above the archwire stained with TriPlaque ID gel. Right: Regions of the site mapped according to whether they are plaque free (white), young plaque (pink), mature plaque (violet/blue), or acid producing plaque (light blue)

To explore the influence of product usage rates on outcomes, product use was coded as categorical variable. Subjects were classified as either high or low users. The expected maximum weight of product (based on the volume and density of the crème for a dose of a pea size amount) was 0.5 grams, thus a daily use rate of less than half of this (0.25 grams/day) was considered low use.

To determine reliability, measurements for total area and individual plaque areas for pink, violet/blue and light blue were compared between the initial and the repeated measurements from the same images. Data points for initial and repeat measurements were subjected to linear correlation, and linear regression analyses using the least squares methods.

2.8 Microbial Analysis

Analysis of dental plaque biofilm samples from the site with the highest levels of acid production in each subject using qPCR followed the previously published protocol (19,24). In brief, DNA was extracted from the plaque samples, then the 6S rRNA genes were amplified using PCR, as described previously (30), and the DNA was stored at -20 °C until analysis. The bacterial load of 14 bacterial species/species groups (*Actinomyces odontolyticus, Actinomyces viscosus, Bifidobacterium dentium, Capnocytophaga gingivalis, Lactobacillus acidophilus/helveticus* group, *Lactobacillus casei/paracasei/zeae* group, *Neisseria flava/subflava* group, *Scardovia wiggsiae, Steptococcus salivarius/thermophilus* group, *Streptococcus mitis/oralis/pneumoniae* group, *Streptococcus mutans, Streptococcus sanguinis/pseudopneumoniae* group, *Streptococcus sobrinus, Veillonella dispar/parvula* group) was determined using a custom-made microbial DNA qPCR array kit (16 x 24 format) (Qiagen, Venlo, Netherlands), as described previously [24].

Each assay targeted the 16S rRNA gene of the relevant bacterium, and was designed using the GreenGene database for 16S sequences. Each array also contained pan-bacteria assays to detect total bacterial load, positive PCR controls to test for PCR inhibitors, and non-template controls to account for assay background.

2.9 Statistical Analysis of Microbial Data

Statistical analysis of microbial data followed the same approach as used in previous work [24]. Data were analysed using the QuantStudio™ Real-time PCR software v1.1 (Life Technologies). In brief, for bacterial identification, cycle threshold (CT) values <40 were considered positive calls, and CT values ≥ 40 were considered negative calls. The fold change of each bacterial amplicon over time for each treatment group was calculated.

Chi-square tests were used to assess for differences between the visits in the proportion of patients testing positive for each bacteria. The same tests were also used to test for differences among the treatment groups at each visit. Univariate general linear models with Tukey adjustment for multiple comparisons were used to test for differences in CT values among the treatment groups. Statistical analyses were carried out using SPSS v24.0 (IBM Corp., Armonk, NY, USA). The threshold used was $p < 0.05$.

3. RESULTS

No subjects reported adverse effects from the active or placebo products, and no adverse hard tissue or mucosal adverse effects were observed.

3.1 Demographics

A total of 66 orthodontic patients were assessed for eligibility, and after 2 refusals, 64 subjects were enrolled into the study. From these, 21 were allocated to the active product, 22 to the placebo product and 21 to the control group. Across each group, there were similar numbers of subjects excluded from the study at the 4 week time point because of failure to comply exactly with the tooth brushing instructions (i.e. brushing immediately before the appointment), with a total of 3 in the active product group, 4 in the placebo group, and 6 in the control group. The final cohort sizes, after these exclusions, were comprised of 18 subjects in the active group (11 F, 7 M with a median age of 14.80 years, range 11.4-47.9), 18 in the placebo group (9 F, 9 M with a median age 13.95 years, range 9.8-36.2), and 15 in the control group (10 F, 5 M with a median age 14.80 years, range 10.8-30.3). There was no significant difference between the groups for age distribution ($p = 0.747$) or gender distribution ($p = 0.607$). As a result of small

differences in the number of teeth (i.e. missing or unerupted maxillary anterior teeth) in different subjects and the numbers of subjects in each group, the number of tooth surfaces (sites) assessed varied between the groups, from 105 surfaces each for active and placebo groups, to 79 surfaces in the control group.

3.2 Product use

For all product users, the target use was ~0.5 g/day based the on the manufacturer's recommendations to use a pea size amount. In the active group, the mean usage was 0.31+ 0.19 g/day, with no significant difference compared to the placebo group (0.30 + 0.20 g/day, p = 0.870). To assess dose response, the active group was divided according to product usage. There were 9 high users (mean 0.47 + 0.07g/day) and 7 low users (mean 0.11 +0.08 g/day).

3.3 Plaque Parameters at Baseline

When the plaque area coverage of the designated tooth surfaces at baseline between the groups was compared using the Kruskal-Wallis test, there were no significant differences for thin (pink) plaque (P = 0.110), mature (violet) plaque, and acid producing (light blue) plaque (P = 0.147) (Table 1).

3.4 Intra-examiner Reproducibility

Data for 61 sites was compared across two independent assessments conducted 3 weeks apart in a blinded manner. Data points for initial and repeat measurements of total area and individual plaque areas for pink, violet/blue and light blue were subjected to linear correlation analyses using the least squares methods. Summary data are shown in Table 2.

Based on the coefficient of determination (r squared), the extent of random variation in the pixel measurements was low – for the total area 0.94%, for pink 1.54%, for violet/blue 3.52%, and for light blue 4.12%.

3.5 Plaque Area at 4 Weeks

In the active group, there was an overall 10.6% reduction in plaque area, and the median plaque-free area increased from 57.2 to 67.8%. This effect was significant according to the Wilcoxon matched-pairs signed-ranks test ($p < 0.0001$). There was a strong association between the paired values, with a nonparametric correlation coefficient of 0.294 ($p = 0.001$). This same pattern of effective pairing was seen in all subsequent data sets.

Table 1. Plaque parameters at baseline

Stained area in %	Active	Placebo	Control
Sites (N)	105	105	79
Pink stain	57.16	63.90	59.51
Blue/violet stain	5.71	4.43	5.36
Light blue stain	4.29	2.65	2.46

Data show the median percentage area scores for plaque in the experimental groups. There were no significant differences between any of the three plaque parameters at baseline (p = 0.110, p = 0.390, and p = 0.108 for pink, blue/violet and light blue, respectively.

Table 2. Reproducibility analyses

	N	r	r squared	slope and CI
Total area	61	0.995	0.991	0.978 (0.953-1.003)
Pink	55	0.992	0.985	0.952 (0.920-0.985)
Violet/blue	61	0.982	0.965	1.076 (1.022-1.130)
Light blue	61	0.979	0.959	0.946 (0.895-0.997)
All data	238	0.998	0.995	0.988 (0.979-0.997)

Data are for least squares linear regression, plotting initial score (X) versus repeated measurement (Y). The values for the linear correlation coefficient (r) and the coefficient of determination (r squared) are shown together with the slope and the confidence interval of the slope. In a perfect correlation, the value for r would be 1.0 and the slope would also be 1.0.

Table 3. Plaque area percentage score changes versus baseline

Parameter	Control	Placebo	Active (LO)	Active (X)	Active (HI)
Total plaque area	+2.9	-4.0	-3.5	-10.6**	-11.2**
Blue/violet stain	+28.9*	+16.3	-60.5**	-72.3**	-93.4**
Light blue stain	+22.8	+1.9	-59.6*	-67.1**	-73.9**

Data show median changes in the percentage area scores for plaque in the experimental groups, versus the corresponding baseline, when matched for site. A positive value indicates a greater plaque area, and a negative value a reduced area. Asterisks show changes that are significantly different from baseline (= p <0.05; ** = p <0.01). The Active group is shown as an aggregate (Active X), and then divided into low users (Active LO) and high users (Active HI), to demonstrate dose response effects*

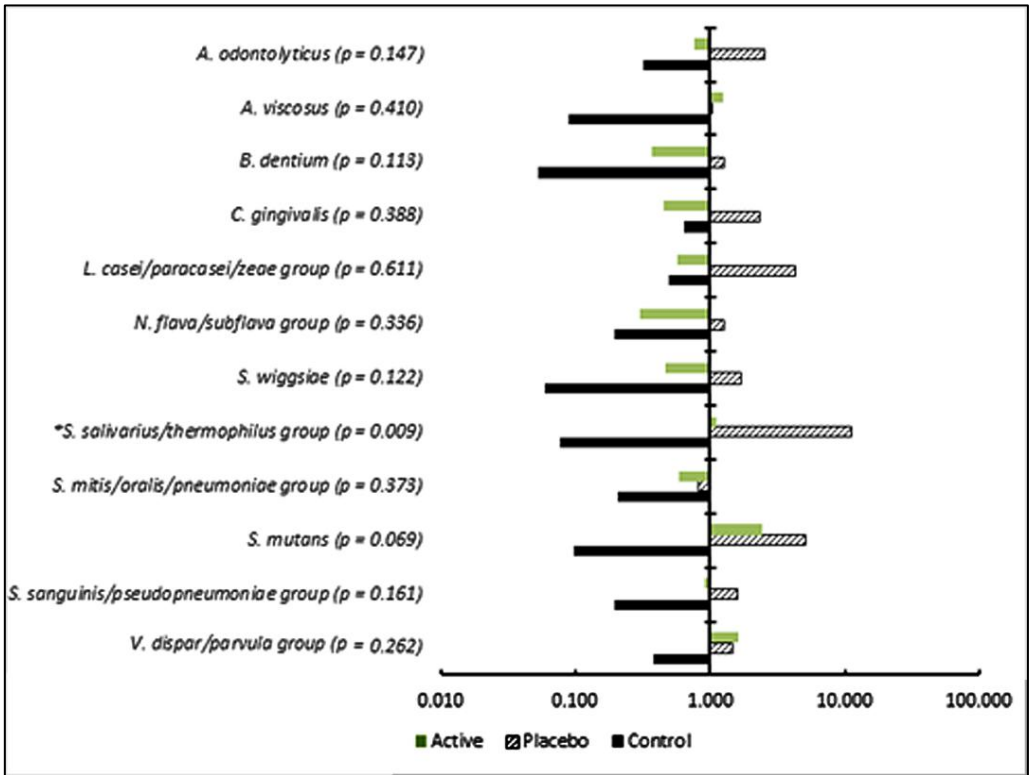

Fig. 2. Fold change in relative bacterial abundance over time for each treatment group. A fold change below 1 indicates a decrease in relative abundance over time and a fold change above 1 indicates an increase in relative abundance over time

To determine whether this increase in plaque free area was dose related, i.e., those who used the active crème regularly benefited more than low users, the active group was separated into low and high product users (Table 3).

There was a dose response effect. Low users had a 3.5% reduction in plaque area which was not significant (p = 0.146), while high product users showed an 11.2% reduction in plaque area (p = 0.007). There was a small reduction in plaque area in the placebo group (4.0%), but this was not significant (p = 0.804). In contrast, in the control group, there was a 2.9% increase in plaque area, but this also was not significant (P = 0.056). In total, 17 of 18 patients (94%) in the active group showed increases in the plaque free area over time. The single non-responder was in the low product use group.

3.6 Plaque Composition

The prevalence of bacterial species in the most fermenting region of dental plaque (light blue stained plaque) is summarized in Table 4. Across all 51 samples that were analyzed, the prevalence of *A. odontolyticus, A. viscosus, C. gingivalis, N. flava/subflava, S. mitis/oralis/pneumoniae, S. mutans, S. salivarius/thermophilus, S. sanguinis/pseudopneumoniae*, and *V.*

dispar/parvula was high at both visits (>89% of samples, Table 4). *B. dentium* and *L. casei/paracasei/zeae* were detected in 24% and 33% of baseline samples respectively, and in 24% and 47% of visit 2 samples respectively, however *L. acidophilus/helveticus* and *S. wiggsiae* were less prevalent (2% and 12% of baseline samples respectively; 6% and 20% of visit 2 samples respectively). *S. sobrinus* was not detected at all.

Table 4. Prevalence of bacterial species in acidogenic plaque samples

	Active	Control	Placebo
Caries associated bacteria			
Streptococcus mutans	100, 94	87, 100	100, 94
Actinomyces odontolyticus	100, 100	100, 100	100, 100
Veillonella parvula	100, 100	100, 100	100, 100
Capnocytophaga gingivalis	100, 100	100, 100	100, 100
Actinomyces viscosus	89, 94	100, 87	94, 89
Lactobacillus casei/paracasei/zeae	39, 33	13, 53	44, 55
Lactobacillus acidophilus/helveticus	0, 17	7, 0	0, 0
Bifidobacterium dentium	27, 22	13, 13	27, 33
Scardovia wiggsiae	11, 22	13, 20	11, 17
Streptococcus sobrinus	0, 0	0, 0	0, 0
Health associated bacteria			
Neisseria flavescens	100, 100	100, 100	100, 100
Streptococcus sanguinis	100, 100	100, 100	100, 100
Streptococcus mitis/oralis	100, 100	100, 100	100, 100
Streptococcus salivarius	100, 100	93, 100	100, 100

Paired numbers show prevalence in percent at baseline and after treatment, for plaque sites showing the greatest acid production (light blue)

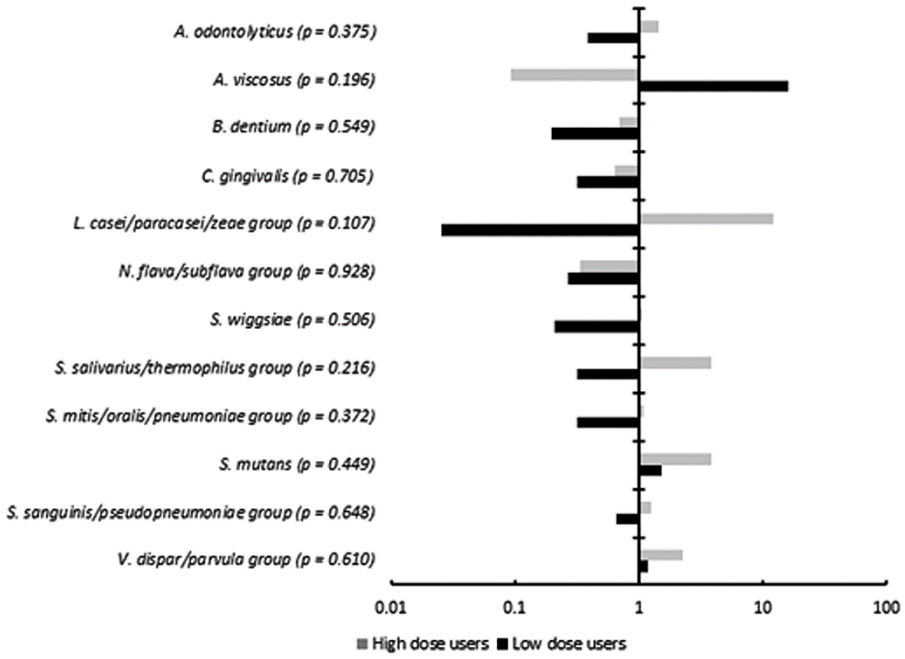

Fig. 3. Fold change in relative bacterial abundance over time in the active group according to dose (low dose users 0.11 g/day; high dose users 0.30 g/day). A fold change below 1 indicates a decrease in relative abundance over time and a fold change above 1 indicates an increase in relative abundance over time

In the control group, there was a higher prevalence of *L. casei/paracasei/zeae* at visit 2 compared with baseline (53% vs. 13%, p = 0.020). No other differences were found between the visits in each treatment group. Among the treatment groups, there were no differences in the prevalence of any bacterium at any time point (Table 4).

S. salivarius was the only bacterial group where the relative fold change differed among the treatment groups (p = 0.009; Fig. 2). The relative fold change of *S. salivarius* in the control group was similar to that found in the active group (p = 0.203) but different to that found in the placebo group (p = 0.006). In the control group, the abundance of *S. salivarius* decreased over time [mean fold change of 0.08, 95% CI 0.01, 0.94]; whereas, it increased over time in the placebo group [mean fold change of 11.15, 95% CI 1.22, 101.95)]. No other statistically significant differences were found between the treatment groups. Within the active group, there were no differences in relative fold change between low and high dose users for any of the bacterial species examined (Fig. 3).

4. DISCUSSION

The present study shows that daily use of CPP-ACP with fluoride in the form of self-applied Tooth Mousse Plus topical crème influences dental plaque, by reducing the area of mature (violet) plaque, and of acid producing plaque. This was accompanied by changes in the microbial composition of the regions within the dental plaque which showed the greatest acid production, with an increase in health-associated *S. sanguinis*. This species is an indicator species for a health-associated plaque microbiome [19-21].

These changes were not seen with the placebo crème which contained the same concentration of fluoride, nor with the commercial fluoride dentifrice. In fact, those subjects receiving the placebo crème also showed an increase in the plaque maturity and plaque fermentation between the two assessments, which indicates that the level of fluoride and ingredients other than CPP-ACP present did not significantly suppress the growth of dental plaque. Together, the results of the plaque and microbial analyses indicate that a useful prebiotic action is exerted by CPP-ACP, in this clinical setting. This result is also consistent with the recent clinical trial of a dentifrice containing CPP-ACP, which was found to cause a species-level shift in the ecology of the dental plaque biofilm in orthodontic patients, resulting in a microbial community less associated with dental caries [24].

The present clinical study has certain limitations. While patient compliance was monitored and reinforced, it could not be controlled absolutely. As with tooth brushing and other self-administered preventive approaches, ensuring compliance over time with professional recommendations remains a challenge. There may be benefits from using CPP-ACP delivery systems that are not influenced by patient compliance, such as CPP-ACP incorporated with fluoride into a varnish, since this could be painted around brackets. Likewise, a fluoride dentifrice that includes CPP-ACP would simplify compliance for high caries risk patients by reducing the number of steps necessary to one, rather than two as in the present study. Studies of the effects of interventions could be a fruitful area for further clinical studies.

The methods adopted in the present study may have wider applicability to studies of dental plaque biofilm in orthodontic patients. The current investigation used a dye-substrate combination liquid, GC TriPlaque ID gel ™, to stain and identify the area, maturity and metabolic activity of dental plaque deposits. This method can be applied to the individual patient level, as part of an overall preventive approach [6]. Standardized digital images were taken using fixed exposure conditions, and calculations for the total area of dental plaque and the individual regions within it were all performed in a blinded manner. In the present study, all subjects had comparable levels of plaque and plaque composition at baseline, and were given the same oral hygiene instructions. Thus, the starting point from which to measure the various treatments was not significantly different for the three groups. Likewise, there were no significant differences between age and gender between the study groups. The technique used for assessment of plaque regions from the images was shown to have high reproducibility, as seen by linear correlation coefficients above 0.98 and values for slope comparing repeat measurements approaching the ideal value of 1.0.

The results of the present study expand upon previous work from the laboratory setting where CPP-ACP was shown to have prebiotic effects on multi-species biofilms. A laboratory study

using a 6-species polymicrobial biofilm model demonstrated that CPP-ACP had a prebiotic effect, which would contribute to slowing the rate of enamel lesion progression [21]. Levels of *S. sanguinis* and significantly increased when CPP-ACP was applied twice-daily over 19 days. Similar results were found in a laboratory study using more complex multi-species biofilms derived from saliva [22]. *S. sanguinis* is a commensal species that uses the arginine deaminase system, and which can generate ammonia from peptide metabolism, and elevate plaque biofilm pH. Elevated levels of *S. sanguinis* will help foster an environment that is not conducive to aciduric microorganisms [22].

The time frame of the present study was short, being only 4 weeks. Longer periods of use of TMP are hypothesized to give sustained prebiotic actions, which will add to its enhancement of overall oral health [25]. Prebiotic effects of CPP-ACP have been demonstrated in several recent studies, including a 2019 study that used a biofilm model of caries with 6 bacterial species that were grown on sound human enamel. The biofilm was exposed to sucrose 4 times each day to provide a cariogenic challenge. CPP-ACP had strong prebiotic actions, suppressing the levels of *Actinomyces naeslundii* and *Lactobacillus casei*, and increasing the level of *S. sanguinis* in the biofilm. Combining stannous fluoride and CPP-ACP resulted in a greater suppression of the acidogenic and aciduric bacteria [26]. Clinical studies have shown prebiotic actions when CPP-ACP is delivered using a chewing gum [27] or a fluoride varnish [28,29]. In terms of compliance, using a varnish to deliver CPP-ACP could be desirable as it removes the need for patients to use a particular chewing gum, crème or dentifrice every day.

A key finding in the present study was that daily use of a CPP-ACP topical crème could reduce the area of acid producing plaque, with a dose-dependent relationship according to the pattern of use. The implication of this is that it should be possible to assess the metabolic properties of the plaque biofilm of a dental patient, and then titrate the frequency of use to achieve the desired outcome.

The present study did not examine the effects of GC Tooth Mousse™, the commercial form of CPP-ACP crème that does not contain fluoride. According to the product labelling information and the manufacturer's instructions for use, this version is recommended for young children (aged 6 or lower), and all subjects in the present trial were aged at least 9 years. GC Tooth Mousse™ used daily has been shown to reduce the levels of mutans streptococci in young children [24]. The present results show evidence of a dose response to CPP-ACP, with high users showing greater benefits than low users. This effect is similar to that seen in a clinical trial which assessed the reduction in mutans streptococci with daily use of Tooth Mousse. Mutans streptococci were present in only 8% of regular Tooth Mousse users, but were present in 28% of irregular users, and in 47% of non-users [24].

As the present study had a duration of only 4 weeks, a key question is whether effects of CPP-ACP on dental plaque translate to fewer white spot lesions or frank cavitations at the end of a prolonged period of orthodontic treatment. Evidence that this may be the case can be found in a 2011 clinical trial within which 60 orthodontic patients used either CPP-ACP with fluoride, or a placebo creme (Tom's of Main, Salisbury, UK) [30]. Subjects in the active group showed a 53.5% reduction in enamel decalcification, whereas enamel decalcification increased by 91.1% in those using the placebo crème. This study also showed that CPP-ACP with fluoride helped to prevent the development of new white spot lesions during orthodontic treatment. In contrast, the placebo crème had no preventive actions on white spot development, and the number of white spot lesions actually increased. A positive result was also seen in a 2021 study of TMP effects on growth and biofilm formation by *S. mutans* [31], which revealed lower biofilm formation without killing the bacteria. Lowering the levels of this pathogen is suggested, to reduce the risk of caries development during orthodontic treatment.

5. CONCLUSIONS

Daily use of a topical crème containing 10% CPP-ACP with 900 ppm fluoride by orthodontic patients results in a lower plaque area, and less mature acid producing plaque compared to a placebo crème and to untreated controls, where the effects on plaque regrowth are in the opposite direction. This suggests that the CPP-ACP component of GC Tooth Mousse Plus exerts prebiotic ecological effects on dental plaque composition. Such actions could be useful in caries prevention in orthodontic patients.

ETHICAL APPROVAL AND CONSENT

The study was conducted in a university orthodontic postgraduate clinic, with institutional ethics approval (No. 2013001324) and informed written consent.

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COMPETING INTERESTS

Author LW is a co-inventor of TriPlaque ID gel, but does not have a financial interest in this product. He has no other conflicts of interest to declare. The other authors have no other conflicts of interest to declare. The products used for this research are in common use in our country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Publication of this work was funded by author LJW.

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