Journal of Dentistry 106 (2021) 103582

Contents lists available at ScienceDirect

Journal of Dentistry

journal homepage: www.elsevier.com/locate/jdent

Casein phosphopeptide–amorphous calcium phosphate fluoride treatment enriches the symbiotic dental plaque microbiome in children

Armelia Sari Widyarman [a,*], Nadeeka S. Udawatte [b], Citra Fragrantia Theodorea [c], Anie Apriani [d], Mario Richi [e], Tri Erri Astoeti [f], Chaminda Jayampath Seneviratne [b,*]

[a] Department Head of Microbiology, Faculty of Dentistry, Trisakti University, Jln. Kyai Tapa 260, Grogol Jakarta Barat 11440, Indonesia
[b] Singapore Oral Microbiomics Initiative, National Dental Research Institute Singapore (NDRIS), National Dental Centre Singapore, Oral Health ACP, Duke NUS Medical School, Singapore
[c] Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Indonesia
[d] Department Pediatric Dentistry, Faculty of Dentistry, Maranatha University, Bandung, Indonesia
[e] MiCORE Laboratory, Faculty of Dentistry, Trisakti University, Indonesia
[f] Department of Preventive and Public Health, Faculty of Dentistry, Trisakti University, Jln. Kyai Tapa 260, Grogol Jakarta Barat 11440, Indonesia

ARTICLE INFO

Keywords:
CPP-ACP
Early childhood caries
Oral microbiome

ABSTRACT

Objectives: The dysbiotic oral microbiome plays a key role in the pathogenesis of caries in children. Topical application of casein phosphopeptide–amorphous calcium phosphate containing fluoride (CPP-ACP/F) is an effective treatment modality for children with caries (CC). Hitherto the mechanism by which CPP-ACP/F modules the oral microbiome in CC has not been investigated. The study aimed to examine the CPP–ACP/F effect on the dental plaque microbiome of children group with caries.

Methods: This preliminary prospective clinical cohort included 10 children with caries. The children received topical fluoride CPP–ACP/F once-a-week for one month. Plaque samples were collected before and after treatment and subjected to 16S rDNA-based next-generation-sequencing. Microbial composition, diversity and functional roles were analyzed in comparison to the clinical characteristics of cohort using standard bioinformatics tools.

Results: CPP-ACP/F treatment modulated dysbiotic oral microbiome towards healthier community as the higher proportion of Proteobacteria and certain microbial protective species were enriched following CPP-ACP/F treatment. Despite overall uniformity of community structure in children with caries between the groups, some bacterial species were differentially represented in a statistically significant manner between pre- and post-treatments. Three bacterial species were found to be predictive of strongly sensitive to the CPP-ACP/F treatment, marked by decreased abundance of *Lautropia mirabalis* and increased abundance *of Gemella haemolysans* and *Schwartzia succinivorans*.

Conclusion: Within the limits of the current study, it could be concluded that the CPP-ACP/F varnish treatment modulated the microbial composition of the dental plaque microbiome towards symbiosis. These symbiotic changes may demonstrate the potential clinical significance of CPP-ACP/F varnish treatment.

1. Introduction

Caries in children is a significant global problem [1]. The studies have reported the prevalence of children with caries in, United States, Europe and Asia as 28 %, up to 32 %, and 36–85 %, respectively [2]. In particularly, in Southeast Asian (SEA) countries has been reported to be higher than the United States and the United Kingdom [1]. According to the 2018 Indonesian Basic Health Research (RISKESDAS) report, the deciduous decayed, missing, or filled (dmf) tooth caries index in children under the age of 5 was 8.1, and the permanent dentition caries index (DMFT) in children aged 5–12 years was 7.1 [3]. The foregoing report is indicative of the severe caries in children throughout the

* Corresponding authors.
E-mail addresses: armeliasari@trisakti.ac.id (A.S. Widyarman), udawatte.madugalle.g.n.s@ndcs.com.sg (N.S. Udawatte), citra.fragrantia02@ui.ac.id (C.F. Theodorea), anieapriani25@gmail.com (A. Apriani), mario.richi1995@gmail.com (M. Richi), erriastoeti@yahoo.com (T.E. Astoeti), jaya.seneviratne@ndcs.com.sg (C.J. Seneviratne).

https://doi.org/10.1016/j.jdent.2021.103582
Received 16 November 2020; Received in revised form 6 January 2021; Accepted 7 January 2021
Available online 12 January 2021

country. Hence, preventive treatments are urgently needed to reduce caries in children and associated risks in Indonesia [3,4].

In microbiological perspective, dysbiosis of the dental plaque microbiome that lead to overgrowth of cariogenic bacteria is the aetiology of caries in children. Hence, careful monitoring of the changes of dental plaque is important for evaluating the caries risk of children. Traditionally, microbial monitoring has been limited to major cariogenic pathogens such as *Streptococcus mutans* [5].

Topical administration of fluoride has been shown to be an effective treatment for dental caries [4]. Fluoride products prevent demineralization and promotes remineralization on the crystal surfaces of the tooth substance by replacement of hydroxyl (OH) ions by fluoride ions. This interaction forms fluorapatite in the tooth enamel in place of hydroxyapatite which is more stable and acid resistant [6]. Moreover, topical fluoride inhibits bacterial enzymes [7]. Casein phosphopeptide–amorphous calcium phosphate containing fluoride (CPP-ACP/F) has been shown to reduce the levels of *S. mutans* in children's saliva and lower the risk of dental caries [8]. CPP–ACP/F acts as a reservoir of calcium phosphate that releases calcium and phosphate ions into the dental plaque, which reduces enamel demineralization and promotes remineralization [9]. Some studies have demonstrated that CPP–ACP/F is more effective than fluoride varnish in reducing the caries risk [10].

CPP–ACP/F has virulence-attenuating properties that can contribute to a beneficial ecological change in oral biofilms, as it has been shown to significantly reduce cariogenic bacteria [11]. The ecological cariostatic effects of CPP–ACP/F are believed to be predominantly mediated by its anti-adhesion, buffering, and biofilm-disrupting actions [12]. For example, dentifrices containing CPP–ACP/F and polyphenol-rich cranberry extracts was found to positively modulate the microbial ecology of dental plaque bacteria such as *S. mutans*, *Veillonella parvula*, *Corynebacterium durum*, *Neisseria flavescens*, and *Streptococcus sanguinis* [13].

Advent of next generation sequencing (NGS) has shifted focus from mono-species pathogens to boarder microbiome-based analysis for the aetiopathology of infectious diseases, including caries in children. Alterations of the oral microbiome including changes in microbial composition and the metabolic functional profile has been associated with the development of caries in children [14]. Dashper et al. (2019) examined the oral microbiome of children in their first four years of life using the 16S rDNA based next generation sequencing. The study demonstrated that oral microbiome profile with the knowledge of bacterial composition, and the existence of particular species in children is a useful tool to predict risk of caries in children [15]. Hitherto, no studies have examined the effect of CPP-APP/F on dental plaque microbiome of children using the NGS in clinical cohorts. Taking this research gap into consideration, this preliminary prospective clinical study aimed to investigate the effect of CPP–ACP/F varnish on the dental plaque microbiome of children with caries.

2. Materials and methods

2.1. Design and participants

This preliminary study included 10 children aged 8–9 years who were treated with CPP-ACP/F varnish. The necessary sample size was obtained using multistage cluster random sampling. Plaque samples were collected from 10 children before and after the treatment procedures using standard methodology. The children received topical fluoride CPP–ACP/F (MI varnish; GC Corporation, Tokyo, Japan) once a week for one month. The project was approved by the Ethics Committee of the Faculty of Dentistry (EC number 228/S2-Sp/KEPK/FKG/11/2018). Informed consent was obtained from all subjects through their parents.

2.2. Plaque sampling

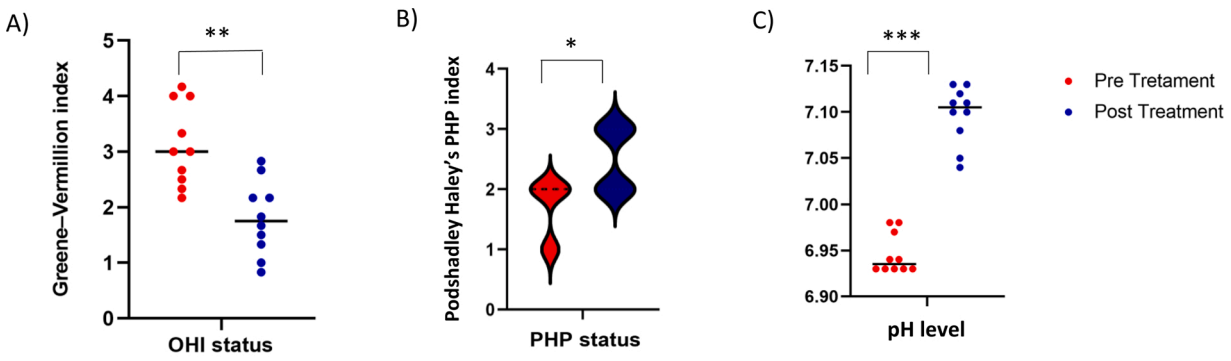
Plaque samples were collected according to the standard protocol [16]]. The participants were asked not to eat or drink anything two hours prior to sample collection. Samples were collected each on buccal and lingual surfaces of first molar teeth of the lower jaw using swabs between 9 and 11 a.m. and placed in sterile falcon tubes with 5 mL of phosphate-buffered saline (PBS). pH measurement was carried out using a digital pH meter (Orion Star A221/Stara 2215-Thermo Scientific) on a test tube containing samples [17]. Oral hygiene practice standards were explained to the patients and reinforced at each visit. Demographic information and clinical parameters of the subjects such as caries index (DMFT), pH level, Oral Health index (OHI index), patient hygiene performance (PHP index) were recorded (Fig. 1D).

2.3. Sample preparation and DNA extraction

Microcentrifuge tubes containing plaque samples were diluted in 1 mL of PBS and centrifuged at 4000 rpm for 10 min. Genomic DNA extraction was performed using a Geneaid Presto Buccal Swab gDNA Extraction Kit (Geneaid, Taipei, Taiwan). The quantity and purity of extracted DNA were measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and a BioDrop DUO UV/Vis spectrophotometer (BioDrop, Cambridge, UK). The extracted DNA samples were stored at −80 °C until further use.

2.4. 16S rDNA V3-V4 region amplification, library preparation and sequencing

Polymerase chain reaction (PCR) amplifications were performed for library preparation prior to sequencing. Primers were specifically designed to target the V3-V4 region of 16S rDNA and modified with Illumina overhang adapters was amplified by PCR using the primers F (5′ -TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG-3′), R (5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG ACTACHVGGGTATCTAATCC -3′). The PCR mixture was composed of 5 μl of each forward and reverse primer, 2.5 μl of DNA template from each sample, and 12.5 μl of 2x KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), amounting to a total volume of 25 μl. The PCR amplification conditions were set as follows: initial denaturation at 95 °C for 3 min, 25 cycles; denaturation at 95 °C for 30 s; primer annealing at 55 °C for 30 s; elongation at 72 °C for 30 s; and final elongation at 72 °C for 5 min. The PCR products were visualized by gel electrophoresis with 1% agarose in 1x TAE buffer at 110 V for 15 min using a 1-kb DNA ladder as a control. The amplified library was purified using AMPure XP beads (Beckman Coulter Genomics, MA, USA). After purification, another PCR was performed to attach Illumina sequencing adapters to the overhang sequence using a Nextera UD Indexes Kit (Illumina, San Diego, CA, USA). The mixture consisted of 5 μl of purified DNA, 10 μl of Nextera UD Indexes Set A, 25 μl of 2x KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), and 10 μl of nuclease-free water, amounting to a total volume of 50 μl. The PCR amplification conditions were set as follows: initial denaturation at 95 °C for 3 min, 8 cycles; denaturation at 95 °C for 30 s; index annealing at 55 °C for 30 s; elongation at 72 °C for 30 s; and final elongation at 72 °C for 5 min. The amplified library was also purified using AMPure XP beads (Beckman Coulter Genomics, MA, USA), visualized by agarose gel electrophoresis, and quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The amplified library after the second purification was eluted, normalized, and pooled to the appropriate concentration according to the recommendation for the sequencing apparatus. A PhiX Control v3 library (Illumina, San Diego, CA, USA) was combined with the amplicon library, expected at 5% spike-in. Sequencing was performed using an iSeq 100 NGS System (Illumina, San Diego, CA) using paired-end sequencing with 2 × 151 cycles, with 10 cycles for each index, according to the manufacturer's protocols [18].



D)

Variable	Subjects (n=10)	p-value
Age (years)	8-9	
Gender		
Male	7 (70%)	
Female	3 (30%)	
pH level		
Pre- treatment	6.9 (SD±0.021)	0.0001***
Post- treatment	7.1(SD± 0.031)	
OHI status (Green – Vermillion - Hirschman index)		
Pre- treatment	3.12(SD± 0.733)	0.0022**
Post- treatment	1.8(SD± 0.67)	
PHP status (Podshadley Haley's PHP index)		
Pre- treatment	1.7(SD± 0.674)	0.0107*
Post- treatment	2.3(SD± 0.483)	
Caries Index Pre-Treatment		
def	1.0(SD± 0.570)	
DMF	1.0(SD± 1.4)	

Fig. 1. Demographic and clinical evaluation of children with ECC between prior and post varnish application of casein phosphopeptide–amorphous calcium phosphate (CPP–ACP/F) for one-month. A) Oral Health index (OHI) for determining the soft sediments classified with microbial signatures denoted as no plaque (0), mild [1- (1/3) covered with soft plaque], moderate [2-(1/3-2/3) covered with soft plaque], bad [3- (>2/3) covered with soft plaque] according to Green – Vermillion - Hirschman index. Each sample achieves lower OHI Status, which is better oral hygiene after CPP-ACP/F varnish treatment. B) Podshadley Haley's PHP index was used to assess each patient's degree of dental plaque deposition and patient hygiene performance. Examinations were performed on a total of 5 areas by dividing each tooth surface into 3 parts corresponding to the mesial, central, and distal areas and then further dividing the central area into the gingival, central, and occlusal surface. Scores were given according to persistence of colored area after staining the plaque with plaque dye denoted as 1 = bad, 2 = moderate and, 3 = good. The better grades of PHP index were following CPP-ACP/F varnish treatment indicated less plaque in the children's teeth C) pH differences between prior and post CPP-ACP/F varnish application. It was shown that generally, each sample's pH reached neutral pH after CPP-ACP/F varnish treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.5. *Bioinformatic analysis*

Once sequencing was finalized, the primary analysis of the obtained sequences was performed using BaseSpace Sequence Hub, the Illumina genomics computing environment. The 16S Metagenomics App performs taxonomic classification of 16S rRNA targeted amplicon reads using the version of the GreenGenes taxonomic database curated by Illumina. The sequences were grouped in operational taxonomic units (OTUs) with 97 % similarity. Common alpha diversity descriptors (including the observed OTUs, Shanon's richness, Chao's richness and, Simpson's evenness), taxonomic diversity profiling, sample clustering and heatmap analysis were calculated MicrobiomeAnalystR Platform with GreenGenes OUT IDs [19,20]. according to the relative abundance of OTUs. The beta diversity was also assessed with the respective algorithms implemented in MicrobiomeAnalystR package and evaluation of the community structure across the sample groups was assisted by principal coordinate analysis (PCoA) and Bray–Curtis dissimilarity indexes retrieved from sample pairwise comparisons. Significance testing was performed using the permutational multivariate analysis of variance (PERMANOVA) test [21].

LEfSe (version 1.0) was used to detect differentially abundant genera in the cohorts of pre and post treatment along with clinical variables of oral hygiene status for biomarker discovery using the online Galaxy workflow framework (http://huttenhower.sph.harvard.edu/galaxy/) [22]. Briefly, the algorithm first used the non-parametric factorial Kruskal-Wallis (KW) sum-rank test to detect the taxa with significantly different abundances, followed by pairwise Wilcoxon tests to detect biological consistency between the two groups. Finally, a linear discriminant analysis (LDA) score was used to estimate the effect size of each differentially abundant feature. A size-effect threshold of 2.0 on the logarithmic LDA score was used for discriminative functional biomarkers. Univariate differential abundance of OTUs was also tested using a negative binomial noise model for over dispersion as implemented by MicrobiomeAnalystR. DESeq2 was run under default settings and q values were calculated using the Benjamini-Hochberg procedure to control for false discovery rates.

To further validate the bacterial OTUs that differentiate following ACP-CPP/F treatment and oral hygine status of the children with Caries; the machine-based learning algorithm Random Forests model was used in the caret package (v.6.0.81); R v3.2.5 (Random forest package v4.6.x)

based on significantly different species derived from LEfSe. To ensure reliability, we adopted 5 times repeated 10-fold cross validation [23] when calculating the variable importance score. The average value of variable importance scores in 10-fold was used to rank the variables. Because the variable importance score of RF considers not only the impact of an individual feature on the response variable but also the interaction of multiple features on the response variable, the feature selection method based on the variable importance score of RF can select more distinguishing features to characterize the sample and improve the prediction performance of the model [23]. The final model accuracy is taken as the mean from the number of repeats. Accuracy was used to select the optimal model using the largest value. The classification performance of the final model was assessed with OOB error rate.

2.6. Statistical analysis

Descriptive statistics were applied to assess com-positional differences between pre and post treatment groups regarding clinical variables. The Shapiro-Wilk test was used to measure normality of the explored continuous variables before comparison using parametric (*t*-test, ANOVA). Statistical comparisons were performed using one-way ANOVA with post hoc Turky's multiple comparisons test or paired sample *t*-test as appropriate. All statistical significance was accepted at $P < 0.05$. All analyses were performed with Prism 8 software (GraphPad software, RRID: SCR_002798).

3. Results

3.1. Clinical evaluation with the CPP-ACP/F treatment

From this pilot study, it was found that the topical application of CPP-ACP/F within the span of a month improved the oral health of children with caries. The level of pH significantly raised to a more neutral level. Oral hygiene status of the children were significantly improved as indicated by better OHI and PHP index scores (Fig. 1).

3.2. The dynamics of oral microbiome changes following CPP-ACP/F treatment

In order to assess the effect of CPP-ACP/F treatment on dental plaque microbiome, we examined the composition and diversity changes across groups. Initial assessment of the microbial diversity and the microbial community structure between pre and post treatment groups were performed with alpha- and beta-diversity using the abundance and prevalence information of OTUs recovered, respectively. Alpha and beta diversity parameters indicated that post-treatment samples had no significant differential distribution of diversity descriptors and community structural changes, compared to their pre-treatment counterparts (Supplementary Table:1 and Supplementary Figs. 1 & 2). Similar findings were observed by classification with oral hygiene status (PHP and OHI) of pre and post treatment samples.

The bacterial distribution was characterized in terms of the actual species abundances. Fig. 2 shows the actual abundances of top 10 predominant species between pre and post CPP-ACP/F treatment (relative abundance >7% of the total sequences) (Fig. 2A). The five most abundant species increased (>1%) with CPP-ACP/F treatment were *Gemella haemolysans, Gemella morbillorum, Streptococcus gordonii, Prevotella shahii, and, Porphyromonas pasteri* together accounting for 76 % of the total sequences. The noticeable difference of shift in prevalence following CPP-ACP/F treatment was corresponding to the slight increase of *G. morbillorum* (1.5 %), *S. gordonii* (1.05 %) and, *G. haemolysans* (8%) (Fig. 2B). However, the correlation of these species *G. morbillorum and S. gordonii* between two cohorts was not significant. Furthermore, we identified two of these species tightly associated with oral PHP and OHI hygiene status of the children. For instance, higher prevalence of *G. morbillorum and G. haemolysans* species was moderately attributed to

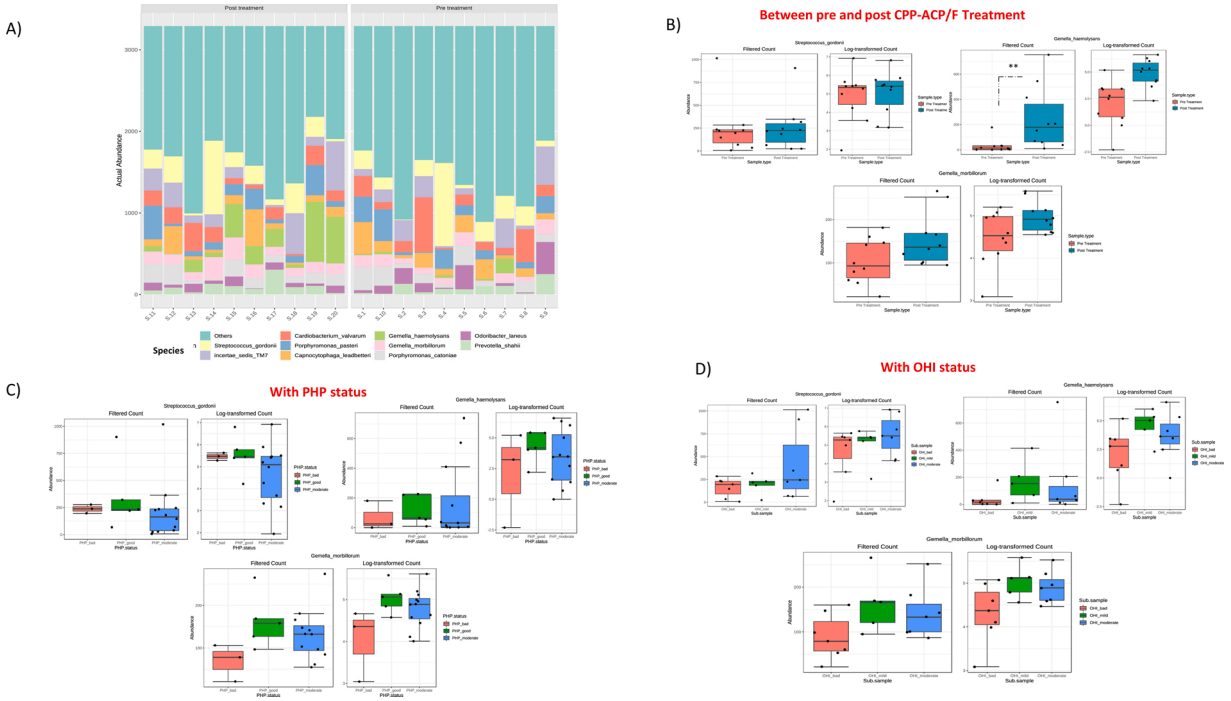


Fig. 2. Distribution of 10 most abundant bacterial species between pre and post CPP-ACP/F treatment and most prominently changed species with advancing level of oral hygiene status. A) Abundance level of top 10 predominate species between prior and post CPP-ACP/F treatment. Most predominant species associated in two cohorts was shown at the bottom; other species were combined as "Others. B) Abundance of three prevalent species sorted in magnitude of change following CPP-ACP/F treatment were shown named as *Gemella morbillorum, Gemella haemolysans* and, *Streptococcus gordonii.* C) The magnitude in change of same three species with the level of soft sediments indication (OHI index) in children with CC. C) The magnitude in change of same three species with degree of dental plaque deposition (PHP) in children with CC. Abbereviation: CC- Childeren with Caries, OHI -Oral Health index, PHP- patient hygiene performance.

PHP_good (Fig. 2C) with OHI_mild (Fig. 2D) oral hygiene status of children.

Fig. 3 shows relative abundances and clusters of OTUs between pre and post treatment by correlating with different oral hygiene status of both PHP and OHI indicators. At phylum level, Bacteroidetes (2%) and Firmicutes (2%) significantly decreased and Proteobacteria significantly increased post-CPP ACP/F treatment. The differentially represented proteobacteria phylm (7%) was predominantly over-represented following CPP-ACP/F treatment. Proteobacteria was correlated with the classification of good oral hygiene status in children with caries (PHP shown as good and OHI shown as mild) (Fig. 3A & B). This indicates that the CPP-ACP/F treatment has modulated dysbiosis in oral microbiome towards healthier community as the higher proportion of Proteobacteria being the major phylum in the improved oral hygiene status with the treatment.

At species level, different patterns of OTU abundance were observed with CPP-ACP/F treatment and the oral hygiene status of the children (Fig. 3C & D). In order to identify the significant differently abundant species in both cohorts and their rank of contribution to oral hygiene indicators, we used complementary and validating method to identify differentially expressed species by linear discriminant analysis effect size (LEfSe) [21]. First, we generated a bar plot of the effect size of (LDA score >2) taxa with differential relative abundance of pre and post treatment followed by classification with oral hygiene status (PHP and OHI) of children. This strategy yielded new candidates with different levels of abundance in two groups. A number of new seven species (LDA score >3) differentially abundant between pre and post treatment sample groups were identified (Fig. 4A and Supplementary Table 2). The similar finding was observed with PHP status of the samples in two cohorts (Fig. 4A). The most conspicuous examples included higher abundances of *G. haemolysans*, and *Schwartzia succinivorans* in post-treatment samples and highest abundances of *Lautropia mirabillis* in pre-treatment samples (Fig. 4B). When classified with OHI status of samples (Fig. 4C), genera *L. mirabillis* and *S. succinivorans* (Fig. 4D) previously found to have a different relative level were; re-identified. The significant differences of these species were further tested using the DESeq2 method. DESeq2 method found overall 6 OTUs in the study cohort that were significantly differentially abundant and passed FDR (Supplementary Table 3), including a new species *Leptotrichia shahii.* Among these, 5 OTUs belonging to the genera were congruent with those produced by the LEfSe analysis (*L. mirabilis., L. buccalis, L. wadei G. haemolysans,* and *S. succinivorans)*.

Further, to determine these different species optimally classified with their level of contribution to treatment and their oral hygine status, we conducted Random Forest (RF) regression algorithm as described in methodology. Seven differently abundant species identified based on LEfSe were selected for 10-fold cross validation model as their abundance notably differed following CPP-ACP/F treatment. As a result, we first obtained a RF regression model that could predict the level of importance of each bacteria in terms of abundance from pre to post treatment groups (Supplementary Fig. 3A). *L. mirabillis* was considered the highest abundant in the pre- treatment wherease *G. haemolysans* was consdiered the highest abundant in post-treatment and vice versa. Then, in the last step, we trained a RF prediction model to select the optimal subset of bacteria discriminating between pre and post treatment cohorts and their level of contribution to oral hygiene status of children with caries. The higher the importance of the subset, the greater the likelihood of association with the featured bacteria (Supplementary Fig. 3C). As expected, only three bacterial genera were found to be predictive of strongly sensitive to the ACP-CPP treatment regardless of

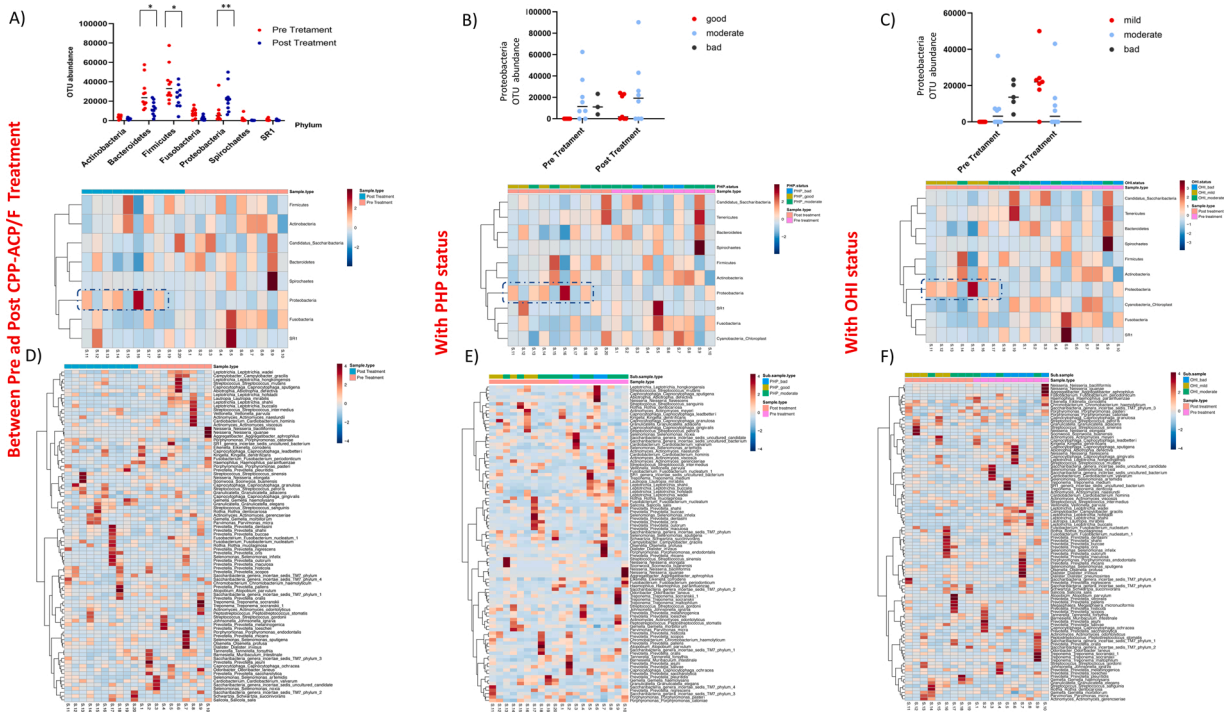


Fig. 3. Heatmap and cluster analysis in the relative abundance of taxa-level OTUs following CPP-ACP/F treatment in children with Caries A) Abundances of phyla significantly associated with CPP-ACP/F treatment (dark blue dashed oval in the cluster heatmaps) are shown; Proteobacteria observed being the major phylum increased following treatment. B) & C) Magnitude of change in phylum Proteobacteria with advancing level of oral hygiene status (PHP and OHI; respectively) in children with ECC between prior and post CPP-ACP/F treatment. Significance denoted as "*'' phyla with $p < 0.05$ and "**'' indicates taxa with $p < 0.01$ after the false discovery rate correction was applied. D), E) and, F) Shift and cluster in the relative abundance of species-level OTUs with treatment and different status of PHP and OHI indicators. The samples ($n = 10$) are arranged by hierarchical clustering using the average method and Bray-Curtis dissimilarity. Abundance as percentage of the total community is indicated by the color scale. The bar along the left side indicates treatment cohort and different stages of oral hygiene indexes. Abbereviations : OHI -Oral Health index, PHP- patient hygiene performance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

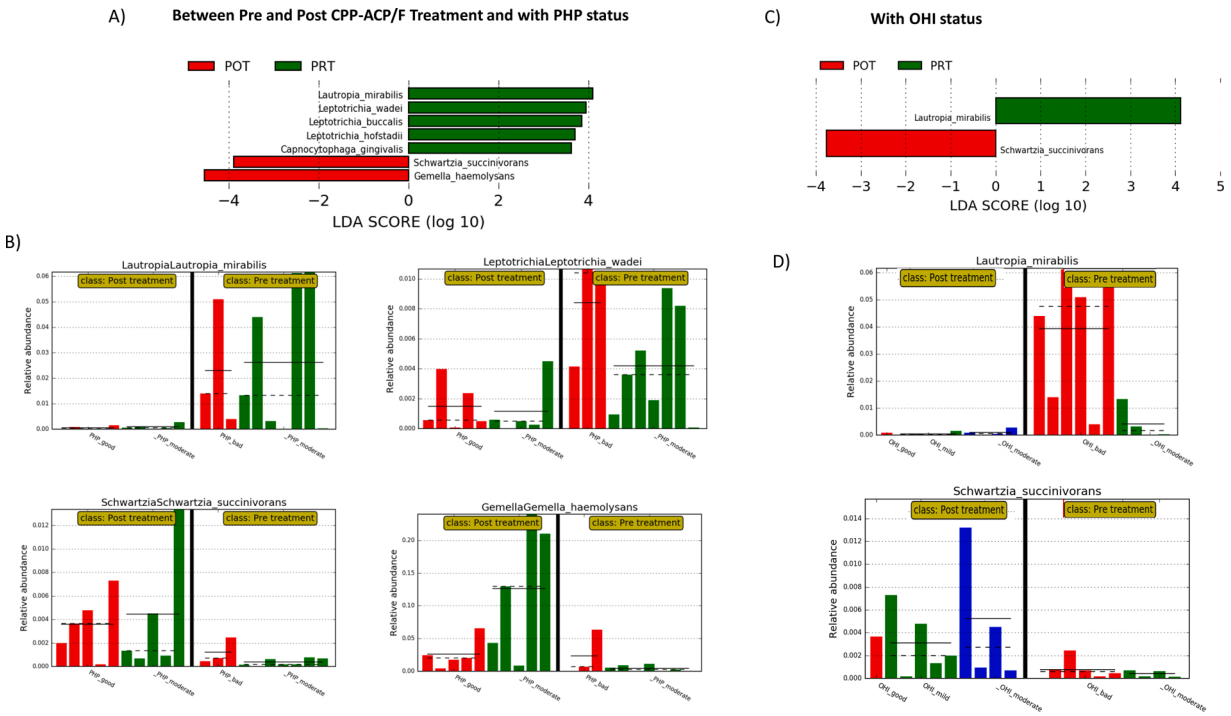


Fig. 4. Differential bacterial abundance between pre and post CPP_ACP treatment using LEfSe analysis. The top 7 species with the largest effect sizes (LDA Score > 3.0) with treatment and with PHP status are presented in the figure A, wherease the top 2 species with the largest effect sizes (LDA Score > 2.0) with OHI status are presented in the figure C. The length of the histogram represents the LDA score; i.e., the degree of influence of species with significant difference between pre (PRT) and post (POT) treatment groups. Relative abundance of the four biomarker microbes with PHP cohort represented in B and, OHI cohort in D. The x-axis represents different level of oral hygiene status (PHP and OHI indexes; respectively) and the y-axis represents relative abundance of most significant species between pre and post treatment. Abbreviations: PRT- Pre treatment, POT- Post treatment, OHI -Oral Health index, PHP- patient hygiene performance.

personal hygiene status of children, marked by decreased abundance of *L. mirabillis* and increased abundance *of G. haemolysans* and, *S. succinivorans* (Supplementary Fig. 3C).

4. Discussion

The present study, for the first time, examined the modulation of dental plaque microbiome by CPP–ACP/F treatment in a prospectively cohort of young children with caries. Although the community structure of the dental plaque microbiome remained stable, certain taxa significantly changed by CPP–ACP/F treatment. It was observed that post-treatment microbiome contained higher level of bacteria belong to Proteobacteria, a phylum in general associated with health. In contrast, Firmicutes, phyla that consists of major cariogenic pathogens was reduced by CPP-ACP/F treatment. Previous studies have reported the comparative oral microbiome profiles between healthy individuals and patients with dental caries. Healthy individuals, especially children, had higher level of Proteobacteria and lower level of Firmicutes in the oral microbiome [24,25]. Hence, overall, active dental caries in children is likely to be associated with higher Firmicutes and lower Proteobacteria. The findings of the present study provides new evidence that CPP-ACP/F treatment could not only reduce the cariogenic bacteria, but also enrich the healthier dental plaque microbiome in treated children.

S. gordonii and *G. morbillorum* are considered as health associated species against caries activity from the longitudinal studies [26,27]. Foregoing studies found that these species predominated in the indigenous bacterial flora of caries- free subjects, suggesting a beneficial role. *S. gordonii* [28] and *G. morbillorum* [27,29] have also been associated with health in number of other studies too. According to the study by Fernando et al. (2019), CPP-ACP/F chewing gum increase the proportion of *S. gordonii* compared to the non-treatment period [30] which in line with our study observation. *S. gordonii* has been consistently associated with healthy dental plaque and low caries incidence by metabolizing arginine to ammonia [31,32] potentially raising oral pH, inhibiting biofilm formation [33] and conducting bacteriocin production [33,34]. Moreover, the present study indicated the *G. morbillorum* was predominant in subjects with better oral hygiene (mild) and PHP status (good).

It has been shown that application of CPP–ACP/F on children's tooth surface once a week for one month increases the salivary pH [35]. The findings of our study are in line with this observation. We found that mean salivary pH of this cohort increased from 6.9 (SD ± 0.021) to 7.1 (SD ± 0.031) following CPP-ACP/F treatment. Although the change in overall pH is small, micro- environment of the oral cavity with higher pH has shown to inhibit growth of acidophilic, caries-causing bacteria, such as *S. mutans* and *Lactobacillus* sp., and promotes remineralization of tooth elements [36]. Similar observation have been made by Fernando et al; (2019). No mutans streptococci or lactobacilli were observed among most abundant species detected from individuals treated with CPP-ACP/F chewing gum [30]. A previous study reported that CPP–ACP application for one month reduced caries lesions by 77 % [37]. The present study demonstrated an improvement in plaque indices and pH by treatment with the CPP-ACP/F varnish that may also be consistent with a reduction in caries activity.

Significantly enriched microbial taxa in the pre- treatment groups include taxa, *Lautropia genus; Leptotrichia* sp./*wadei; Leptotrichia shahii,* whereas taxa *G. haemolysans* and *S. succinivorans* were enriched in the post-treatment group. These findings are in line with Fernando et al., (2019) which showed CPP-ACP/F gum significantly decreased the proportion of *L. mirabilis*/sp., *L. buccalis, L. shahii,* and *L.* sp./*wadei* when compared to the non- chewing treatment period [30]. According to the study by Richards et al. [2017], *Leptotrichia* species (in particular L. *wadei*) were found more frequently in plaque from caries active sites [38], whereas L. *mirabilis,* L. *buccalis* have been implicated as opportunistic pathogens, particularly in the immunocompromised individual [39,40]. Moreover, Johansson et al., (2016) reported that the oral microbiomes of individuals with high caries levels are dominated by

Streptococcus, *Alloprevotella*, *Leptotrichia*, *Neisseria*, *Prevotella*, and *Porphyromonas* genera, whilst caries-free microbiomes are dominated by *Gemella* [41]. *G. haemolysans* is one of the core species of microbes that colonizes *in situ* dental plaque biofilms of healthy individuals. Moreover, *G. haemolysans* is an aero-tolerant and commensal oral bacterium; therefore, it is highly adaptable to all human hosts, both before and after varnish treatment [42]. No reports of *S. succinivorans* are known for causative role in oral disease and are found to colonize in healthy individuals.

The certain microbial changes appear to be more exclusive to CPP-ACP/F treatment corroborated by prediction model based on random forests. The relationship is potentially symbiotic due to its presence in dental plaque as well as its significant change typically towards better oral hygiene status of children with caries. Therefore, it will be interesting to examine the role of *G. haemolysans, L. mirabalis* as well as *S. succinivoran* in children with caries and how they are modulated by CPP-ACP/F treatment.

The relatively smaller sample size is a limitation of the present study. Future studies with larger sample size in different geographical locations will provide conclusive evidence on the new findings. Inclusion of wider age group to examine the effect of CPP–APP/F should also be considered.

5. Conclusion

The present study found certain bacterial phylotypes of the dental plaque microbiome were significantly modulated by CPP-ACP/F treatment in children with caries. It was observed that health-associated species such as *G. haemolysan* and *S. succinivorans* are enriched in the dental plaque microbiome following treatment with fluoride-containing CPP–ACP/F. The new findings highlight the need of better understanding of the oral microbiome in the aetiopathology of caries in children and evaluating the efficacy of dental treatments such as CPP-ACP/F.

Authorship contribution statement

ASW, CTF, CJS and TEA designed the study. AA and MR collect the samples. ASW, CFT and MR performed the laboratory experiments. NS and CJS analysed the data. ASW, CFT, NS and CJS wrote the paper. All authors discussed the results and commented on the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

The authors thank Faculty of Dentistry, Trisakti University for funding this study and Microbiology Center of Research of Education (MiCORE) Laboratory, University of Trisakti, for invaluable support in this study.

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.2021.103582.

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