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Original Article

# Topical fluoride varnish application shifts dysbiotic dental plaque microbiome towards eubiosis in children with dental caries

Armelia Sari Widyarman [a,**], Nadeeka S. Udawatte [b], Idham Tegar Badruzzaman [c], Caesary Cloudya Panjaitan [d], Anie Apriani [e], Jeddy [c], Tri Erri Astoeti [d], Chaminda Jayampath Seneviratne [b,*]

[a] Department of Microbiology, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia
[b] School of Dentistry, The University of Queensland, Australia
[c] Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia
[d] Department of Preventive and Public Health, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia
[e] Department of Pediatric Dentistry, Faculty of Dentistry, Maranatha University, Bandung, Indonesia

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ABSTRACT

*Objective:* This study used high-throughput amplicon sequencing to examine the impact of long-term continuous fluoride treatment on the dental plaque microbiota of children aged 8 to 9 with mixed dentition.
*Design:* The study population consisted of twenty 8–9-year-old children with dental caries. Topical application of fluoride-varnish was weekly administered for one month to all subjects. Clinical indicators and anthropological data, such as the caries index (DMFT and dmft), were documented for every participant at baseline. A baseline assessment and a month after the fluoride varnish treatment were conducted for the salivary pH level and the Patient-Hygiene-Performance (PHP) index. Following application of the fluoride varnish, plaque samples were obtained both one month later and before (baseline) and were then used for 16S rRNA gene-based Next Generation Sequencing.
*Results:* The results showed significant differences in the community composition structure ($p < 0.01$). Notable caries-associated pathogens in the dental plaque microbiome were depleted whilst health associated phylum Proteobacteria was increased in the abundance following fluoride-varnish application. In children with mixed dentition, this study found that after one month of fluoride-varnish treatment, there was a significant decrease in the prevalence of the dominant pathogenic genera, *Fusobacterium, Porphyromonas, Capnocytophaga, Neisseria*, and *Leptotrichia*, along with an increase in certain genera related to healthy oral condition, mostly from the phylum Proteobacteria, such as *Areinmonas*, *Pseudoxanthomonas*, and *Luteimonas*.
*Conclusions:* Fluoride-varnish application may shift the community level microecology from dysbiosis to eubiosis. Moreover, application of fluoride-varnish with weekly intervals for one month reduced the caries-causing bacteria while enriching the rise of unique, ubiquitous genera primarily belonging to the Proteobacteria, which may plaque a defensive role against progression of caries. Furthermore, a rising pH level towards neutrality (pH 7) indicated a healthier oral environment following the application of fluoride varnish.

## 1. Introduction

Dental caries is caused by sugar-driven, biofilm-mediated disease processes that cause the dental hard tissues to gradually demineralize (Pitts et al., 2017). It is the most common oral disease, affecting 60–90 % of school-aged children globally (Oral Health Database 2020). Without timely intervention, dental caries may progress to more severe stages, resulting in pain and discomfort in the teeth, feeding difficulties, and eventually tooth loss. Especially for low-income countries like Indonesia, dental caries is not only a clinical issue but also a substantial

*Abbreviations:* DMFT, Decayed, Missing, and Filled surface for permanent Teeth; dmft, Decayed, Missing, Filled surfaces for primary Teeth; PHP, Patient – Hygiene – Performance; PBS, Phosphate-buffered Saline; PCR, Polymerase Chain Reaction; NGS, Next Generation Sequencing.

* Corresponding author at: School of Dentistry, The University of Queensland, QLD4006 Australia.
** Corresponding author at: Department of Microbiology, Faculty of Dentistry, Universitas Trisakti, Indonesia.
*E-mail addresses:* armeliasari@trisakti.ac.id (A. Sari Widyarman), jaya.seneviratne@uq.edu.au (C. Jayampath Seneviratne).

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financial burden (Ministry of Health of The Republic of Indonesia, 2018; Nomura et al., 2019; Kumar et al., 2017).

Fluoride-containing products, including fluoride-varnish have been commonly used as a strategy to prevent early childhood caries (ECC) (Motallaei et al., 2021). The efficacy of fluoride-varnish in preventing dental caries in both primary and permanent teeth has been extensively investigated. Several Cochrane systematic reviews including 200 trials and over 80,000 participants have provided compelling evidence in this regard (Abou Neel et al., 2016; Chenicheri et al., 2017; Ribeiro et al., 2017). Topical fluoride application converts hydroxyl groups in the hydroxyapatite component of the enamel into calcium fluoride and fluorapatite by replacing them with fluoride ions. Fluorapatite is more resistant to acids, resulting in enamel becoming less prone to demineralization in a cariogenic acidic environment. Moreover, fluoride ions interfere with acid production of cariogenic bacteria, and thereby inhibiting their cariogenic activity (Buzalaf et al. 2011).

A recent study showed that the application of fluoride varnish modulates in dental plaque microbiology and physicochemical effects, providing preventive effect on ECC (Zhang et al., 2022). However, the effect of fluoride-varnish on the dental plaque microbiome has not been adequately investigated. Thus, in this work, we employed high-throughput amplicon sequencing to examine the impact of one-month fluoride application on the dental plaque microbiome of children with mixed dentition.

## 2. Materials and methods

### 2.1. Study population and clinical examination

The present study analyzed the dental plaque samples from our earlier clinical trial on ECC (Apriani et al., 2020). Hence, the samples were derived from a cohort consisting of twenty children aged 8 to 9 years who had dental caries. In the original clinical trial, the subjects with systemic diseases, those undergone orthodontic treatment, those who had antibiotics or antifungals within six months, or not cooperative for clinical examination were excluded. Ethical approval was obtained from the Faculty of Dentistry's Ethics Committee (EC number 228/S2-Sp/KEPK/FKG/11/2018). At the baseline, clinical parameters, and demographic information, including the caries index (DMFT and dmft), were recorded for all subjects (Smith and Packer 1971). Salivary pH level and the Patient-Hygiene-Performance (PHP) index were also assessed at both the baseline and one month following the topical application of fluoride-varnish (Podshadley & Haley 1968). For the final analysis of the index score, six teeth were considered, namely buccal 16, labial 11, buccal 26, ligual 36, labial 31, and lingual 46. Overall PHP index scoring flow chart can be seen in Supplementary Figure 1.

### 2.2. Fluoride varnish application and plaque sampling

The Clinpro™ White Varnish (3 M ESPE, Loughborough, Leicestershire, U.K), a topical fluoride white varnish in gel form containing 5 % sodium fluoride was used for the study. The children were advised to avoid rinsing, brushing, eating, or drinking for 30 min after the application. The fluoride-varnish was reapplied once a week for a total of one month. Participants were instructed to follow general oral hygiene practices, such as brushing their teeth twice a day with toothpaste, to maintain uniform oral health conditions during the fluoride varnish treatment period, which lasted from baseline to one month after the application.

The plaque samples were collected at the baseline and one-month post-fluoride varnish application. The samples were obtained from the buccal and lingual surfaces of the mandibular first molars using a sterile swab and placed in 15-ml Falcon tubes filled with 5-ml of sterile phosphate-buffered saline (PBS). To ensure standard collection protocol, all samples were collected between 9 and 11 AM, which were transported to the laboratory immediately for the purpose of this sequencing study, twenty dental plaque samples were randomly selected from the fluoride application group in the original study.

### 2.3. Sample preparation and DNA extraction

The dental plaque samples in the 15 ml Falcon tubes were homogenized using a vortex, and then centrifuged at 4,500 rpm for 10 min. The supernatant was discarded and replaced with one mililiter of sterile PBS. The new mixture was homogenized and transferred into 1.5 ml microcentrifuge tubes. Genomic DNA was isolated using a Geneaid Presto Buccal Swab gDNA Extraction Kit (Geneaid, Taipei, Taiwan). Quality control of the extracted DNA was evaluated using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

### 2.4. PCR amplification and 16S rRNA sequencing

Library preparation was conducted by amplifying the V3-V4 region of 16S rRNA. The relevant primers were selected as described previously (Widyarman et al., 2021; Widyarman et al., 2022). The resultant PCR products were cleaned utilizing AMPure XP Beads (Beckman Coulter Genomics, Brea, California). The final PCR products were diluted, pooled, and mixed with phi-X (Phi-X Control V3 library; Illumina, San Diego, CA, U.S.A.) with a 5 % spike-in control. Sequencing was conducted using the iSeq 100 NGS system (Illumina, San Diego, CA, U.S.A), with paired-end sequencing and a total of 2 × 151 cycles and 10 cycles for each index, as per the manufacturer's instruction (Mandal et al., 2015).

### 2.5. Bioinformatic analysis

Pre-processing of the crude sequencing data was performed according to the previously published protocol (Widyarman et al., 2021; Widyarman et al., 2022). In brief, a clustering algorithm based on 97 % similarity was used to identify operational taxonomic units (OTUs), and taxonomic information was annotated with Illumina's Greengenes database (greengenes.secondgenome.com/downloads/data-base/13_5). Alpha diversity indices were calculated to assess microbial community richness and diversity using MicrobiomeAnalystR platform and vegan package in R. The files were obtained from GitHub (https://github.com/xia-lab/MicrobiomeAnalystR) (Chong et al., 2020; McMurdie & Holmes 2013). To evaluate the contrasts in the microbial community composition and structure among groups before and after fluoride-varnish application; beta diversity indices based on weighted UniFrac distance were calculated using Bray–Curtis's dissimilarity indexes and principal component analysis (PCA) using R software (version 4.2.2) (Zhao et al., 2021). To identify taxon contributing to the distinction between pre and post fluoride-varnish application, supervised multivariate statistical analysis, sparse partial least squares discriminant analysis (sPLS-DA) was employed (Rohart et al., 2017). For this purpose, the multivariate method with 5 × 10-fold cross-validation was performed by R package mixOmics v6.6.231, including sequence variants present at ≥ 0.05 % relative abundance in ≥ 1 samples. Multilevel decomposition in mixOmics enables observation of subtle differences which would otherwise be masked by the individual variation in order to obtain better discriminative potentials between pre and post fluoride-varnish treatment. Differentially abundant microbial biomarkers were further designated by linear discriminant analysis effect size (LEfSe) based on both significance and biological relevance (Segata et al., 2011; Blankenberg et al., 2010). Additionally, we used Microbiome Multivariable Association with Linear Models [MaAsLin2] (Mallick et al., 2021) and DESeq2 (Love et al., 2014) to identify key species abundances that reach a statistically significant association between pre and post-fluoride-varnish application. The Sparse Inverse Covariance Estimation for Ecological Association and Statistical Inference (SPIEC-EASI) uses graphical network models (Kurtz et al., 2015). Therefore, this method was used to infer the microbial association (at genus level)

network within and between pre and post fluoride-varnish treatment groups. We focused on co-occurrence network analysis by agglomerating at the genus level to mitigate some of the potential issues associated with confounding factors (such as species-level annotation errors from 16S amplicon sequencing, and variations in patient selection). Correlations with an R-corr absolute value only above 0.6 and a $p < 0.05$ were plotted.

### 2.6. Statistical analyses

The Wilcoxon rank sum test was used to evaluate variations among pre and post fluoride-varnish application groups of the oral microbiota's diversity indices. Normalization and log transformation were used to standardize the data and reduce the effect of outliers. The Bonferonni correction was used to conform the p-values to account for numerous measurements. Finally, correlations between species and metadata were identified to determine statistically significant relationships. The Student's *t*-test was used to compare the difference between the plaque pH and PHP values before and after fluoride varnish application following normalization of the data. Spearman's correlation test was used in measuring the strength of the association between PHP score and DMFT/dmft in the baseline group. Finally, the test was evaluated using the R programming language to determine if the correlation was statistically significant, with a p-value of $< 0.05$ indicating a significant correlation.

## 3. Results

### 3.1. Study demographics and clinical characteristics

The present study selected twenty dental plaque samples derived from 10 male and 10 female participants for subsequent sequencing. The mean age of this cohort was 9 ± 0.36 years. The PHP scores of the participants were significantly lower compared to the baseline following fluoride varnish treatment (PHP index: from 2.26 to 1.6; $p = 0.002$). The mean DMFT and dmft scores for the participants were 0.9 ± 0.96 and 4.45 ± 3.13; respectively. In addition, salivary pH level was significantly raised closer to neutral pH i.e. from pH- 6.52 to pH −7.04 ($p < 0.001$) following the fluoride-varnish application. The demographic and oral health data of the study population are shown in Table 1.

### 3.2. Fluoride-varnish application modulated the dental plaque microbiome diversity and community structure

Overall, 1910 sequence variants were distinguished across all 20 plaque samples, out of which 478 sequence variants present in the samples accounted for the majority of the microbial diversity found in the plaque samples. The remaining sequence variants were found to be rare, accounting for only a small proportion of the total microbiota in the plaque samples. This indicates that a few predominant species are responsible for most of the microbial diversity in the plaque samples. Additionally, the filtering process ensured that the sequence variants were not false positives or outliers identified in at least two samples with a minimum relative abundance of 0.05 %. The Good's analysis for all samples was detected to be over 99.9 %, suggesting that the sequencing depth was ample enough to capture the microbial diversity in the plaque samples.

**Table 1**
The demographic and oral health data of the study population.

| Variables | Subjects (n = 20) | *p*-value |
|---|---|---|
| **Age (years)** | 9 ± 0.36 | |
| **Gender** | | |
| Male | 10 | |
| Female | 10 | |
| **PHP Index (Patient Hygiene Performance)** | | |
| Pre fluoride-varnish treatment | 2.26 ± 1.09 | 0.002*** |
| Post fluoride-varnish treatment | 1.6 ± 0.53 | |
| **pH Level** | | |
| Pre fluoride-varnish treatment | 6.52 ± 0.04 | <0.001*** |
| Post fluoride-varnish treatment | 7.04 ± 0.03 | |
| **DMFT Index** (decayed, missed, filled surfaces for permanent teeth) | 0.9 ± 0.96 | |
| **dmft Index** (decayed, missed, filled surfaces for primary teeth) | 4.45 ± 3.13 | |
| **Spearmant Correlation b/n PHP Index and DMFT/dmft scores at baseline** | | |
| DMFT vs PHP | $r^2 = 0.63$ | 0.002*** |
| dmft vs PHP | $r^2 = 0.60$ | 0.004*** |

Alpha diversity metrics were utilized in order to examine the overall richness, relative abundances, and evenness of the species in the pre- and post-treatment groups using, i) the Chao index, ii) observed species (OTU count), iii) the Simpson index and the iv) Shannon index. The results of all metrics indicated that the diversity of the microbiota associated with the post-treatment group was significantly altered and reduced when compared to the pre-treatment group (pShannon = 0.0073, pSimpson = 0.0079, pChaoI = 0.013). (Fig. 1A).

In order to determine the degree of relatedness between the microbiome composition from the pre- and post-fluoride varnish treatment samples and to examine the clustering of samples with similar microbial composition, PCA plots derived from the Bray Curtis dissimilarity distance were employed. The Bray Curtis plot (Fig. 1B and C) shows clear separation ([PERMANOVA] F-value: 9.0838; R-squared: 0.19293; p-value: 0.001 of Bray–Curtis distances) with only minimal overlap of samples between these groups based on PC axes 1 and 2 (Fig. 1B and Fig. 1D red-dotted square) in PCA analysis and X variate1 and 2 (Fig. 1C) axes in sPLS-DA analysis.

sPLS-DA was further used to identify the taxon variants that contribute to the differences between pre- and post-treatment groups by taking into account the concentrations of the various taxon variants, as well as their interactions with each other, to identify which variants are most influential in distinguishing the two groups (sPLS-DA, Fig. 1D and E-red-dotted square). Of which, few genera commonly of interest in pediatric dental caries research such as *Fusobacterium*, *Porphyromonas* were found to be decreased in the post-treatment group (Fig. 1E-red-dotted square) and several genera highly abundant in the post-treatment group belonging to phylum *Proteobacteria* (i.e. *Luteimonas*, *Arenimonas*, *Pseudoxanthomonas, Cellvibrio*) were identified distinctively differentiated between pre and post treatment (Fig. 1E-red-dotted square and Fig. 1F, Supplementary Table. 1). However, few samples (n = 3/20) were discovered not to be affected by fluoride-varnish treatment suggesting that other external factors influencing the taxon composition, that could not be considered in this study (Fig. 1A and B). This observation is illustrated graphically by hierarchical clustering on the heatmap based on the dominant taxa and as well as their abundance between the outliers identifiable in the PCA/s-PLSDA plots and in the heatmaps (s.22,s.23, and, s.24) (Fig. 1A, B and F). Nevertheless, the foregoing results indicated that one month of fluoride-varnish application significantly modulated the dental plaque bacterial diversity and community structure.

### 3.3. Fluoride application significantly changed the community structure and abundance of specific bacteria

To determine the genera and species that significantly contributed to the difference in plaque community structure after fluoride-varnish application, the univariate and multivariate techniques such as DESeq2, MaAsLin2 were used (Mallick et al., 2021). Thew LEfSe analysis as employed to identify the differentially abundant species and genera. 164 species belonging to 44 genera were identified as either significantly enriched or depleted between pre- and post-fluoride-varnish treatment counterparts (Supplementary Table. 2). The key genera identified inclusive of *Streptococcus* (8.5 %), *SR1_genera_incertae_sedis* (7.35 %),

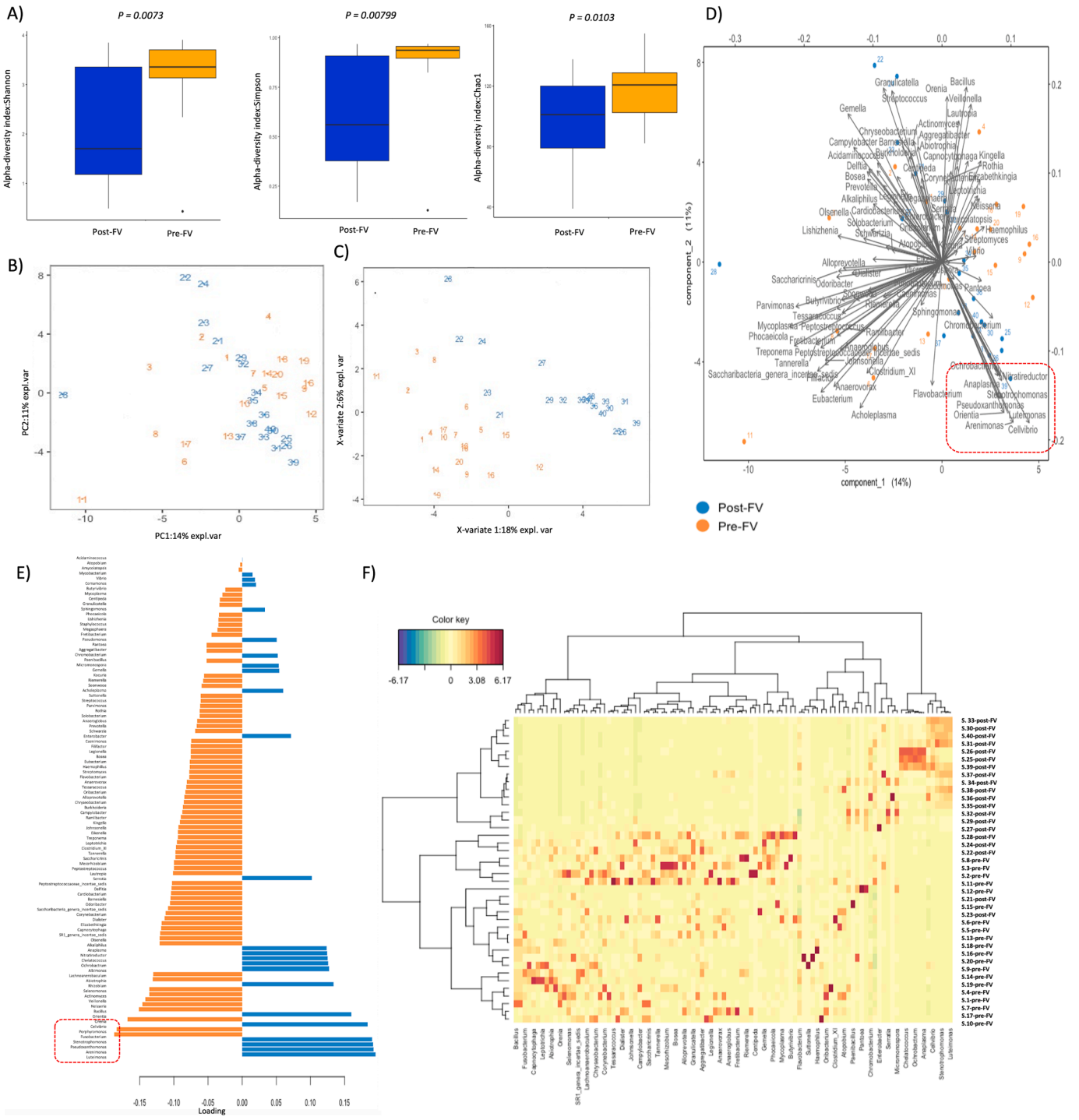


**Fig. 1. Plaque microbiota community characteristics of post-fluoride-varnish treated children (8–9 years old) with dental caries (n = 20) can be distinguished from that of pre-fluoride-varnish counterparts using 16S rRNA gene amplicon sequencing.** A) The Shannon diversity index, Simpson index, and Chao1 index were used to calculate alpha diversity and revealed distinct features following the application of fluoride-varnish. PC analysis of read counts transformed using log-cumulative-sum scaling at the sequence variant level. (C) Multivariate sparse partial least-squares discriminant analysis (sPLS-DA) of read counts transformed with log-cumulative-sum scaling at the level of genus variants. Genera contributing to separation with component-1 of sPLS-DA (red dotted squares indicating most discriminative genera). Rank of the importance of loading coefficients at the genus level. (most discriminative genera are shown in red dotted squares), bottom to top; the colour of the bar indicates the group where the sequence variant has the highest median abundance based on component 1. Orange is for pre-fluoride-varnish, and blue is for post-fluoride-varnish. The heat map of read counts is transformed using log-cumulative-sum scaling to identify discriminatory sequence variants along with component −1 of sPLS-DA.

*Prevotella* (5.72 %), *Vellinolla* (6.7 %), *Fusobacterium* (11.63 %), *Porphyromonas* (4.25 %), *and Capnocytophaga* (6.88 %), *Selenomonas* (2.9 %), and *Neisseria* (4.95 %). They were among the top 10 predominant species that account for more than 67 % of the total sequences in pre-treatment group. Of these, *Fusobacterium, Neisseria, Porphyromonas, Capnocytophaga,* and, *Selenomonas* species were significantly depleted in the post-treatment group ($p < 0.05$; Fig. 2B. and 2C, Supplementary Table. 2). Following one month of fluoride treatment, the core plaque

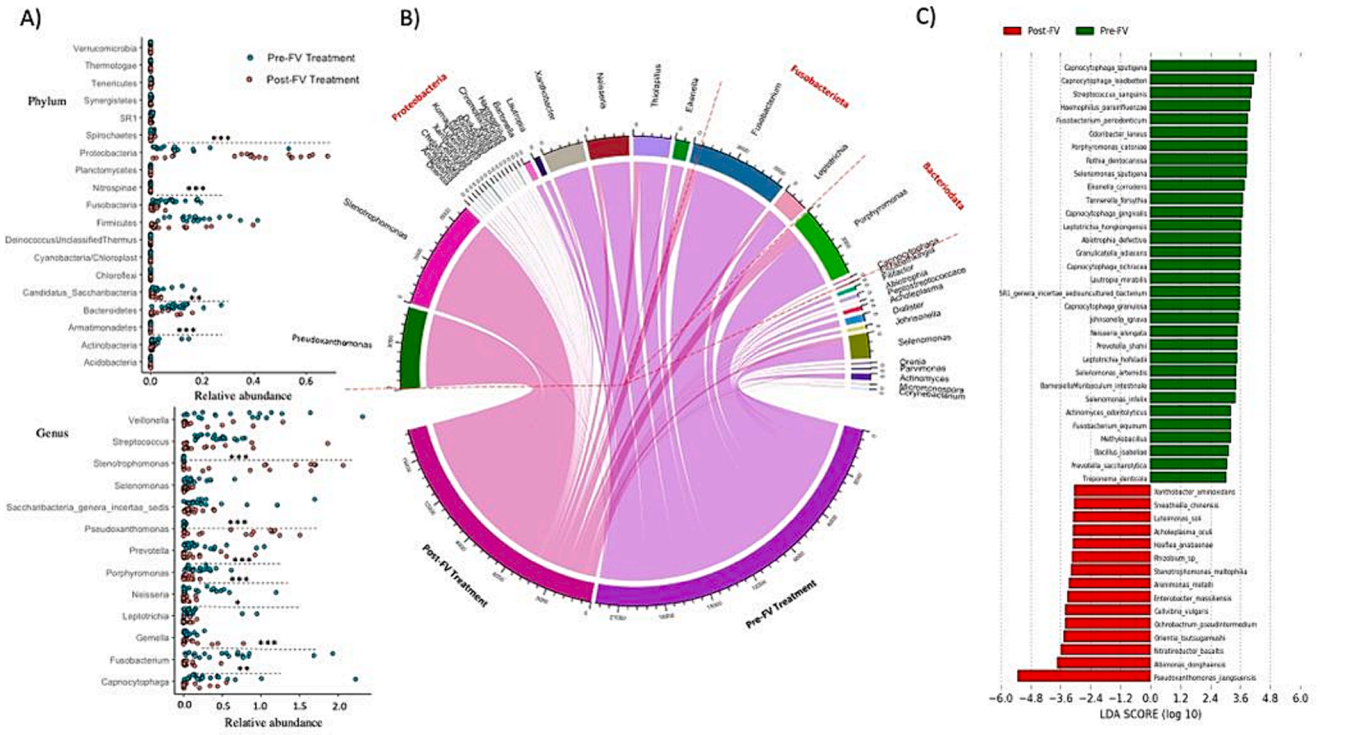


**Fig. 2. Most significantly influenced taxa contribute to one month of fluoride-varnish application in children with dental caries** (A) Change of top abundant taxonomic composition at phylum and genus level following fluoride-varnish application. Letters indicate statistical differences between samples are *P < 0.05, **P < 0.01, and ***P < 0.001 detect taxa with the most statistically significant differences filtered using a q-value of 0.05 and effective size of 0.05 threshold in DefSeq2 analysis. The chord diagrams (B) depict the key taxa identified by the two groups (Baseline-pre-fluoride-varnish application, post-fluoride-varnish application). Taxa identified in two or more genera are connected by arcs (chords). (C) Species-level differences in taxonomic biomarkers contribute with the highest significance to the plaque microbiome composition change identified by LEfSe analysis (,LDA > 3, p < 0.005). Histogram of the LDA scores for differentially abundant features among groups. The threshold on the logarithmic LDA score for discriminative features was set to 3.0.

microbiome has significantly altered along with distinguished taxonomic assignment of genera belongs to *Stenotrophomonas* and *Pseudoxanthomonas,* accounting for 37 % of the variation (p < 0.01; Fig. 2A and 2B). Additionally, the majority of these bacterial communities represented by the top 10 genera (>14 %) have greatly contributed to a significant change in the dominant phyla of the plaque core microbiome (Proteobacteria-18.5 %, Fusobacteria-13.4 %, Bacteroidetes-7.79 % followed by Actinobacteria-0.7 %; p < 0.01; Fig. 2A, Supplementary Table. 2). Among known disease associated species consistently identified by all three analysis methods represented in Fig. 2C and 3A (p < 0.05; LDA > 3 / MaAsLin2; coef- from 3.0 to −3.0/ DESeq2;log2 fold change values ranged from 3.0 to −3.0). *Fusobacterium, Capnocytophaga, Leptotrichia, Porphyromonas catonie, Streptococcus sanguinis, Treponema denticola, Actinomyces odontolyticus, Selenomonas infelix, Tannerella forsythia* and *Eikenella corrodens* were significantly reduced in post-fluoride-varnish (Fig. 2C; LDA > 3 and 3A-blue dotted squares, Supplementary Table. 2). In contrast, species *Pseudoxanthomonas jiangsuensis, Albimonas donghaensis, Nitratireductor basaltis, Orientia tsutsugamushi, Ochrobactrum pseudintermedium, Cellvibrio vulgaris, Enterobacter massiliensis, Chromobacterium haemolyticum, Arenimonas metallic, Acholeplasma oculi, Stenotrophomonas maltophilia, Sneathiella chinensis, Luteimonas soli, and Xanthobacter aminoxidans* were significantly enriched in post-fluoride-varnish treatment group (Fig. 2C and 3A; p < 0.05; LDA > 3 / MaAsLin2; coef- from 5.0 to −5.0/ DESeq2;log2 foldchange values ranged from 8.0 to −6.0, Fig. 2C, Supplementary Table. 2). From the species that were significantly elevated in the post-treatment group, over 73 % of them belong to the Proteobacteria (Fig. 2C; LDA > 3 and 3A- green dotted square).

The foregoing results showed that the substantial differences in the community composition structure in the dental plaque were accounted by the depletion of certain bacteria associated with dental caries and the replenishment in the abundance of species belonging to the phylum Proteobacteria, as a result of one month of fluoride-varnish application.

### 3.4. *Proteobacteria interactions enriched in post-fluoride varnish application microbial association networks*

The co-occurrence network approach was utilized to gain a deeper understanding of the plaque microbiome community associations and changes occurring internally and compared between pre- and post-fluoride-varnish application. The SPIEC-EASI is a standard method that measures the strength of the relationships between genera (represented as nodes) and their interactions (represented as edges). *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* were the most prominent phyla in the microbial association networks. Interestingly, *Proteobacteria* was detected as the most abundant phylum, approximately 61.21 % (Fig. 3B-boxplot-red dotted square) of the total nodes found in the post-fluoride-varnish group. Comparatively, *Firmicutes*, *Actinobacteria,* and, *Bacteroidetes* represented 27.88 % of the total nodes of SPIEC-EASI association networks in the pre-fluoride-varnish group. The average degree of an undirected graph in a circle plot (Fig. 3B-circle plot) was used to calculate the number of edges compared to the number of nodes; representing the higher number of connections to each other in a network. The highest number of edges were represented by genera belonging to *Proteobacteria,* exclusively within the post-fluoride-varnish group (degree > 20, Supplementary Table. 3). Additionally, most of the network edges among identified genera belong to phylum *Proteobacteria* which is aligned regarding its relative abundances in the post-treatment group (Fig. 3B- red-dotted square, Supplementary Table. 3).

In order to differentiate the positive and negative edges in the

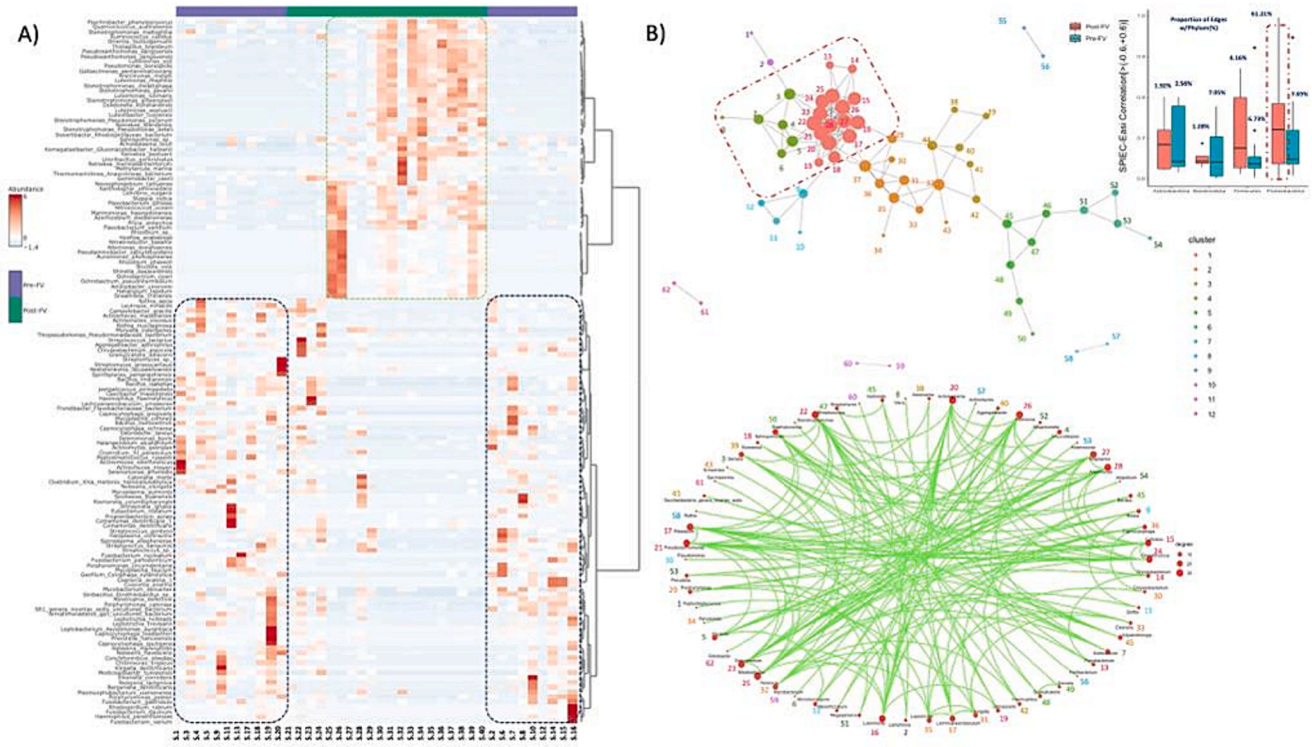


**Fig. 3.** **Distinctively differentiated species and microbiome association network following fluoride-varnish application** Heatmap of most abundant species among children with dental caries differentiates between post-fluoride-varnish application and baseline counterparts. Species are highlighted in dotted green square-pre fluoride-varnish group and blue square-post fluoride-varnish group. (B) Co-occurrence interaction networks, colored by module resilience. The significant clustered interaction was given in caries groups stratified by different colors of clusters. The observed smaller average path length in the post-fluoride-varnish group indicated compact network properties and strong microbial interactions (hub distance in clusters 1[red] and 4[dark green]. The red-dotted square indicates clusters of genera in the post-fluoride-varnish group that exclusively belong to the phylum Proteobacteria. Distribution of the fractions of interactions that involve taxa at phylum level between pre and post-fluoride-varnish application-associated microbiome networks depicted in a boxplot. The Y-axis represents the proportion of interactions involving a phylum in terms of SPIEC-EASI correlation. The solid green line represents edges with positive (>90 %)/negative interactions in the circle network plot. Nodes at the end of each line represent the genera of interaction among the interacting nodes. The average degree of an undirected graph in a circle plot (Fig. 3B-circle plot) was used to measure the number of edges compared to the number of nodes presenting a higher number of connections to each other in a network. Analysis of interacting microbes revealed the highest number of edges. (See also Supplementary Table. 3).

network (Positive |coefficient correlation (=corr)| $\geq$0.6 and $p < 0.01$ and negative correlations (corr)| $\geq -0.6$ ($p < 0.01$), we analyzed the differences within and between the microbiome networks of pre- and post-treatment groups (Fig. 3B-boxplot, Supplementary Table. 3). The post-fluoride-varnish network showed the highest clustering coefficient (0.72), followed by the pre-fluoride-varnish group (0.63), indicating that microbial interactions are strongest in the post-fluoride-varnish group. The observed smallest average path length in the post-fluoride-varnish group indicating compact network properties and strong microbial interactions (1.18–1.6 hub-distance in cluster 1[red] and 4[dark green] in Fig. 3B- red dotted square, Supplementary Table. 3).

Of notable observations, *Bacteroidetes-Bacteroidetes*, *Firmicutes-Firmicutes*, *Firmicutes- Bacteroidetes-Actinobacteria-Proteobacteria* interactions were enriched in caries active pre-fluoride-varnish treatment compared to their post-treatment counterparts. These phyla exhibit considerable mean abundance differences in most caries active pre-treatment groups compared to the post-fluoride-varnish treatment counterparts, suggesting that these interactions play an important role in modulations of caries-associated microbiota. On the other hand, we found that *Proteobacteria-Proteobacteria* (Fig. 3B-boxplot, Supplementary Table. 3) associations were extensively enriched in the post-treatment network, which could indicate the return to eubiosis of dental plaque microbiome following fluoride-varnish treatment.

## 4. Discussion

The present study examined the modulation of dental plaque microbiome of children with mixed dentition following one-month fluoride varnish application. Next-generation sequencing and comprehensive bioinformatic analysis of dental plaque samples, derived from pre- and post-fluoride application, clearly showed differences in the diversity, composition, and structure of the dental plaque microbiome. The majority of the differentially expressed abundant taxa belonged to the dominant phyla found in the core plaque microbiome such as *Proteobacteria*, *Fusobacteria, Bacteroidetes*, and *Actinobacteria*. The alpha diversity in the post treatment group was significantly lower compared to the baseline. This finding suggests a shift from a caries-promoting dysbiotic state to a health-associated eubiotic state of the dental plque microbiome. Higher diversity present in the baseline dental plaque samples could be due to the presence of cariogenic species. Similarly, the Chao diversity index for the post-fluoride-varnish treatment group was significantly lower compared to the baseline microbiome. This suggests that, even at low abundance, exclusive rare taxa, such as *Areinmonas*, *Pseudoxanthomonas*, and *Luteimonas* i.e can have a strong protective role against the progression of caries following fluoride-varnish application.

The sPLS-DA analysis showed that low abundant rare taxa that were initially present in the baseline plaque microbiome significantly contributed to the distinctive clustering patterns following fluoride application. Hence, response of the dental plaque microbiome to the fluoride-varnish treatment is affected by the relative abundance of rare, low-abundant taxa. Moreover, the majority of the significant edges in the plaque microbiome association networks in the post-treatment group were rare taxa. This supports the notion that, in comparison to their more prevalent counterpart, rare species may play a significant

influence in microbiome community dynamics (Banerjee et al., 2018; Jousset et al., 2017; Lynch & Neufeld 2015). Interestingly, *Proteobacteria* was significantly more abundant in the post-treatment group. Moreover, *Proteobacteria* was determined as the phylum that mostly dominating in terms of network edge participation, with many of the edges in the post-treatment network involving one or both vertices being Proteobacteria. Hence, increased inter-interactions of Proteobacteria may be a hallmark of eubiosis of dental plaque microbiome following fluoride-varnish application.

The pre-treatment group was predominantly composed of *Bacteroidetes* and *Firmicutes*, which made up the majority of the plaque microbiome. These two phyla were found to participate in significant network associations, both in terms of nodes and edges, proportional to their high relative abundances. There were several notable differences in the post-treatment plaque microbiome networks compared to the baseline. The baseline group showed an enrichment of Bacteroidetes-Bacteroidetes and Firmicutes-Firmicutes interactions, while the post-treatment group showed an enrichment of Proteobacteria-Proteobacteria interactions. Zhang et al. (2022) reported that two weeks after fluoride treatment could significantly influence the dental plaque microecology, thereby inhibiting the progression of early enamel caries. Fluoride application significantly disrupted the structure of the supragingival plaque microbiome. However, it had no influence on the diversity and richness of the microbiome. The composition of the community, notably the taxa Capnocytophaga, Rothia, and an unidentified Prevotellaceae, was disrupted. Comparatively, in the present study, which employed one month of fluoride-varnish treatment found that the prevalence of the dominant genera—*Fusobacterium, Porphyromonas, Capnocytophaga, Neisseria*, and *Leptotrichia*—were significantly lower following fluoride treatment. Aditionally, some genera, primarily from the phylum Proteobacteria, such as *Areinmonas, Pseudoxanthomonas*, and *Luteimonas*, increased.

There are several studies in the literature which have identified certain foregoing bacteria as being linked to the cariogenic process, particularly the *Capnocytophaga* species (Zhang et al., 2021), *Leptotrichia* species (Eribe et al,. 2004; Eribe & Olsen 2008; Eribe & Olsen 2011; Lim et al., 2016), *E. corrodens* and *T. forsythia* (Inquimbert et al., 2019). *Capnocytophaga* is known to produce acid from d-galactose, d-glucose, and d-fructose (Zhang et al., 2021). *Leptotrichia* also acts as a pioneer colonizer, allowing the adhesion of other microorganisms and promoting biofilm formation, highly saccharolytic, implying that it ferments a wide variety of mono- and disaccharides to lactic acid (Eribe et al., 2004; Eribe & Olsen 2008; Eribe & Olsen 2011; Lim et al., 2016). E. corrodens is a relatively early colonizer in biofilm formation and could permit cariogenic bacteria to adhere to biofilm (Inquimbert et al., 2019). Moreover, T. forsythia was correlated with T. denticola, which mediates its adherence to other potential periodontal and caries risk pathogens in oral biofilms (Inquimbert et al., 2019). A complete understanding of the cariogenic properties of other microorganisms is remains to be determined. In addition, most of the significant enriched abundant species in the post fluoride varinish application group i.e. *Pseudoxanthomonas jiangsuensis, Cellvibrio vulgaris, Luteimonas soli* belong to phylum *Proteobacteria*.

The present study discovered that fluoride-varnish treatment may play a significant role in shifting from dysbiosis to eubiosis of dental plaque microbiome by affecting the diversity, composition of community structure of acidogenic and aciduric bacteria. This change was likely due to the reduction of plaque pH to a neutral level ($p < 0.001$) and the reduction of plaque deposits (PHP; $p < 0.01$). Previously, *Marquis* et al., in 2003 observed a similar trend in dental plaque samples from children with ECC. Anti-cariogenic effect of fluoride was mainly due to the reduction of acid-tolerant, cariogenic bacteria found in plaque. The study proposed that decrease in acid production facilitate in demineralization, preventing the progression of ECC. Consequently, the beneficial bacteria may increase during the acidification–alkalinization cycles in plaque (Marquis et al., 2003). Furthermore, fluoride application is known to significantly change the dental plaque ecology by reducing acidity and fostering a microbial population that is less acid-tolerant and less cariogenic in the long run (Marsh, 1994). Our observations are in line with the foregoing studies. However, further investigations at the functional level are necessary to understand the mechanism by which caries-causing bacterial communities are depleted and new and rare abundant genera such anaerobes like *Pseudoxanthomonas*, *Luteimonas*, and *Arenimonas* are enriched in plaque biofilm following fluoride-varnish treatment, representing a transition from dysbiosis to eubiosis.

Interestingly, *S. mutans* was not identified in dental plaque microbiome of the majority of the subjects with caries active status at the baseline. Although *S. mutans* was considered the major cariogenic bacteria in the past, emerging evidence from sequence-based studies has clearly demonstrated that dental caries is a poly-microbial disease (Fechney et al., 2019). Moreover, Fechney et al. in 2019 documented the result of their study showing no association of *S. mutants* with ECC. This indicates that other bacteria may be equally or even more involved in caries formation, as *S. mutans* is not necessarily present in individuals who have caries. The microbiome of an individual may have a greater effect on caries formation than mere presence of *S. mutans*.

One of our main research limitations was the small sample size, which might have prevented us from extrapolating the findings to a population level. Further studies with bigger sample sizes are warranted for firm conclusions at the microbiome level. Another drawback is the lack of a control group consisting of supragingival plaque samples from teeth that have not had fluoride applied. To gain a deeper understanding of the role that bacteria play in impeding the use of fluoride varnish to ameliorate dental caries, more extensive research at the functional level is required.

## 5. Conclusion

In conclusion, our study demonstrated that the application of fluoride varnish to 8–9-year-old children caused a significant change in the community level dental plaque biofilm microecology from dysbiosis to eubiosis. Interestingly, the enrichment of health-associated species belonging to the phylum Proteobacteria following fluoride varnish application may play a protective role in preventing the progression of ECC, resulting in a healthier oral environment. Long-term studies with larger sample size are warranted to solidify these interesting findings.

## Ethical statement

This study was approved by The Ethics Commission of Faculty of Dentistry, Universitas Trisakti (IRB number 228/S2-Sp/ KEPK/FKG/11/ 2018).

## CRediT authorship contribution statement

**Armelia Sari Widyarman:** Conceptualization, Methodology, Investigation, Supervision, Writing – original draft, Writing – review & editing. **Nadeeka S. Udawatte:** Software, Validation, Formal analysis, Data curation, Writing – original draft. **Idham Tegar Badruzzaman:** Investigation, Visualization, Writing – review & editing. **Caesary Cloudya Panjaitan:** Investigation, Visualization, Writing – review & editing. **Anie Apriani:** Methodology, Investigation, Resources, Data curation. **Jeddy:** Investigation, Visualization, Writing – review & editing. **Tri Erri Astoeti:** Resources, Visualization, Supervision, Project administration, Writing – review & editing. **Chaminda Jayampath Seneviratne:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sdentj.2024.07.010.

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