Effect of silver diamine fluoride on plaque microbiome in children

May Lei Mei [1,2], Zejun Yan[3], Duangporn Duangthip[2], John Yun Niu[2], Ollie Yiru Yu[2], Meng You[4], Edward CM Lo[2], Chun Hung Chu[2].

1. Faculty of Dentistry, University of Otago, Dunedin, New Zealand
2. Faculty of Dentistry, The University of Hong Kong, Hong Kong SAR, China
3. Department of Research & Development, Aviagen Ltd, Edinburgh, United Kingdom.
4. West China Hospital of Stomatology, Sichuan University, Chengdu, China.

Correspondences

Dr ML Mei

Faculty of Dentistry

University of Otago

PO Box 56, Dunedin 9054

New Zealand

may.mei@otago.ac.nz

Prof CH Chu

Faculty of Dentistry

The University of Hong Kong

34 Hospital Road

Hong Kong SAR

China

chchu@hku.hk

Abstract

Objective: To investigate the microbiome profile and relative abundance changes of the plaque on silver diamine fluoride (SDF)-treated caries in children with early childhood caries.

Methods: A single trained examiner assessed the caries as being arrested or active and then collected plaque samples from the caries lesions from fourteen 5-year-old children immediately before, 2 weeks after, and 12 weeks after a one-off application of 38% SDF. We assigned 16S rRNA gene sequences to operational taxonomic units (OTUs) using a 98.5% identity cut-off. We also used a variety of taxonomy- and phylogeny-based statistical approaches to compare the biodiversity and relative abundance among different groups.

Results: The caries arrest rate were 90% and 83% after 2 and 12 weeks, respectively. We studied 46 plaque samples and identified 388 OTUs (254 identified at the species level, 129 identified at the genus level, and 76 identified at the family level). There was no significant change in the diversity in the arrested caries before and 12 weeks after SDF treatment ($p=0.71$). The diversity in active caries reduced significantly 12 weeks after SDF treatment ($p=0.006$). The relative abundance of certain caries-related species (e.g., *Streptococcus mutans* and *Lactobacillus sp*.) was reduced in arrested caries but was increased in active caries after SDF treatment.

Conclusion: There was no overall microbiome changes in the caries arrested by SDF. The relative abundance of some caries-related species is reduced in arrested caries, while increased in active caries.

Clinical Significance: This study provides information on microbiome changes on SDF-treated caries of children.

Keywords

silver diamine fluoride; microbiology; early childhood caries; plaque; microbiome.

1. Introduction

Early childhood caries (ECC) refers to the presence of decayed, missing, or restored teeth in the primary dentition of children younger than 6 years old. ECC is one of the most common chronic childhood diseases globally, affecting up to 73% of socioeconomically disadvantaged children [1]. Treatment in young children has been challenging because it requires advanced clinical skills and high-cost general anaesthesia for patient management [2]. An untreated carious lesion can progress, cause pain, and form a dental abscess, leading to severe infection [3]. ECC treatments differ from adult treatment, as an atraumatic approach for children can slow the progression of caries so that the arrested decayed tooth will exfoliate before causing oral pain [4].

Silver diamine fluoride (SDF) is a topical fluoride solution for managing caries. Clinical studies have shown that 38% SDF prevents and arrests coronal caries in preschool children [2, 5]. A systematic review reported that 81% of active caries lesions in ECC became arrested after SDF treatment [6]. Various mechanisms of action have been proposed to explain SDF's promising caries-arresting efficiency. Some researchers suggested that SDF hardens a caries lesion by regaining calcium and phosphate from saliva [7, 8], and it reduces dentine collagen degradation by inhibiting collagenases like MMPs and cathepsins [9]. In addition, SDF has shown intense antibacterial effects on certain cariogenic biofilms in mono-cariogenic species biofilm [10] or multi-cariogenic species biofilm [11, 12]. However, these studies were conducted in *in vitro* biofilm models, which by no means accurately represent the sophisticated microbiological environment in the oral cavity. In contrast, the biofilm in the human mouth involves 30 genera, representing at least 700 species that interact with one another [13]. Two studies have investigated the microbiome changes after SDF treatment, but they are either investigated limited sample size [15] or on root caries [16]. Previous findings demonstrating that there are

microbiome profiles change with the progression of caries and differ from the primary to the secondary dentition [14]. It remains to be determined *in situ* whether the SDF's caries-arresting effects on caries lesion stem from the suppression of a single or a specific consortium of bacteria species or whether dental plaque as a whole undergoes a more complicated shift in terms of multiple bacteria groups. Moreover, it is also critical to discern what happened in the caries lesions that are still active even after SDF treatment.

Conducting quantitative and qualitative analyses of polymicrobial ecosystems such as plaque biofilms is complicated because the ecosystems' complexity makes the potential pathogenic species difficult to study and because a large proportion of oral bacteria cannot be cultured. Metagenomics, or the study of the genetic material directly recovered from environmental samples, has been intensively and broadly used to investigate the microbial composition of samples collected from various ecosystems and consequently to answer relevant biological questions [17]. Next generation sequencing (NGS) technology with subsequent metagenomic analysis has improved our knowledge of oral bacterial communities, as compared with traditional culture-based research. Therefore, we aimed to (a) describe the effects of SDF treatment on the microbiome profile present in plaque from ECC subjects and (b) compare the microbiome profiles present in plaque between arrested and active caries after SDF treatment. The null hypotheses were that (a) SDF has no effect on microbiome profile changes on the plaque from ECC subjects and (b) no difference can be observed in the microbiome profile present in plaque between arrested and active caries.

2. Materials and methods

2.1. Subject recruitment

This study received approval from the Institutional Review Board (The University of Hong Kong) under process number IRB UW13-555. Children from Hong Kong kindergarten III (all are 5 years old) who were involved in an HKU preschool oral health program were recruited. Inclusion criteria: Children aged 5, who have at least one active caries with dentine exposed, had never been treated with SDF. Exclusion criteria: At subject level, children who had major systemic illnesses and took any drugs including oral or systemic antibiotics were excluded. At tooth level, a tooth with non-vital signs and symptoms including pulpal exposure, discoloration, mobility, and dental abscess etc. were excluded. Written consent forms were collected from the participants' parents or guardians. A lesion was recorded as active if it was soft on probing [5, 18]. The examinations were performed by a single examiner (DD) using the same clinical procedures published in previous study [18]. The examiner is a specialist in Dental Public Health and has been calibrated and trained by EL and CHC. At baseline and follow-up examinations, duplicate examinations were conducted on approximately 10% of the study children. Cohen's Kappa statistics was used to assess the intra-examiner reliability.

2.2. Sample size calculation

For quantitative analysis, a major outcome of this study was the Shannon index, which represents a measure of the biodiversity (richness and evenness) in a given sample. The sample size calculation was performed with reference to a similar study that investigated plaque bacterial microbiome diversity in children, which detected a mean (±SD) of Shannon index as 3.7 (±0.1) and 3.5 (±0.1) for caries-free and caries-affected subjects, respectively [19]. In order to detect such a difference at a significance level of 5% and a statistical power of 0.90, minimal 6 subjects in each group were required. We selected 14 subjects and the total sample size was 42 at three time points.

2.3. Plaque sample collection at the baseline (T0)

Plaque samples were collected in the morning, and the children were asked not to brush their teeth or eat the night before the examination. In brief, a sterile spatula was used to collect a pooled plaque sample from the teeth's active lesion surfaces. The collected plaque sample was released from the spatula by agitation in 1.0 ml of phosphate-buffered saline (PBS) solution. The plaque samples were put into an ice box and immediately transported on ice to a microbiology laboratory at the Faculty of Dentistry, The University of Hong Kong.

2.4. Topical SDF treatment

After the plaque samples were collected, the surface of each caries lesion was cleaned with a cotton rod. Subsequently, a one-off 38% SDF solution (Saforide; Toyo Seiyaku Kasei Co. Ltd., Osaka, Japan) was topically applied to the caries lesions using a microbrush (Micro Applicator – Regular, Premium Plus International Ltd., Hong Kong, China). The concentration of silver and fluoride ions of 38% SDF solution from Saforide has been tested in our previous study [20]. The bottle of SDF was freshly opened before the treatment. An independent operator applied the SDF [5].

2.5. Plaque sample collection after treatment (T1 and T2)

Plaque samples were collected at two time points; namely, 2 weeks (T1) and 12 weeks (T2) after the SDF treatment. All the cases were collected in the same day. To assess lesion activity, a caries lesion was diagnosed as arrested if its surface was smooth and hard on probing. A lesion was recorded as active if it was soft on probing [21]. Plaque formed on both arrested and active (if there was any) caries lesions was collected. The same equipment and procedure were adopted to collect the plaque samples from the same caries lesion as used in the baseline collection.

2.6. DNA extraction

Total bacterial DNA was extracted and purified using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. First, 180 µL of lysis buffer (20 mg/mL lysozyme, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 1.2% Triton) was added to suspend the bacterial pellet, which was incubated for 1 h at 37 °C. Then, 20 mL of proteinase K and 200 µL of buffer AL were added to the treated plaque samples, which were incubated for 90 min at 56 °C and then for a further 15 min at 95 °C. After the addition of 200 µL ethanol (96%–100%), the mixture was transferred to the QIAamp Mini spin column and centrifuged for 1 min at 8000 rpm. Afterward, the DNA pellets were sequentially washed with 500 µL Ethanol Wash 1 and then 500 µL Ethanol Wash 2. Finally, the DNA was eluted in 200 ml buffer, specifically Elution (AE). The DNA quality was evaluated by the absorbance measurements at A260/280 using a NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The DNA samples were stored at −20 °C before analysis.

2.7. PCR amplification of 16S rRNA genes and MiSeq sequencing

The V3-V4 hypervariable region of 16S rRNA genes was amplified using universal primers (338 F 5'- ACTCCTACGGGAGGCAGCAG-3'; 806 R 5'-GGACTACHVGGGTWTCTAAT-3') via the polymerase chain reaction (PCR). The PCR reaction Transgen AP221-02 was performed using a 20 µl reaction mixture containing TranStart Fastpfu DNA Polymerase (TransGen Biotech, Beijing, China), 4 µl of 5×FastPfu Buffer, 2 µl of 2.5 mM dNTPs, 0.8 µl of 5 µM forward primers, 0.8 µl of 5 µM reverse primers, 0.2 µl of BAS, and 10 ng of template DNA and ddH_2O. The reaction was carried out in a GeneAmp® 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) using the following program: 95 °C for 3 min followed by 27 cycles of 95° C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. The final extension was performed at 72 °C for 10 min, and the reaction was halted at 10 °C. The PCR product was extracted from 2% agarose gel and purified using

the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions, and it was quantified using a QuantiFluor™ -ST (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2 ×300) on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

2.8. 16S rRNA data analysis

The raw 16S rRNA gene-sequencing reads were demultiplexed. Adapter sequences were removed by Trimmomatic and quality-filtered and merged using the FLASH software with the following criteria: (i) The 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and truncated reads shorter than 50 bp were discarded. Then, (ii) exact barcode matches, two-nucleotide mismatch in primer matching, and reads containing ambiguous characters were removed. Finally, (iii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. Reads that could not be assembled were discarded. OTUs that were 98.5% similar were clustered using UPARSE (version 7.1, http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each OTU's representative sequence was analysed by RDP Classifier (http://rdp.cme.msu.edu/) against the 16S rRNA database (Human Oral Microbiome Database [HOMD]) using a 0.7 confidence threshold. The data were analysed by MOTHUR (Version1.30.1) on Majorbio I-Sanger Cloud Platform's (www.i-sanger.com) free online platform. Customized Python programming scripts and Adobe Illustrator were used to construct diagrams.

2.9. Statistical analysis

Cohen's Kappa statistics was used to assess the intra-examiner reliability. All of the data were assessed for a normal distribution via the Shapiro–Wilk test for normality. One-way ANOVA and Mann-Whitney *U*-tests, as appropriate, were used to compare the means of the diversity indices and the richness and relative abundance of the salivary microbiome. Student's *t*-tests and Wilcoxon signed-rank tests, as appropriate, were used to compare the percentages of abundance of the same species between arrested caries and active caries at the same time point. Analyses were performed with the SPSS (version 23.0, SPSS, Chicago, IL, USA) software. The level of statistical significance was set at 0.05.

3. Results

Regarding intra-examiner reliability, the kappa value for the duplicate examinations of caries lesion activity was 0.9. Dental plaque samples from 14 children were collected before and after SDF treatment at three time points (T0, T1, and T2) (Table 1). Their clinical details are shown in Table 1. The children's mean decay teeth (DT) was 4.2. The clinical evaluation of lesion texture showed that 90% of the lesions were hardened after 2 weeks of SDF treatment and that 83% of the lesions were still hardened after 12 weeks (Table 1). Of 46 plaque samples (14 subjects at three time points; plaque from subjects 9, 10, 13, and 14 was collected from arrested caries and active caries at T1), 388 OTUs were identified (254 identified at the species level, 129 identified at the genus level, and 76 identified at family level).

One-way ANOVA was performed to determine whether significant differences existed in microbiome diversity between the three time points, namely pre-SDF treatment (T0), 2 weeks after SDF treatment (T1), and 12 weeks after SDF treatment (T2). The Shannon index was calculated to represent the community diversity. No significant diversity changes were

observed in arrested caries before or after SDF treatment ($p = 0.71$) at all time points. However, in active caries, significantly reduced diversity was observed after 2 weeks (T1) when compared to the original diversity (T0) ($p = 0.006$). A principal coordinate analysis (PCoA) plot based on unweighted UniFrac of microbiota from arrested caries showed a temporal shift in the positive direction after 2 weeks (T1), but returned to original after 12 weeks (T2) (Figure 1). A PCoA plot on the microbiota of active caries was not performed due to the limited sample size.

Figure 2 shows the relative abundances and clusters of OTUs at a genus level at different time points. Thirty predominant OTUs representing the bacterial genus deposited in the HOMD with a high (98.5%) identity were commonly detected. It showed that the OTUs from arrested caries (T1 and T2) are closer to the original profile (T0) than the ones from active caries (T1 and T2) are). Of all the OTUs, 20 interesting species were selected from Figure 2 based on the criteria that either the species is predominant or the level of the species notably changed between different time points or caries statuses.

The abundance of these species is shown in Figure 3. Different patterns were observed in arrested caries versus active caries. In arrested caries, the abundance of predominant species such as *Neisseia sp.*, *Veillonella sp*., and *Leptotrichia sp*. was relatively stable across the three time points, whereas the abundance of *Streptococcus mutans* was reduced after SDF treatment (T1 and T2) when compared to pre-SDF treatment (T0) (Figure 3A). In contrast, in active caries, the abundance of *Neisseia sp.* was reduced after SDF treatment, whereas the abundance of *Veillonella sp.* and *Rothia sp*. significantly increased after SDF treatment. Interestingly, neither *Streptococcus sobrinus* nor *Lactobacillus sp*. could be detected at T0, but they appeared

in active caries in nonnegligible abundance after SDF treatment (T1 and T2), especially after 2 weeks (T1) (Figure 3B). In addition, *Streptococcus mutans* was less abundant in arrested caries but more abundant in active caries after SDF treatment.

Furthermore, 10 species or species groups were selected for further analysis because they are either well known as caries-/health-related species or their abundance notably differed between arrested caries and active caries. The mean percentage of abundance of each of these species is shown in Figure 4. Overall, the difference in the species level after 2 weeks (T1) was pronounced between arrested caries and active caries. However, the species level tended to return to original levels after 12 weeks (T2) in both arrested and active caries. *Campylobacter sp.*, *Nesseria sp.*, *Lautropia mirabilis*, *Kingella oralis*, and *Actinomyces naeslundii* levels were higher in arrested caries than in active caries (Figures 4A-E). By contrast, *Veillonella sp*., *Lactobacillus sp*., *Rothia sp.*, *Streptococcus mutans*, and *Streptococcus sobrinus* levels in arrested caries were lower than those in active caries (Figures F-J). Statistically significant changes in species levels between arrested caries and active caries were observed for *Lactobacillus sp.* and *Rothia sp.* at T1 and T2 ($p < 0.05$) (Figure 4G&H) and *Streptococcus mutans* and *Streptococcus sobrinus* at T1 ($p < 0.05$) (Figures 4I&J).

4. Discussion

This study was the first study to investigate the microbiota changes in both arrested and active caries after SDF treatment in ECC subjects. According to the results, two hypotheses can be rejected: that (a) SDF affected the microbiome profile of caries and (b) the microbiome profile present in arrested caries was significantly different from that of active caries. To assess caries activity, a caries was recorded as active when it was soft on probing, which means when light

force is applied, it could easily penetrate the dentine. In contrast, a caries was recorded as arrested when it was hard on probing, which means the dentine could not be penetrated with light force. This method has been shown to be a simple and practical way to evaluate caries activity in clinical trials [5, 22].

Chu et al. reported successful caries arrest after three applications of SDF at 2-week intervals [23]. We aimed to observe the short-term (2 weeks) and intermediate (12 weeks) effects on microbiota changes. One limitation was that we did not collect long-term samples because the kindergarten III students graduated soon after the 3 months follow-up. Additionally, 16S rRNA gene amplicon sequencing, as an application of NGS, only provides information about relative taxa-specific gene abundances. However, relative gene abundances are commonly used to analyse the relationship between microbial community composition and environmental parameters [24].

Up to now, two studies have investigated the microbiota changes after SDF treatment. In a clinical trial, Milgrom et al. collected plaque samples from three children before and 14 days after 38% SDF application. RNA sequencing analysis identified that there are no consistent changes in relative abundance of caries-associated microbes or microbiota diversity [15]. Mitwalli et al. investigated the effects of 38% SDF on the microbiome profile present in plaque from root caries [16]. They found that the relative abundance of some acid-producing species tended to decline after SDF treatment, while the overall microbiota diversity did not significantly change. This is consistent with the findings on SDF-arrested caries in this study. We did not find significant changes in microbiota diversity on arrested caries. The microbiota of arrested caries showed a temporal shift in the positive direction after 2 weeks (T1) but returned to their original status after 12 weeks (T2) (Figure 1). *Streptococcus mutans* tended to

decline in arrested caries (Figure 4F). Some species showed an increasing tendency in arrested caries (e.g., *Neisseria sp.* and *Actinomyces naeslundii*).

An interesting finding of the current study was that the microbiota changed in active caries. First, the microbiota diversity was reduced after 2 weeks (p = 0.006). Second, the relative abundance of some acid-producing species was higher in active caries than that of pre-SDF treatment caries and was higher than that of arrested caries (trend, not all statistically significant). We found that *Veillonella sp*., *Lactobacillus sp*., *Rothia sp.*, *Streptococcus mutans*, and *Streptococcus sobrinus* levels tended to increase. *Streptococcus mutans* and *Streptococcus sobrinus* are the most well-known cariogenic species that are actively involved in the caries progress. Laboratory studies have shown that this species' strains can produce lactic acid, which causes dental caries. *Streptococcus sobrinus* is also a lactate-producing species, but its prevalence is distinctly low, and it always co-exists with *Streptococcus mutans* [14, 25]. In the current study, *Streptococcus sobrinus* was not found in either original caries lesions (T0) or arrested caries after SDF treatment. This is consistent with Becker et al. [25]. Interestingly, *Streptococcus sobrinus* had a nonnegligible abundance in active caries after SDF treatment (Figure 4J). A similar trend was observed in *Lactobacillus sp*., which have been implicated as secondary pathogens in deep carious lesions. Lactobacilli's ability to ferment a variety of carbohydrates and survive in a low-pH environment is the major hallmark of the caries paradigm [26]. SDF treatment increased the abundance of *Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus sp*. in active caries cases, which makes the rest of the treatment more challenging (Figures 4G, I, and J).

Veillonella sp. is among the most prevalent species detected in saliva and dental plaque in ECC [27]. *In vitro* studies have shown that the combination of *Veillonella* and *Streptococcus mutans* leads to more acid production and greater demineralization than *Streptococcus mutans* does

alone [28]. *Veillonella* metabolizes the lactic acid produced by other species to form propionic and acetic acids. The mixture of acetic and lactic acid has additive demineralizing effects [29]. Furthermore, *Veillonella spp*. protects acid-producing bacteria in caries through nitrate reduction. A low nitrite concentration (0.2 mM) can cause the complete elimination of *Streptococcus mutans* in acidic environments [30]. High levels of *Rothia sp.* have been isolated from ECC saliva samples [31], but only a few authors have studied this species, and its mechanism is still unclear. Increases in the acid-producing species may induce an acidic microenvironment that suppresses the growth of or even kills certain acidophobic bacteria, and therefore reduces the biodiversity of the microbiota. However, the mechanisms of the active caries after SDF treatment still need further in-depth investigation.

In addition, all of the SDF failure cases were found in the posterior teeth with cavitated lesions, where food is easily trapped and plaque accumulates. In an 18-month clinical SDF trial, Duangthip et al. reported that the lesions in anterior teeth had a 4.21-times higher chance of becoming arrested compared to those in posterior teeth [18]. In a clinical situation, effective plaque control and oral hygiene maintenance are the key to successful SDF treatment [5].

5. Conclusion

No overall microbiome profile changes were observed in the SDF-arrested caries within 14 subjects investigated. The relative abundance of some caries-related species was reduced in arrested caries, but they increased in active caries after SDF treatment. The mechanisms of failure cases after SDF treatment are worth studying in the future.

Acknowledgements

This study was supported by National Natural Science Foundation of China (NSFC) - General Program (2018) 81870812 and General Research Fund (GRF) 17100218 of the Research Grant Council, Hong Kong.

Author Contributions

Mei ML: Conceptualization, Methodology, Formal analysis, Writing - Original Draft. Yan Z and Duangthip D: Methodology, Formal analysis, Writing - Review & Editing. Niu JY: Methodology, Writing - Review & Editing. You M: Formal analysis, Writing - Review & Editing. Yu OY and Lo ECM: Writing - Review & Editing. Chu CH: Conceptualization, Writing - Review & Editing. All authors gave final approval and agree to be accountable for all aspects of the work.

References

[1] J. Xiao, N. Alkhers, D.T. Kopycka-Kedzierawski, R.J. Billings, T.T. Wu, D.A. Castillo, L. Rasubala, H. Malmstrom, Y. Ren, E. Eliav, Prenatal Oral Health Care and Early Childhood Caries Prevention: A Systematic Review and Meta-Analysis, Caries Res 53(4) (2019) 411-421.

[2] C.H. Chu, E.C. Lo, Promoting caries arrest in children with silver diamine fluoride: a review, Oral Health Prev Dent 6(4) (2008) 315-21.

[3] P.S. Casamassimo, S. Thikkurissy, B.L. Edelstein, E. Maiorini, Beyond the dmft: the human and economic cost of early childhood caries, J Am Dent Assoc 140(6) (2009) 650-7.

[4] D. Duangthip, K.J. Chen, S.S. Gao, E.C.M. Lo, C.H. Chu, Managing Early Childhood Caries with Atraumatic Restorative Treatment and Topical Silver and Fluoride Agents, Int J Environ Res Public Health 14(10) (2017).

[5] M.H.T. Fung, D. Duangthip, M.C.M. Wong, E.C.M. Lo, C.H. Chu, Arresting Dentine Caries with Different Concentration and Periodicity of Silver Diamine Fluoride, JDR Clin Trans Res 1(2) (2016) 143-152.

[6] S.S. Gao, I.S. Zhao, N. Hiraishi, D. Duangthip, M.L. Mei, E.C.M. Lo, C.H. Chu, Clinical Trials of Silver Diamine Fluoride in Arresting Caries among Children: A Systematic Review, JDR Clin Trans Res 1(3) (2016) 201-210.

[7] M.L. Mei, L. Ito, Y. Cao, E.C. Lo, Q.L. Li, C.H. Chu, An ex vivo study of arrested primary teeth caries with silver diamine fluoride therapy, J Dent 42(4) (2014) 395-402.

[8] M.L. Mei, F. Nudelman, B. Marzec, J.M. Walker, E.C.M. Lo, A.W. Walls, C.H. Chu, Formation of Fluorohydroxyapatite with Silver Diamine Fluoride, J Dent Res 96(10) (2017) 1122-1128.

[9] M.L. Mei, L. Ito, Y. Cao, Q.L. Li, C.H. Chu, E.C. Lo, The inhibitory effects of silver diamine fluorides on cysteine cathepsins, J Dent 42(3) (2014) 329-35.

[10] C.H. Chu, L. Mei, C.J. Seneviratne, E.C. Lo, Effects of silver diamine fluoride on dentine carious lesions induced by Streptococcus mutans and Actinomyces naeslundii biofilms, Int J Paediatr Dent 22(1) (2012) 2-10.

[11] M.L. Mei, C.H. Chu, K.H. Low, C.M. Che, E.C. Lo, Caries arresting effect of silver diamine fluoride on dentine carious lesion with S. mutans and L. acidophilus dual-species cariogenic biofilm, Med Oral Patol Oral Cir Bucal 18(6) (2013) e824-31.

[12] M.L. Mei, Q.L. Li, C.H. Chu, E.C. Lo, L.P. Samaranayake, Antibacterial effects of silver diamine fluoride on multi-species cariogenic biofilm on caries, Ann Clin Microbiol Antimicrob 12 (2013) 4.

[13] R.J. Palmer, Jr., Composition and development of oral bacterial communities, Periodontol 2000 64(1) (2014) 20-39.

[14] J.A. Aas, A.L. Griffen, S.R. Dardis, A.M. Lee, I. Olsen, F.E. Dewhirst, E.J. Leys, B.J. Paster, Bacteria of dental caries in primary and permanent teeth in children and young adults, J Clin Microbiol 46(4) (2008) 1407-17.

[15] P. Milgrom, J.A. Horst, S. Ludwig, M. Rothen, B.W. Chaffee, S. Lyalina, K.S. Pollard, J.L. DeRisi, L. Mancl, Topical silver diamine fluoride for dental caries arrest in preschool children: A randomized controlled trial and microbiological analysis of caries associated microbes and resistance gene expression, J Dent 68 (2018) 72-78.

[16] H. Mitwalli, M.D.A. Mourao, J. Dennison, P. Yaman, B.J. Paster, M. Fontana, Effect of Silver Diamine Fluoride Treatment on Microbial Profiles of Plaque Biofilms from Root/Cervical Caries Lesions, Caries Res 53(5) (2019) 555-566.

[17] A. Escobar-Zepeda, A. Vera-Ponce de Leon, A. Sanchez-Flores, The Road to Metagenomics: From Microbiology to DNA Sequencing Technologies and Bioinformatics, Front Genet 6 (2015) 348.

[18] D. Duangthip, M.C.M. Wong, C.H. Chu, E.C.M. Lo, Caries arrest by topical fluorides in preschool children: 30-month results, J Dent 70 (2018) 74-79.

[19] Y Li, Y Ge, D Saxena, P.W. Caufield. Genetic profiling of the oral microbiota associated with severe early-childhood caries. J Clin Microbiol 45 (2007) 81-7.

[20] M.L. Mei, C.H. Chu, E.C. Lo, L.P. Samaranayake. Fluoride and silver concentrations of silver diammine fluoride solutions for dental use. Int J Paediatr Dent 23 (2013) 279-85.

[21] M.H.T. Fung, D. Duangthip, M.C.M. Wong, E.C.M. Lo, C.H. Chu, Randomized Clinical Trial of 12% and 38% Silver Diamine Fluoride Treatment, J Dent Res 97(2) (2018) 171-178.

[22] C.H. Chu, E.C. Lo, H.C. Lin, Effectiveness of silver diamine fluoride and sodium fluoride varnish in arresting dentin caries in Chinese pre-school children, J Dent Res 81(11) (2002) 767-70.

[23] C.H. Chu, A.H. Lee, L. Zheng, M.L. Mei, G.C. Chan, Arresting rampant dental caries with silver diamine fluoride in a young teenager suffering from chronic oral graft versus host disease post-bone marrow transplantation: a case report, BMC Res Notes 7 (2014) 3.

[24] F. Bonk, D. Popp, H. Harms, F. Centler, PCR-based quantification of taxa-specific abundances in microbial communities: Quantifying and avoiding common pitfalls, J Microbiol Methods 153 (2018) 139-147.

[25] M.R. Becker, B.J. Paster, E.J. Leys, M.L. Moeschberger, S.G. Kenyon, J.L. Galvin, S.K. Boches, F.E. Dewhirst, A.L. Griffen, Molecular analysis of bacterial species associated with childhood caries, J Clin Microbiol 40(3) (2002) 1001-9.

[26] P.W. Caufield, C.N. Schon, P. Saraithong, Y. Li, S. Argimon, Oral Lactobacilli and Dental Caries: A Model for Niche Adaptation in Humans, J Dent Res 94(9 Suppl) (2015) 110S-8S.

[27] E. Kanasi, F.E. Dewhirst, N.I. Chalmers, R. Kent, Jr., A. Moore, C.V. Hughes, N. Pradhan, C.Y. Loo, A.C. Tanner, Clonal analysis of the microbiota of severe early childhood caries, Caries Res 44(5) (2010) 485-97.

[28] W.D. Noorda, D.J. Purdell-Lewis, A.M. van Montfort, A.H. Weerkamp, Monobacterial and mixed bacterial plaques of Streptococcus mutans and Veillonella alcalescens in an artificial mouth: development, metabolism, and effect on human dental enamel, Caries Res 22(6) (1988) 342-7.

[29] J.D. Featherstone, B.E. Rodgers, Effect of acetic, lactic and other organic acids on the formation of artificial carious lesions, Caries Res 15(5) (1981) 377-85.

[30] L.S. Silva Mendez, R.P. Allaker, J.M. Hardie, N. Benjamin, Antimicrobial effect of acidified nitrite on cariogenic bacteria, Oral Microbiol Immunol 14(6) (1999) 391-2.

[31] S. Jiang, X. Gao, L. Jin, E.C. Lo, Salivary Microbiome Diversity in Caries-Free and Caries-Affected Children, Int J Mol Sci 17(12) (2016).

Table 1. Caries status of the 5-year-old children before, 2-week and 4-week after silver diamine fluoride (SDF) therapy

Child	Sex	Before SDF therapy		2 weeks after SDF therapy			12 weeks after SDF therapy		
		No of teeth with active caries	No of teeth with arrested caries	No of teeth with active caries	No of teeth with arrested caries	Caries arrested rate	No of teeth with active caries	No of teeth with arrested caries	Caries arrested rate
1	F	9	0	0	9	100%	0	9	100%
2	F	8	0	2	6	75%	3	5	63%
3	M	7	0	1	6	86%	2	5	71%
4	F	6	0	2	4	67%	2	4	67%
5	M	5	0	0	5	100%	0	5	100%
6	F	5	0	0	5	100%	0	5	100%
7	M	4	0	0	4	100%	0	4	100%
8	M	4	0	0	4	100%	0	4	100%
9	M	3	0	1	2	67%	3	0	0
10	M	2	0	0	2	100%	0	2	100%
11	M	2	0	0	2	100%	0	2	100%
12	M	2	0	0	2	100%	0	2	100%
13	F	1	0	0	1	100%	0	1	100%
14	M	1	0	0	1	100%	0	1	100%
Total		59	0	6	53	90%	10	49	83%

Figure 1. A principal coordinate analysis (PCoA) plot of plaque from arrested caries showing similar relations among 30 bacterial community samples (3 time points each from 10 subjects).

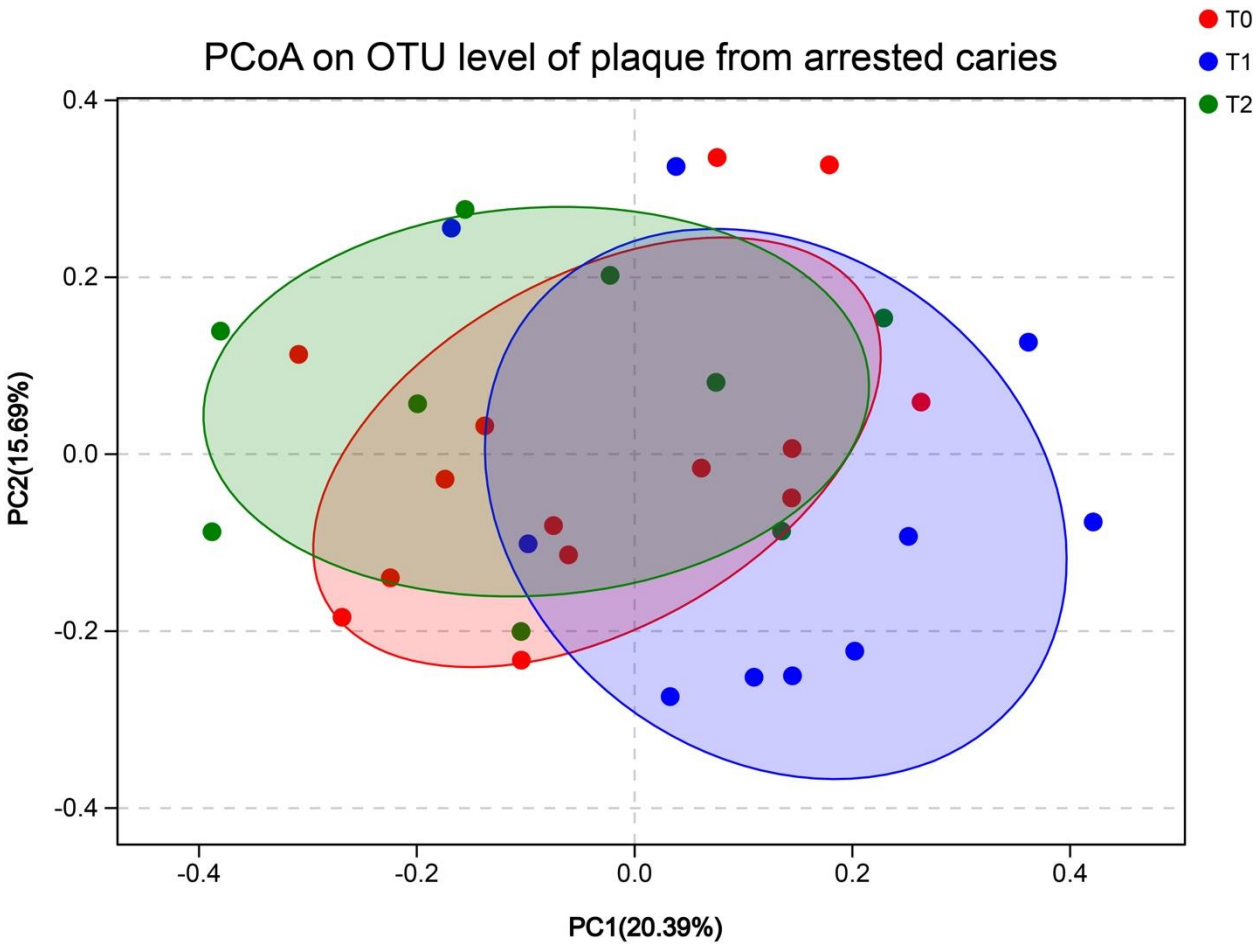


Figure 2. Shift and cluster in the relative abundance of genus-level OTUs at three time points in both arrested and active caries.

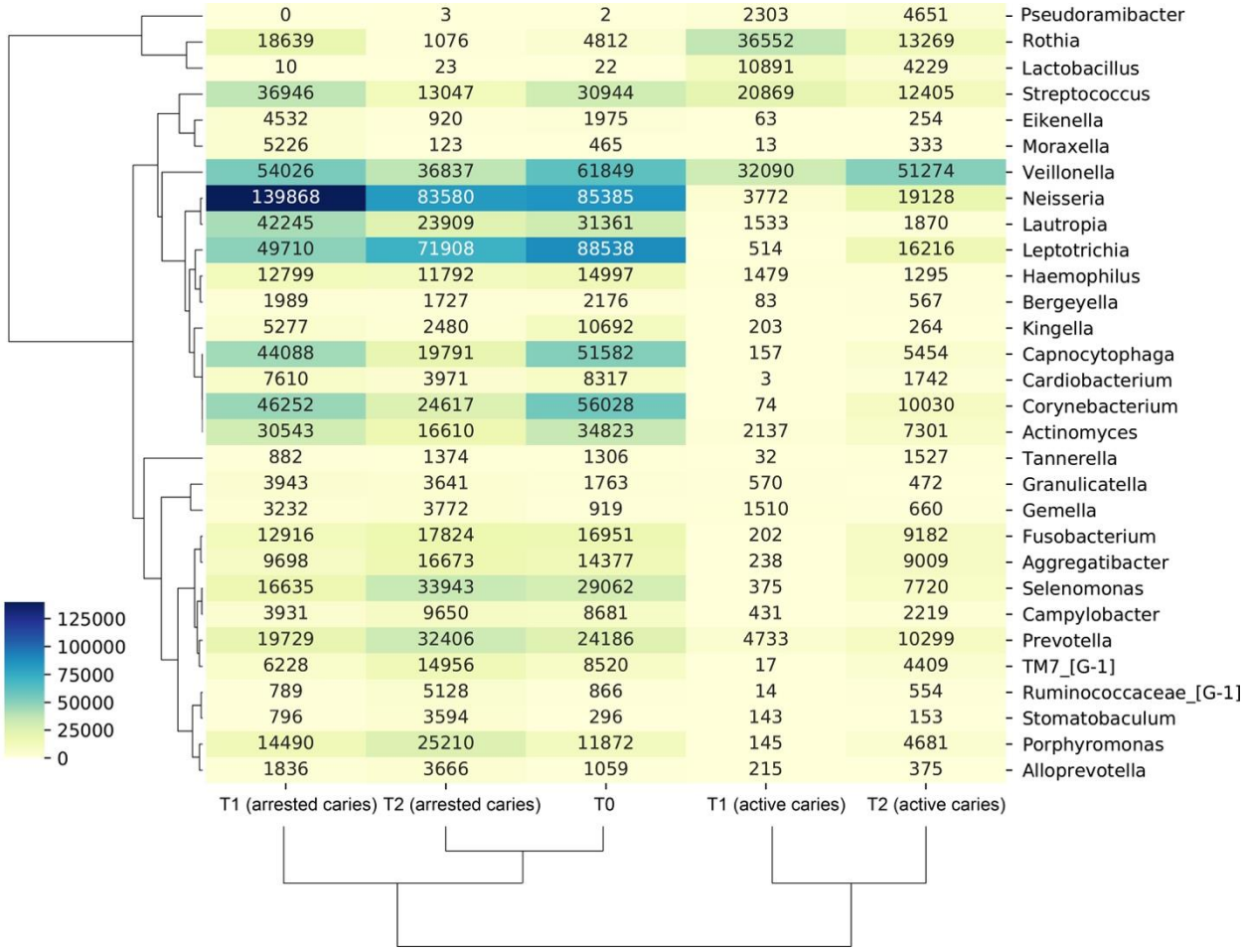
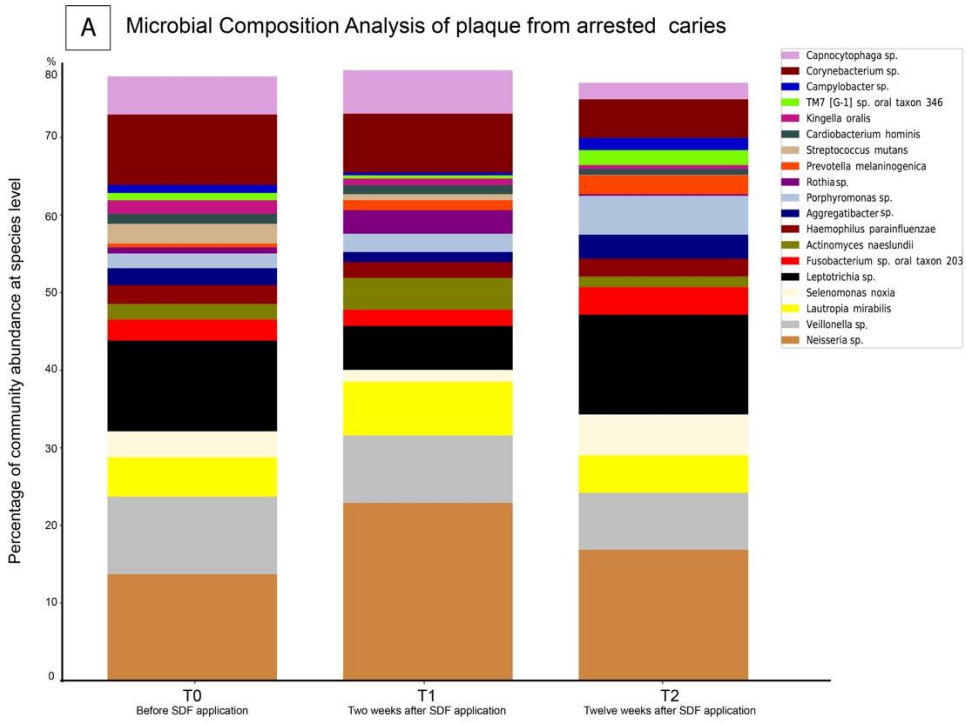


Figure 3. Percentage of community abundance level of predominate species. A. analysis of plaque from arrested caries; B. analysis plaque from active caries.

Capnocytophaga sp. includes Capnocytophaga granulosa and Capnocytophaga sputigena; Corynebacterium sp. Includes Corynebacterium matruchotii and Corynebacterium durum; Campylobacter sp. includes Campylobacter gracilis and Campylobacter concisus; Rothia sp. includes unidentified Rothia and Rothia mucilaginosa; Porphyromonas sp. includes Porphyromonas sp. oral taxon 278 and Porphyromonas sp. oral taxon 279; Aggregatibacter sp. includes unidentified Aggregatibacter and Aggregatibacter sp. oral taxon 898; Leptotrichia sp. includes Leptotrichia sp. oral taxon 225, Leptotrichia shahii, Leptotrichia wadei and Leptotrichia hongkongensis; Veillonella sp. includes Veillonella dispar and Veillonella atypica; Neisseria sp. includes unclassified Neisseria, Neisseria elongate and Neisseria bacilliformis; Lactobacillus sp. includes Lactobacillus salivarius, Lactobacillus reuteri genosp, Lactobacillus fermentum, Lactobacillus gasseri.



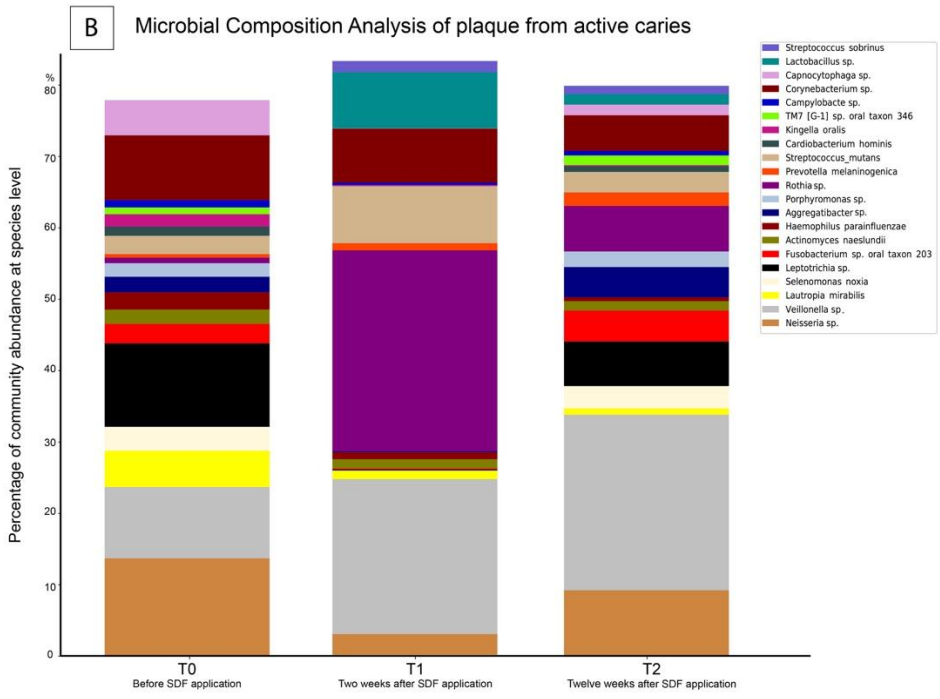


Figure 4. Mean levels of health-related (A-E) and caries-related (F-J) species or species groups in the plaque collected from arrested caries and active caries with time.
P indicate student t test between plaque from arrested caries and that from active caries in the same time point. *P<=0.05. Campylobacter sp. includes Campylobacter gracilis and Campylobacter concisus; Neisseria sp. includes unclassified Neisseria, Neisseria elongate and Neisseria bacilliformis; Veillonella sp. includes Veillonella dispar and Veillonella atypica; Lactobacillus sp. includes Lactobacillus salivarius, Lactobacillus reuteri genosp, Lactobacillus fermentum, Lactobacillus gasseri; Rothia sp. includes unidentified Rothia and Rothia mucilaginosa.