Effects of Silver Diamine Fluoride on Oral Bacteriome and Mycobiome

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ABSTRACT

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Purpose: To investigate changes to the oral bacteriome and mycobiome in young children treated with silver diamine fluoride (SDF) at three different frequency regimens.

Methods: Plaque samples were collected from 45 children with early childhood caries (ECC). Children were recruited into a randomized clinical trial testing three different treatment frequency regimens of SDF (15 from each SDF regimen with two applications 4 months apart (4M), two applications 6 months apart (6M), and two applications 1 month apart (1M)). DNA was extracted and sent to Genome Quebec Innovation Center for library preparation and paired-end Illumina MiSeq PE300 sequencing of the V4 region of bacterial 16S rRNA and fungal ITS1 rRNA genes. Sequencing data was analyzed using QIIME2 2018.11.

Results: A total of 195 carious lesions in 44 children were treated at baseline and followed over two subsequent study visits. The overall arrest rates were 77.69% at Visit 2 and 93.61% at Visit 3 respectively. Arrest rates were higher for all lesions after two applications of SDF. Average arrest rates were higher at Visit 2 and Visit 3 for Regimen 4M and Regimen 1M compared to Regimen 6M.

Bacteria: The alpha diversity analysis and beta diversity analysis showed no significant differences in the supragingival bacteriome between visits for all three regimens. Taxonomic assignment showed that *Streptococcus*, *Corynebacterium* and *Actinomyces* were the most abundant genera overall. At species level, *Streptococcus mutans* and *Veillonella dispar* both showed a decrease in relative abundance from Visit 1 to Visit 3 in all regimens, but these changes were not statistically significant. The differential abundance analysis showed that the supragingival plaque of the children at baseline (Visit 1) was enriched with cariogenic bacteria such as *Lactobacillus spp.* and/or *Bifidobacterium spp.* compared to Visits 2 and 3, regardless of the regimen used. Children in Regimen 6M showed higher abundances of *Lactobacillus salivarius* in Visits 2 and 3, and also showed higher dmft scores at Visits 2 and 3. They also showed lower arrest rates, compared to the other regimens.

Fungi: The alpha diversity analysis and beta diversity analysis showed no significant differences in the supragingival mycobiome between visits for all three regimens. Taxonomic assignment showed that *Candida, Blumeria*, and *Malassezia* were the most abundant genera. *Candida albicans* was highly abundant in all groups regardless of number of visits or regimens. Our study found a vast diversity of fungal species and differential abundance analysis suggested that SDF treatment may have an effect on the abundance of specific fungi.

Conclusion: SDF was an effective modality for arresting dental caries with higher arrest rates for all lesions after two applications of SDF. The overall arrest rate at Visit 2 was 77.69% and the overall arrest rate at Visit 3 was 93.61% respectively. There was no significant difference between all supragingival plaque samples with both alpha diversity analysis and beta diversity analysis. Taxonomic assignment showed that *Streptococcus*, *Corynebacterium* and *Actinomyces* were the most abundant genera overall for bacteria while *Candida, Blumeria*, and *Malassezia* were the most abundant genera overall for fungi. The differential abundance analysis showed significant changes in both bacterial and fungal species, particularly *Lactobacillus spp.*, *Bifidobacterium spp. and Candida spp.* Further studies with a larger sample size are needed to confirm whether the presence or absence of various bacterial and fungal species are the result of SDF application at various frequencies or simply the result of a disrupted microbiome.

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Dedication

For my mother, who carries the weight of all our burdens on her tiny shoulders.

And for my father who always supports us, tirelessly.

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1.0 INTRODUCTION

Silver diamine fluoride (SDF) is a simple and non-invasive agent to arrest caries in children. It is important to study which application regimen of SDF is most effective and appropriate in order to optimize implementation in dental public health programs, high-risk communities and in situations where comprehensive dental treatment under General Anesthesia is delayed, unavailable or unaffordable. It is also important to understand any potential changes to the oral microbiome in young children and whether the frequency of application of SDF is related to changes to the oral microbiome.

2.0 LITERATURE REVIEW

Dental caries is a multifactorial disease caused by a combination of factors including oral colonization with increased levels of cariogenic bacteria, decreased microbial diversity, and teeth that are susceptible due to enamel hypoplasia and/or frequent consumption of fermentable carbohydrates (1-3). Various social, economic, and behavioural factors may also contribute to the dental caries process (4, 5).

> "Early Childhood Caries (ECC) is defined as the presence of one or more decayed, missing, or filled tooth surfaces in any primary tooth in a child under the age of six (1)"

> "Severe Early Childhood Caries (S-ECC) is any sign of smooth-surface caries in a child younger than three years of age, and from ages three through five, one or more cavitated, missing, or filled smooth surfaces in primary maxillary anterior teeth or a decayed, missing, or filled score of greater than or equal to four (age 3), greater than or equal to five (age 4), or greater than or equal to six (age 5)(1)."

The oral microbiome includes more than 700 bacterial species and 100 fungal species (6, 7). Some bacterial species commonly associated with dental caries include *Streptococcus mutans, Streptococcus sobrinus, Streptococcus salivarius, Streptococcus parasanguinis* and *Veillonella parvula* (3). *Candida albicans* is an opportunistic fungal organism frequently found in the oral cavity of children with ECC and S-ECC (8). In vitro studies have shown that interactions involving *Candida albicans* and *Streptococus mutans* were synergistic and associated with dysbiosis of oral microbiome (8, 9), suggesting that mycobiome analysis is important in the study of dental caries. Both *C. albicans* and *S. mutans* develop symbiotic interactions in the presence of sucrose that boost their ability to colonize teeth and synergistically enhance virulence which results in aggressive onset of dental caries similar to ECC lesions (9). Xiao et al (2018) found that *C. albicans* appeared to be associated with highly acidogenic and aciduric species including *Streptococcus*, and certain species of *Lactobacillus, Scardovia*, *Veillonella* and *Prevotella* in children with S-ECC (8).

Cariogenic bacteria are acidogenic and aciduric and result in the demineralization of enamel (2). Sustained demineralization causes cavitation of enamel and dentin which can progress and result in pain, dental abscess, severe infection and loss of teeth if left untreated (10-13). Other consequences include loss of school days, diminished ability to learn, diminished oral health-related quality of life, high treatment costs, higher risk of developing new carious lesions on both primary and permanent teeth and hospitalizations or emergency room visits (1, 12).

Dental caries is the most prevalent childhood disease worldwide (14, 15). Epidemiological data from multiple studies in the US indicate that low-income and minority children are affected the most due to health inequalities and lack of access to oral health services (1, 16-19). Within Canada, day surgery for ECC constitutes 31% of all day surgeries for children aged 1-5 years, making this the major cause for day surgeries within this age group (20, 21). The overall rate for dental surgery to treat ECC was 12.1 per 1000 children aged 1-5 years (20, 21). When rates of dental surgery were compared for neighborhoods with high vs low proportions of Indigenous children, Schroth et al (2016) found that neighbourhoods with a high proportion of Indigenous children had rates that were 7.8 times higher (84.5 vs. 10.9 per 1000) (21). Children from less affluent areas in Canada had rates of dental surgery that were 3.7 times higher than those of children from more affluent areas (25.7 vs. 6.9 per 1000)(21). Within Manitoba, rates

for dental surgery were 26.9 per 1000 children aged 1-5 years (20, 21). In Northern Manitoba, rates for dental surgery were even higher i.e. 116 per 1000 children aged 1-5 years (20, 21).

Treatment of children aged 1-5 often requires high-cost general anesthesia and advanced clinical skills which may be unavailable or unaffordable for many pediatric populations (12). Oral rehabilitation may involve multiple extractions, restorations and crowns (22). Children that are treated under general anesthesia often develop new or recurrent caries within months of surgical rehabilitation with some children requiring subsequent visits to the operating room for dental treatment under general anesthesia (23, 24). Traditional treatment modalities do not address causative factors and have not reduced the incidence of childhood caries and rates for dental surgery in North America (21). Improved public health policies and guidelines for prevention and treatment may help to reduce these numbers. An atraumatic approach may slow the progression of caries allowing the tooth with arrested decay to exfoliate before causing pain or infection (25). Application of SDF in children too young to tolerate restorative procedures can also stabilize active caries until the child is older and able to cooperate for traditional restorative techniques or for minimally invasive techniques (26). In addition to Silver Diamine Fluoride (SDF), minimally invasive techniques also include Atraumatic/Alternative Restorative Technique (ART), Interim Therapeutic Restoration (ITR) and Hall technique (27). Since treatment with SDF is non-invasive and easily performed (13), it can be a useful addition to existing traditional treatment modalities. Primary teeth that have been treated with SDF and atraumatic techniques can sustain function and act as a space maintainer until eruption of the permanent successor (13).

SDF is a topical fluoride solution containing silver and fluoride ions, originally developed by Reiichi Yamaga at Osaka University in the 1960s and used in community dental clinics for managing dental caries by arresting decay through the 1970s and 1980s in Japan (28, 29). A solution of 38% SDF (Advantage Arrest) contains 25% Silver, 5.5% Fluoride, and 8% Ammonia. There are currently no in vivo studies explaining the mechanism of action of SDF. In-vitro studies suggest that SDF has anti-bacterial effects on mono-cariogenic and multi-cariogenic species biofilms while also reducing the demineralization of dentin (30-32). The combination of silver and fluoride in an alkaline solution has a synergistic effect on the arrest of caries and this alkaline nature of SDF also provides an unfavorable environment for collagen enzyme activation (33). The

silver portion of SDF inhibits bacterial growth by interacting with bacterial cell membranes and enzymes (33). Silver also inhibits cathepsins and dentin collagen degradation (33). The fluoride portion of SDF forms fluorohydroxyapatite which has reduced solubility and increases microhardness by increasing the levels of calcium and phosphorus on the surface layer of the arrested dentin carious lesion (33). Fluoride also inhibits matrix metalloproteinases (MMPs) and dentin collagen degradation (33).

SDF has been used in Argentina, Australia, Brazil, China, Japan and the United States to arrest decay (34). In Canada, Advantage Arrest™ received approval for clinical use of SDF (38%) in 2017. Various studies have highlighted the effects of SDF in arresting caries (35-39). Gao et al (2016) found that overall arrest rates with 38% SDF are approximately 81% in primary teeth with variation dependent on tooth location, size of cavity and presence of plaque (13).

Data from Fung et al (2018) shows the following caries arrest rates by type of tooth when using SDF 38% semi-annually (40):

Overall arrest at 30 months all teeth:	75.0%
Lower anterior teeth:	91.7%
Upper anterior teeth:	85.6%
Lower posterior teeth:	62.4%
Upper posterior teeth:	57%

In the pilot study that preceded our current study, Sihra et al. (2020), concluded that at least two applications of SDF are recommended, with lesion arrest rates of 74.1% and 96.2% after 1 and 2 applications of SDF, respectively (41). Contraindications for the use of SDF are allergy to silver compounds (38). Relative contraindications include desquamative gingivitis and mucositis due to risk of increased absorption and pain from disruption of the stratified squamous epithelium (38). Minor side effects include transient metallic taste and transient gingival irritation (38, 42). SDF should not be placed on exposed pulps or used in teeth with evidence of pulpitis and evidence of pulpal necrosis, as these teeth require surgical intervention (43, 44). One major limitation in the use of SDF is the colour change that occurs with successful arrest of

caries. Since arrested carious lesions stain black, the final appearance may not be acceptable to many children and their parents (13). Informed consent with pre-treatment discussion of expected outcomes is essential for patient and parent satisfaction (13).

There is currently no consensus on the frequency of application of SDF for optimal arrest rates. In vitro studies have shown the anti-bacterial effects of SDF (30-33). Initial clinical trials suggest that SDF may have effects on the oral microbiome (bacterial and fungal). Fakhruddin et al (2020) found that SDF has an antifungal effect on oral Candida species from dentinal caries lesions from S-ECC (45). Milgrom et al (2018) studied the effect of SDF on the oral microbiome through RNA analysis of plaque in six children before and after treatment (3 receiving SDF and 3 placebo) and found no consistent changes in the relative abundance of cariogenic bacteria (39). However, the sample size for this study was very small.

The purpose of this study is to investigate the oral bacteriome and mycobiome changes that result from SDF applications randomized to three different application frequency regimens. Specifically, this study aims to determine potential changes to the oral bacteriome and mycobiome in young children, and to determine whether the frequency of applications has any impact on the oral microbiome in young children. This study is part of a larger randomized controlled trial to investigate the effectiveness of using SDF to arrest ECC and S-ECC in children.

3.0 OBJECTIVES AND HYPOTHESIS

Objective #1: To determine potential changes to the oral bacteriome and mycobiome in young children treated with SDF at three different frequency regimens.

Hypothesis: There is no change in the oral bacteriome and mycobiome in young children treated with SDF at three different frequency regimens.

Objective #2: To determine whether the frequency of application of SDF has any impact on the oral microbiome in young children.

Hypothesis: There is no relationship between frequency of application of SDF and the oral microbiome in young children.

4.0 METHODOLOGY

4.1 Study Population, Design, and Sample Collection

The study protocol was approved by the University of Manitoba Biomedical Research Ethics Board (HS24849-B2021:031). Study visits took place at the Children's Hospital Research Institute of Manitoba (**CHRIM**) or at community-based dental clinics in Winnipeg (Access Downtown, Mount Carmel Clinic and SMILE plus). A total of forty-five children < 72months of age were recruited between December 2019 and June 2020 with block randomization by site in order to achieve equal proportions in each regimen by clinic site of recruitment. Children were not randomized based on sex and only the first 15 children in each regimen were recruited for this study from the larger randomized controlled trial.

Table 1: Inclusion and Exclusion Criteria.

Inclusion Criteria	Exclusion Criteria
1) Child is < 72 months of age with ECC with active carious lesions **2)** Child has ≥ 1 primary tooth with caries that is eligible to receive SDF **3)** Eligible primary teeth must have soft cavitated carious lesions extending into dentin **4)** The cavitated lesions must allow for direct application of SDF	**1)** Child is allergic or has sensitivity to silver **2)** Child has hereditary generalized developmental defects of enamel (e.g., Amelogenesis Imperfecta, Dentinogenesis Imperfecta, etc) **3)** Child has severe medical problems that limit participation **4)** Child requires immediate rehabilitation under GA because of severe infection or pain **5)** Antibiotic use within the last 2 weeks **6)** Any teeth that meet PUFA criteria (pulpal involvement, ulceration due to trauma, fistula, abscess). The child may still qualify if other eligible teeth with caries do not meet PUFA criteria

All children had ECC or S-ECC and were randomized to three different treatment regimens (Table 2). Each treatment protocol was assigned a regimen number (1, 2 and 3) for randomization purposes. Regimen 1 was the Winnipeg Regional Health Authority (**WRHA**) Oral Health Program Protocol, advising two applications of SDF four months apart. Our pilot study by Sihra et al (2020), used the WRHA protocol so this regimen was chosen as Regimen 1 (41). Regimen 2 was the protocol suggested by the American Dental Association (**ADA**), recommending two applications of SDF six months apart (46). Regimen 3 was the protocol proposed in the American Academy of Pediatric Dentistry (**AAPD**) Clinical Practice Guideline suggested by Horst, recommending an initial application followed by 2-4 weeks of monitoring, with reapplication if needed (37, 44, 47). We implemented this as two applications of SDF one month apart. For simplicity, Regimen 1 (4 months), Regimen 2 (6 months) and Regimen 3 (1 month) will henceforth be referred to as Regimen 4M, Regimen 6M and Regimen 1M respectively.

Written informed consent, by parents or caregivers, was obtained for each child. A short survey was then completed via interview discussing general and dental health, oral hygiene, dietary intake of sugars, and family demographics along with the Early Childhood Oral Health Impact Scale (ECOHIS) to assess Oral Health-Related Quality of Life (OHRQL). Dental exams and assessment of lesions was completed by Dr. R.J. Schroth. Supragingival plaque sample collection and SDF application was completed by Dr. R.J. Schroth and pediatric dentistry residents under the supervision of Dr. R.J. Schroth. Carious lesions were assessed based on clinical hardness, colour change, and size of lesions. The colour (yellow, brown, black), hardness (very soft, medium, very hard) and the dmft score were recorded for each visit. Hardness was assessed with light pressure within the lesion using a probe. Teeth were also assessed for pain and/or infection at each visit. Lesions were deemed arrested if found to be black and very hard. Supragingival plaque samples were taken by scrubbing a sterile interdental brush on all available tooth surfaces (48, 49). Samples were then dislodged into 1 mL of RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) and then frozen at -80°C until DNA extraction. Dental plaque samples were taken at first (baseline), second, and third (final) study visits. Silver Diamine Fluoride 38% (Advantage Arrest) was applied to cavitated lesions involving dentin followed by 5% sodium fluoride varnish (NaF).

Table 2: Study Stages.

Recruitment & Randomization	• Recruitment • Informed consent • Randomization into one of three SDF treatment regimens using random envelopes		
	Regimen 1 (Regimen 4M) WRHA Oral Health Program protocol 2 applications **4 months apart**	**Regimen 2 (Regimen 6M) Suggested by ADA** 2 applications **6 months apart**	**Regimen 3 (Regimen 1M) Suggested by AAPD** 2 applications **1 month apart**
First Study Visit (V1)	• Baseline questionnaire, including ECOHIS • Dental assessment • Plaque swab sample • First SDF application	• Baseline questionnaire, including ECOHIS • Dental assessment • Plaque swab sample • First SDF application	• Baseline questionnaire, including ECOHIS • Dental assessment • Plaque swab sample • First SDF application
Second Study Visit (V2)	4 months after First Study Visit • Follow-up visit questionnaire, including ECOHIS • Dental assessment • Plaque swab sample • Second SDF application	6 months after First Study Visit • Follow-up visit questionnaire, including ECOHIS • Dental assessment • Plaque swab sample • Second SDF application	1 month after First Study Visit • Follow-up visit questionnaire, including ECOHIS • Dental assessment • Plaque swab sample • Second SDF application
Third Study Visit (V3)	4 months after Second Study Visit • Follow-up visit questionnaire, including ECOHIS • Dental assessment • Plaque swab sample	6 months after Second Study Visit • Follow-up visit questionnaire, including ECOHIS • Dental assessment • Plaque swab sample	1 months after Second Study Visit • Follow-up visit questionnaire, including ECOHIS • Dental assessment • Plaque swab sample

4.2 DNA Extraction, 16S and ITS1 rRNA Amplicon Sequencing and Data Analysis

DNA extraction was completed using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) with one modification. Since plaque samples were in liquid form, they were centrifuged for 10 minutes at 13,000 rpm. The supernatant was then discarded and 800 µL of buffer CD1 was used to re-suspend the pellet. This solution was then added to the PowerBead Pro Tube and manufacturer's instructions were followed for DNA extractions. DNA concentration was measured using NanoDrop OneC Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Extracted DNA was sent on dry ice to McGill University - Genome Quebec Innovation Center for library preparation and paired-end Illumina MiSeq PE300 sequencing of the V4 region of bacterial 16S rRNA and fungal ITS1 (internal transcribed spacer 1) rRNA genes. The forward and reverse primer sequences used to amplify the 16S and ITS1 rRNA genes, respectively, were (16S 515F) GTGCCAGCMGCCGCGGTAA, (16S 806R) GGACTACHVGGGTWTCTAAT, (ITS1 30F) GTCCCTGCCCTTTGTACACA, and (ITS1 217R) TTTCGCTGCGTTCTTCATCG.

4.3 Bioinformatics and Statistical Analysis

Sequencing data was analyzed using QIIME2 2018.11 (Quantitative Insights into Microbial Ecology) (49, 50). DADA2 implemented in QIIME2 was used to filter and merge the pair-end sequences, and to obtain the table of amplicon sequence variants (ASVs) (51). The ITS1 sequences were trimmed using the Q2-ITSxpress QIIME2 plugin before the DADA2 step (52). The Human Oral Microbiome Database (HOMD, version 15.1) and the UNITE database (version 8.2; QIIME developer release) were used for the taxonomic assignment of bacteria and fungi, respectively, with classify-sklearn naïve Bayes taxonomy classifier in QIIME2 (6, 48, 53, 54). The fungal ASVs that were assigned only at kingdom level were submitted to further fungal ASV curation with the R package LULU (55) and the program BLASTN in NCBI (56). The ASVs with non-fungal BLASTN results were discarded and the remaining were repeatedly assigned to new taxonomic assignments using different UNITE databases threshold levels (54, 57, 58) and taxonomy classification methods (q2-feature-classifier classify-sklearn and classify-

consensus-blast) in QIIME2 (49, 59). The data was imported into R using the R package "qiime2R" (version 0.99.13) and additional filtering was performed using "phyloseq" (version 1.30.0) to remove singletons (60, 61). The ASV counts were then normalized using the cumulative-sum scaling (CSS) approach from the R package "metagenomeSeq" version 1.28.2 (62).

The alpha diversity analysis was performed using the Shannon index and raw ASV count data in R ("phyloseq" package, version 1.30.0; "qiime2R" package, version 0.99.13). Alpha diversity comparisons were done by the Kruskal-Wallis test or Friedman test as appropriate. Beta diversity analysis was performed on CSS normalized ASV data, using weighted UniFrac distances and the permutational analysis of variance (PERMANOVA) test with 999 permutations in the R package "vegan" (adonis function; version 2.5.6) (63). It was visualized using principle coordinate analysis (PCoA) in the R package "ggplot2" (version 3.3.3) (64).

A paired DESeq2 negative binomial Wald test was used to detect differentially abundant species between groups, controlling the false discovery rate (FDR) for multiple comparison, within "phyloseq" (65). FDR adjusted $P < 0.05$ was considered significant.

5.0 RESULTS

5. 1 Characteristics of Study Participants

Forty-five children with mean age of 43.5 ± 13.8 months participated. Children had not been randomized based on sex and only the first 15 children in each regimen were recruited for this study from the larger randomized controlled trial. As a result, 17 (37.8%) were female and 28 (62.2%) were male. One child assigned to Regimen 6M was lost to follow up after baseline visit bringing final sample size to 132 plaque samples.

Table 3: Characteristics of study participants.

Age at Baseline (months)	
Mean ± SD	**43 ± 13.8**
[Range]	[21-71]
Sex, n (%)	
Female	**17 (37.8%)**
Regimen 1M	4
Regimen 4M	7
Regimen 6M	6
Male	**28 (62.2%)**
Regimen 1M	11
Regimen 4M	8
Regimen 6M	9

5. 2 Lesion-Level Analysis

A total of 195 carious lesions in 44 children were treated at baseline and followed over two subsequent study visits. One child presented with an abscessed SDF-treated tooth at Visit 3 in Regimen 1M and this lesion was considered a failure i.e., not arrested. Average arrest rates were higher at Visit 2 and Visit 3 for Regimen 4M and Regimen 1M compared to Regimen 6M (Table 4). Arrest rates were higher for all lesions after two applications of SDF. Average dmft rates remained the same between Visit 1, 2 and 3 for Regimen 4M and Regimen 1M while average dmft increased from 6.79 to 7.00 between Visit 1 and 2 and remained at 7.00 at Visit 3 for Regimen 6M.

Table 4: Average Arrest Rates.

	Average Arrest Rate Visit 2	**Average Arrest Rate Visit 3**
Regimen 1M	81.6%	96.7%
Regimen 4M	79.5%	98.3%
Regimen 6M	71.9%	85.8%
Total	**77.7%**	**93.6%**

Table 5: Average dmft.

	Average dmft Visit 1	**Average dmft Visit 2**	**Average dmft Visit 3**
Regimen 1M	6.67	6.67	6.67
Regimen 4M	6.40	6.40	6.40
Regimen 6M	6.79	7.00	7.00
Total	**6.62**	**6.69**	**6.69**

5. 3 Supragingival Plaque Bacterial Community Analysis

A total of 8,041,956 quality-filtered 16S rRNA sequences were used for 132 plaque samples, with a mean of 60,014.6 sequences per sample. A total of 3,915 amplicon sequence variants were assigned to 11 bacterial phyla, 93 genera, and 258 species.

The alpha diversity analysis using Shannon Index showed no significant difference between visits for all 132 samples (Friedman Test, $P = 0.98$). No significant differences were seen within Regimen 4M between First, Second and Third visits (Friedman Test, $P = 0.63$). For Regimen 6M, there was an increase in alpha diversity measure from Second to Third visit but this was not statistically significant (Friedman Test, $P = 0.22$). No significant differences were seen within Regimen 1M between First, Second and Third visits (Friedman Test, $P = 0.63$).

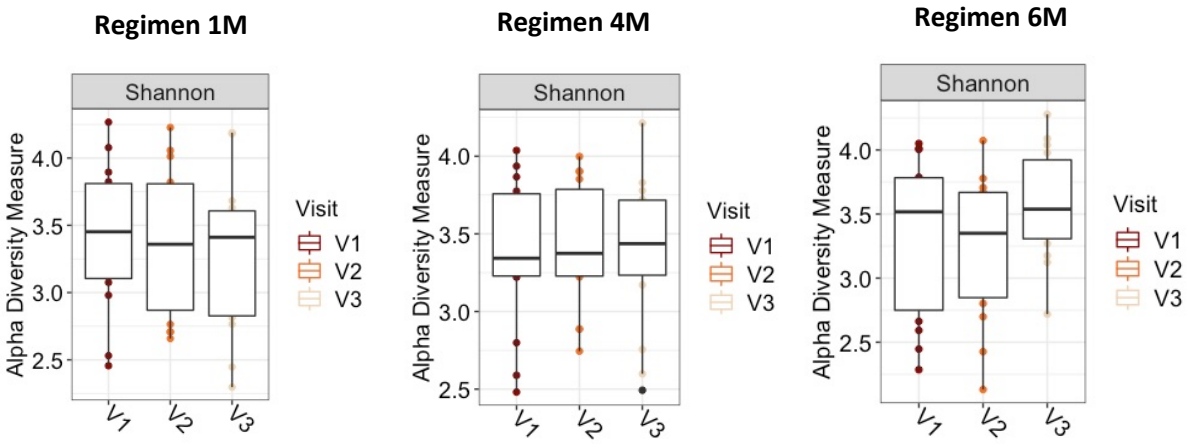


Figure 1. Alpha diversity analysis. Boxplot of Shannon Index for bacterial taxa in Regimen 1M, Regimen 4M and Regimen 6M and subgroup analysis by visit. The line inside the box represents the median. Whiskers represent the lowest and highest values within the 1.5 interquartile range.

Overall, the beta diversity analysis showed no significant differences in the supragingival plaque bacteriome between visits (PERMANOVA, pseudo-F = 0.62, R^2 = 0.01, P = 0.90). No significant differences were seen between First, Second and Third visits within Regimen 4M (PERMANOVA, pseudo-F = 0.82, R^2 = 0.04, *P* = 0.3), Regimen 6M (PERMANOVA, pseudo-F = 0.48, R^2 = 0.02, *P* = 0.71) and Regimen 1M (PERMANOVA, pseudo-F = 0.71, R^2 = 0.03, *P* = 0.5) (Figure 2).

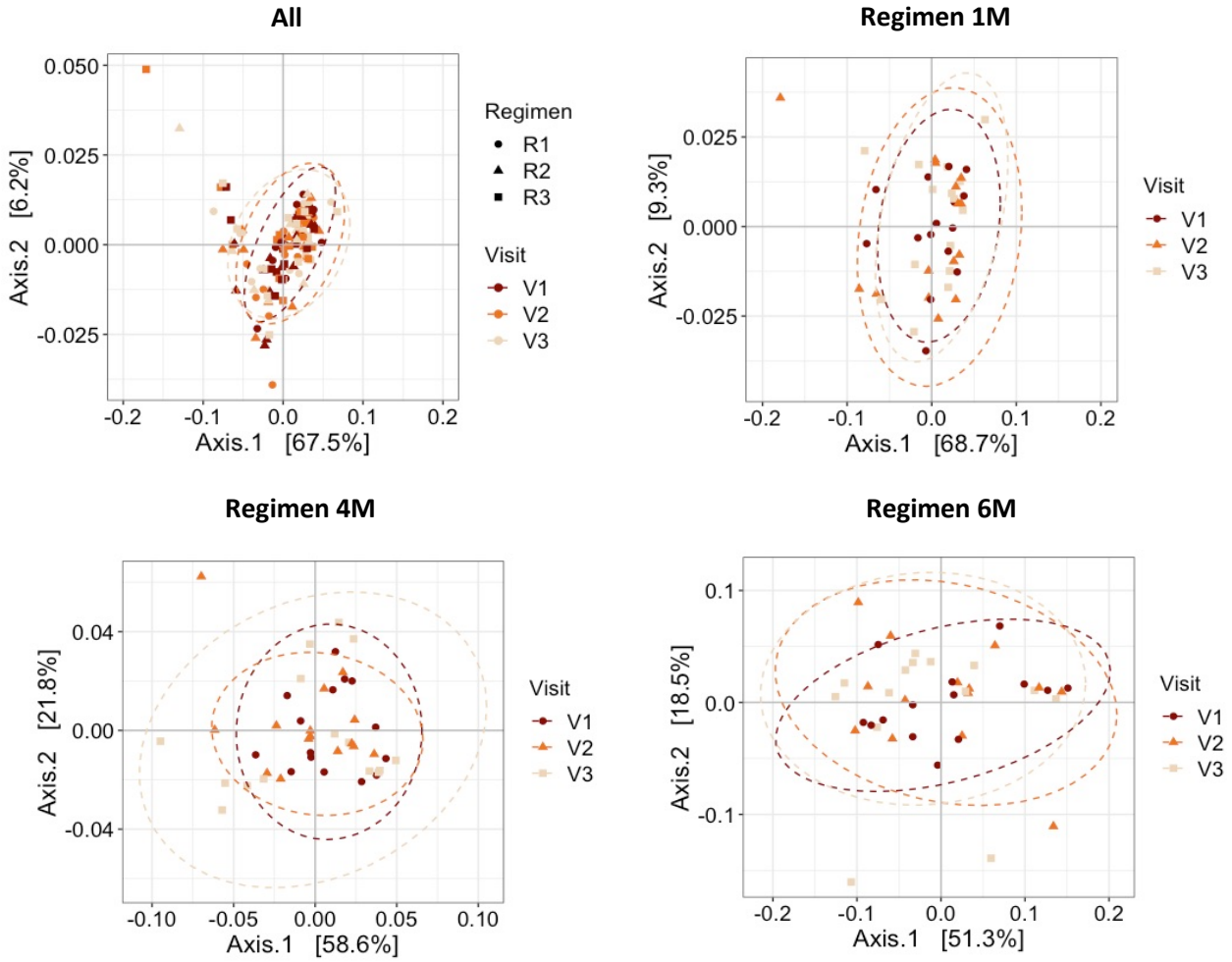


Figure 2. Beta diversity analysis. Principal coordinate analysis plots of weighted UniFrac distances based on the overall structure of the supragingival plaque bacteriome. Each data point represents a sample, coloured according to visit. The ellipses represent a 95% confidence level. No significant separation of samples was noted between regimens or visits.

Taxonomic assignment showed that *Streptococcus*, *Corynebacterium* and *Actinomyces* were the most abundant genera overall. Within Regimen 6M, there was a decrease in relative abundance of *Streptococcus* between Visit 2 and Visit 3 (Figure 3). At species level, *Streptococcus mutans* showed an increase in relative abundance from Visit 1 to Visit 2 and then a decrease in relative abundance from Visit 2 to Visit 3 within Regimen 6M (Figure 4). *Streptococcus mutans* also showed a decrease in relative abundance at Visit 3 compared to Visit 1 for Regimen 4M and Regimen 1M (Figure 4). These changes were not found to be statistically significant (Figure 5). Within Regimen 6M, *Veillonella dispar* showed decrease in relative abundance between Visit 1, 2 and 3 (Figure 4), however, this change was not found to be statistically significant (Figure 6). *Veillonella parvula* did not show much change between visits for all three regimens (Figure 4). *Streptococcus sobrinus* was not detected and though *Streptococcus salivarius* and *Streptococcus parasanguinis* were detected, they were not among the top 25 most abundant species.

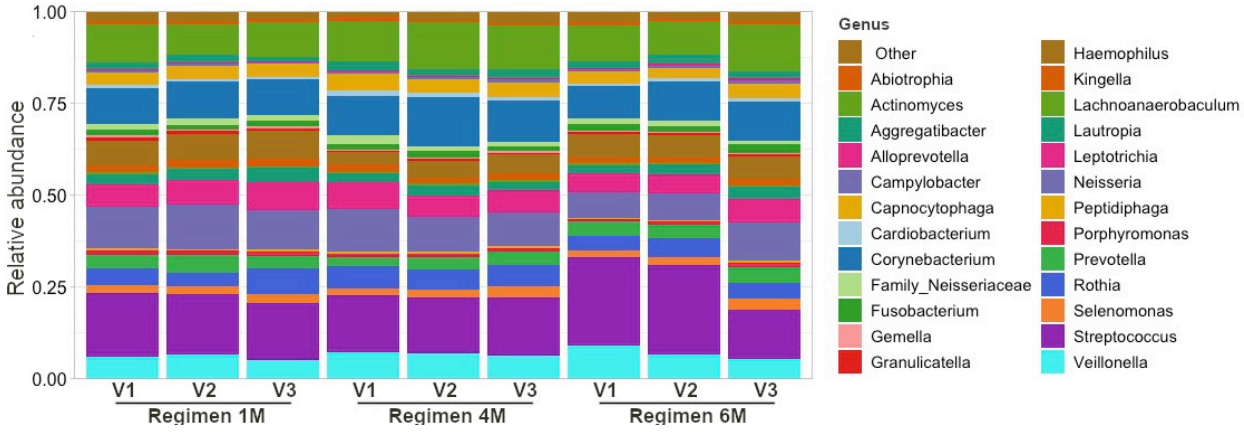


Figure 3. Taxonomic profiles of dental plaque according to regimen and visit at bacterial genus level. Colours were assigned only to the top 25 most abundant taxa.

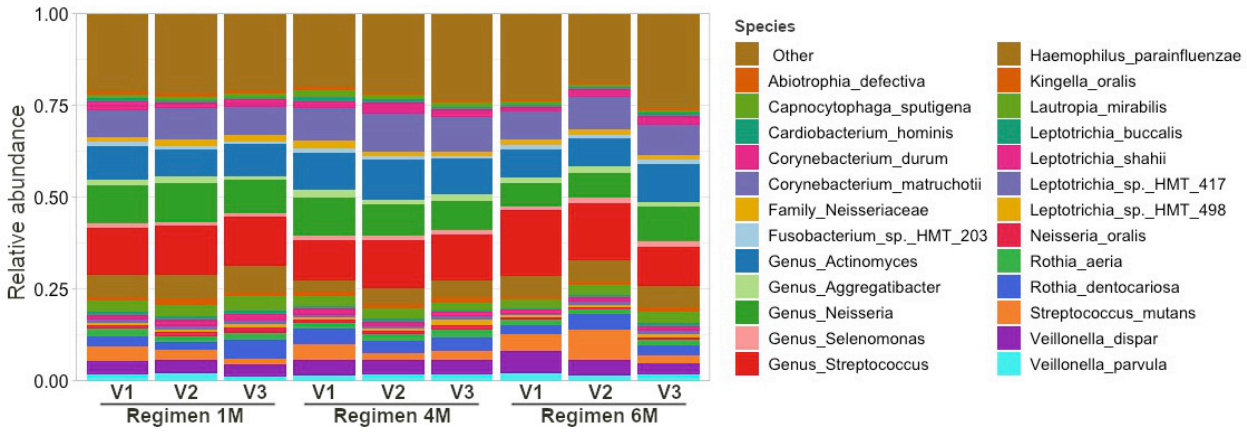


Figure 4. Taxonomic profiles of dental plaque according to regimen and visit at bacterial species level. Colours were assigned only to the top 25 most abundant taxa.

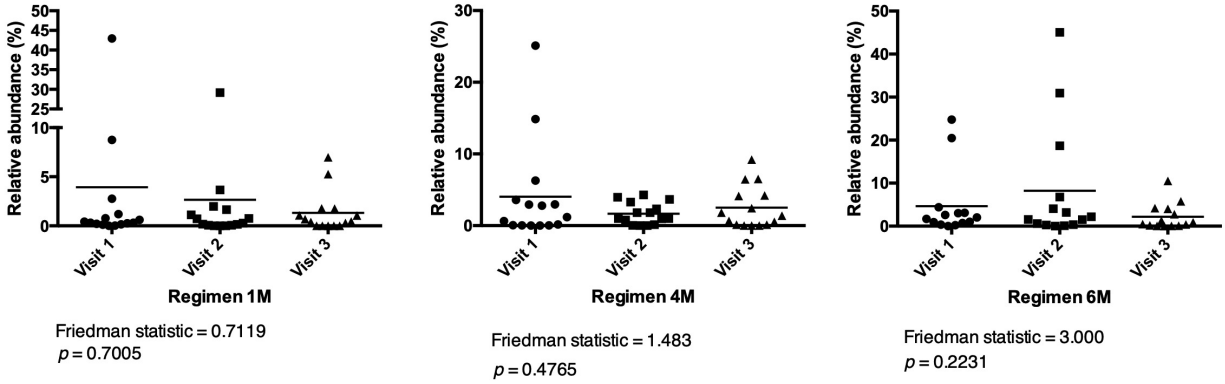


Figure 5. Relative abundance of *Streptococcus mutans* according to regimen and visit.

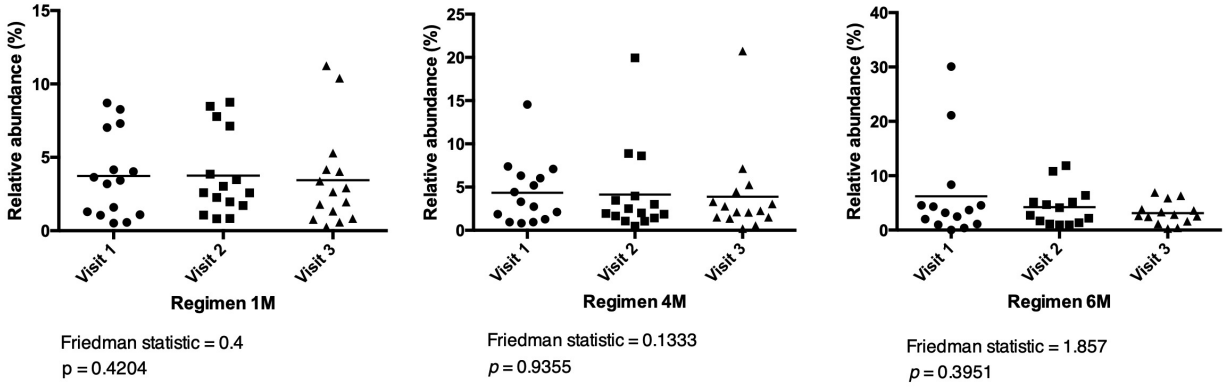


Figure 6. Relative abundance of *Veillonella dispar* according to regimen and visit.

The differential abundance analysis showed that various bacterial species were significantly enriched or depleted between visits 1 and 2 or between visits 1 and 3. Interestingly, compared to visits 2 and 3, the supragingival plaque of the children at baseline (Visit 1) was enriched with cariogenic bacteria such as *Lactobacillus spp.* and/or *Bifidobacterium spp.,* regardless of the regimens (Figures 7-10). However, children in Regimen 6M showed higher abundances of *Lactobacillus salivarius* in Visits 2 and 3 and also higher dmft scores at Visits 2 and 3 and lower arrest rates, compared to the other regimens (Figure 9).

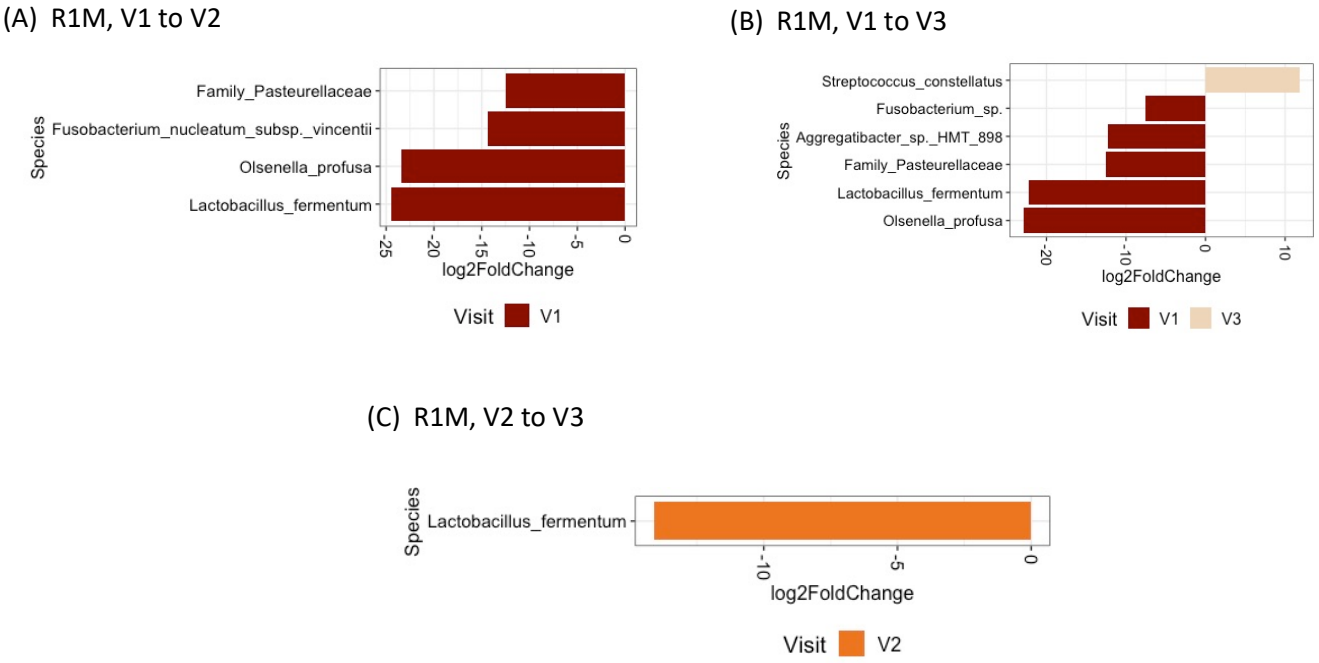


Figure 7. Differential abundance analysis for bacterial species in Regimen 1M. The figure shows the relative fold change in bacterial abundance between (A) visits 1 and 2, (B) visits 1 and 3 and (C) visits 2 and 3. Only showing bacterial taxa that had FDR adjusted $P < 0.05$ are shown.

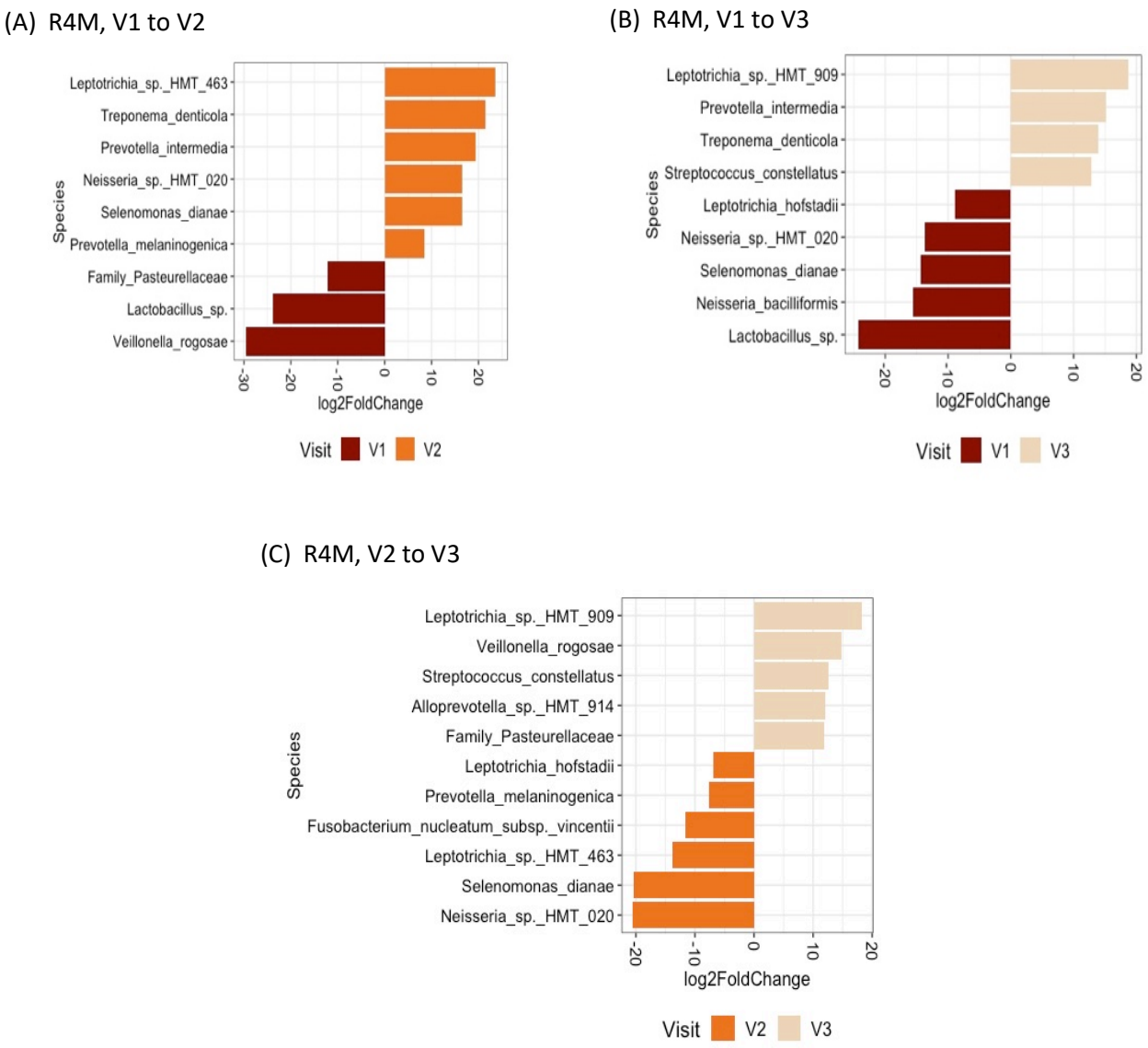
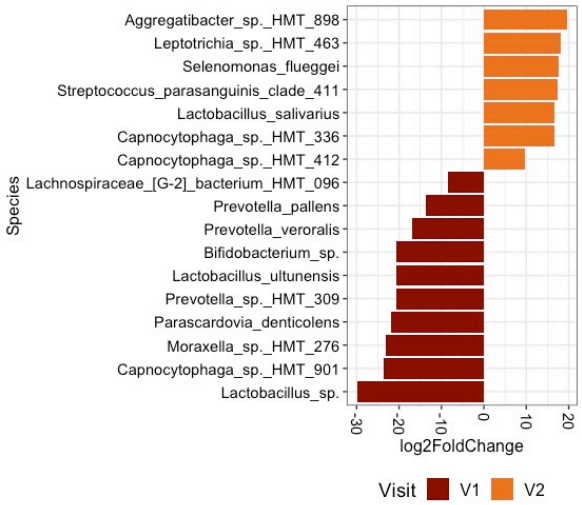
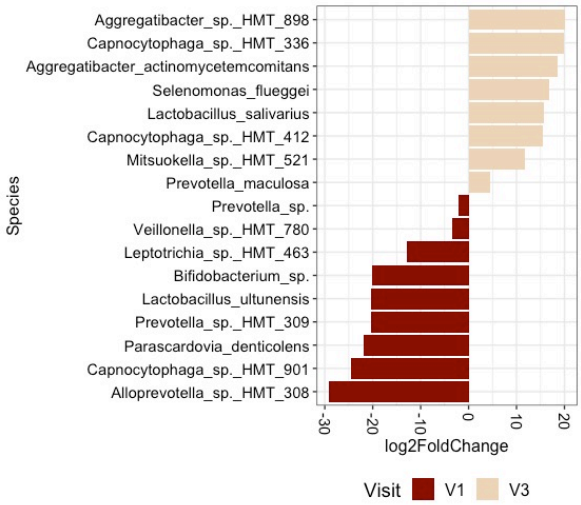


Figure 8. Differential abundance analysis for bacterial species in Regimen 4M. The figure shows the relative fold change in bacterial abundance between (A) visits 1 and 2, (B) visits 1 and 3 and (C) visits 2 and 3. Only bacterial taxa that had FDR adjusted $P < 0.05$ are shown.

(A) R6M, V1 to V2



(B) R6M, V1 to V3



(C) R6M, V2 to V3

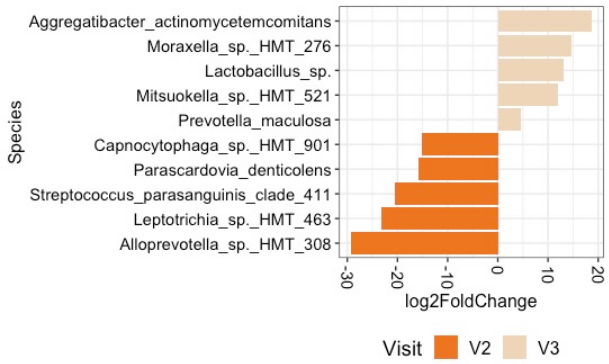
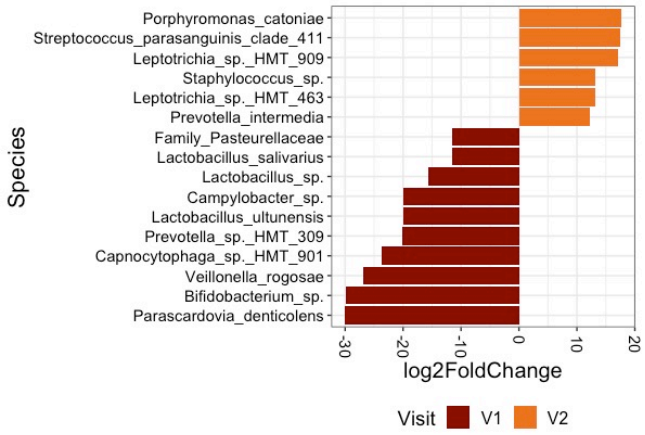
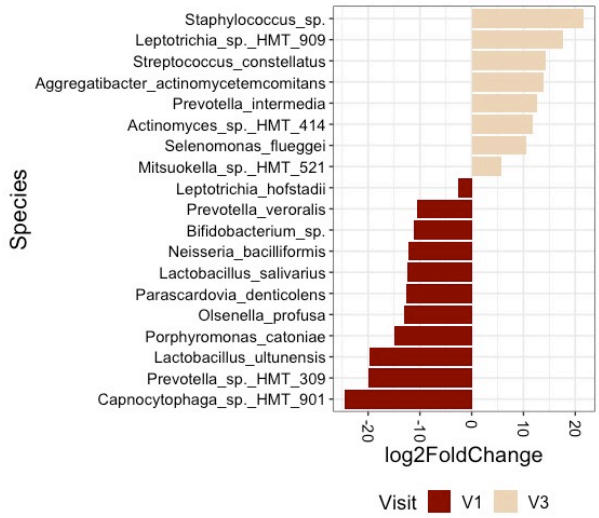


Figure 9. Differential abundance analysis for bacterial species in Regimen 6M. The figure shows the relative fold change in bacterial abundance between (A) visits 1 and 2, (B) visits 1 and 3 and (C) visits 2 and 3. Only bacterial taxa that had FDR adjusted $P < 0.05$ are shown.

(A) All Regimens, V1 to V2



(B) All Regimens, V1 to V3



(C) All Regimens, V2 to V3

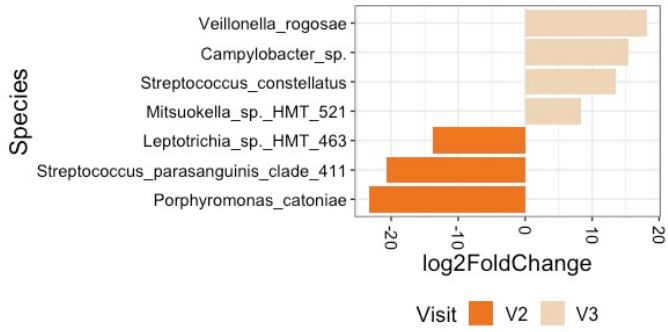


Figure 10. Differential abundance analysis for bacterial species in all regimens. The figure shows the relative fold change in bacterial abundance between (A) visits 1 and 2, (B) visits 1 and 3 and (C) visits 2 and 3. Only bacterial taxa that had FDR adjusted $P < 0.05$ are shown.

5.4 Supragingival Plaque Fungal Community Analysis

A total of 479,857 quality filtered ITS rRNA sequences reads (mean of 3,808 reads/sample) were obtained. Five samples had low quality of sequence reads and were removed. Due to the design of the study, a total of eighteen samples were also removed to allow for paired analysis of the remaining 114 plaque samples. A total of 200 amplicon sequence variants (ASVs) were assigned to 2 fungal phyla, 73 genera, and 82 species.

The alpha diversity analysis using Shannon Index showed no significant differences between visits for all 114 samples (Friedman Test, $P = 0.24$). No significant differences were seen between First, Second and Third visits within Regimen 4M (Friedman Test, $P = 0.19$), Regimen 6M (Friedman Test, $P = 0.53$), and Regimen 1M (Friedman Test, $P = 0.15$).

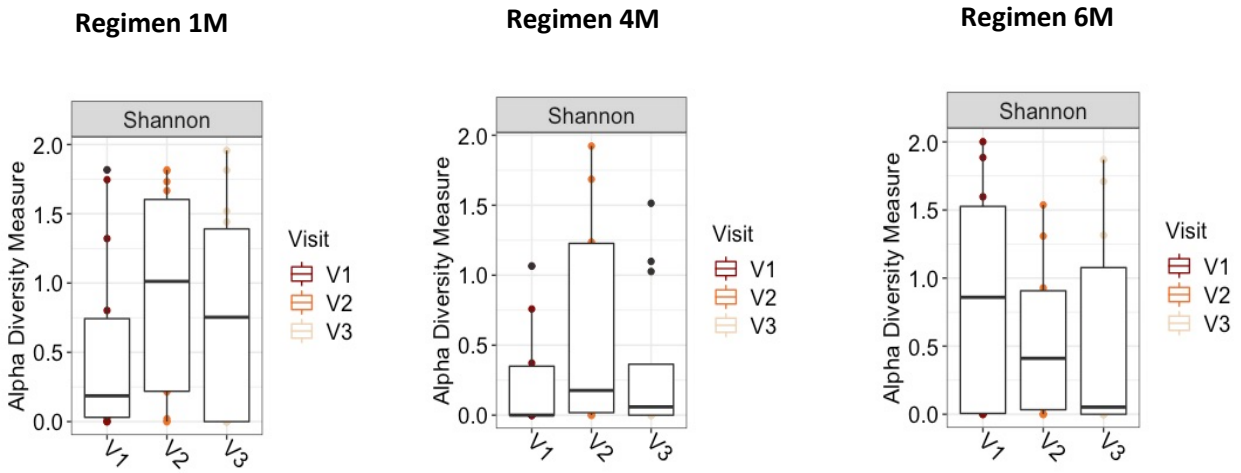


Figure 11. Alpha diversity analysis. Boxplot of Shannon Index for fungal taxa in Regimen 1M, Regimen 4M and Regimen 6M and subgroup analysis by visit. The line inside the box represents the median. Whiskers represent the lowest and highest values within the 1.5 interquartile range.

The beta diversity analysis showed no significant difference in supragingival plaque mycobiome between visits for all groups (PERMANOVA, pseudo-F = 0.67, R^2 = 0.01, *P* = 0.64). No significant differences were seen between First, Second and Third visits within Regimen 4M (PERMANOVA, pseudo-F = 0.78, R^2 = 0.045, *P* = 0.55), Regimen 6M (PERMANOVA, pseudo-F = 0.48, R^2 = 0.03, *P* = 0.72) and Regimen 1M (PERMANOVA, pseudo-F = 0.62, R^2 = 0.03, *P* = 0.81).

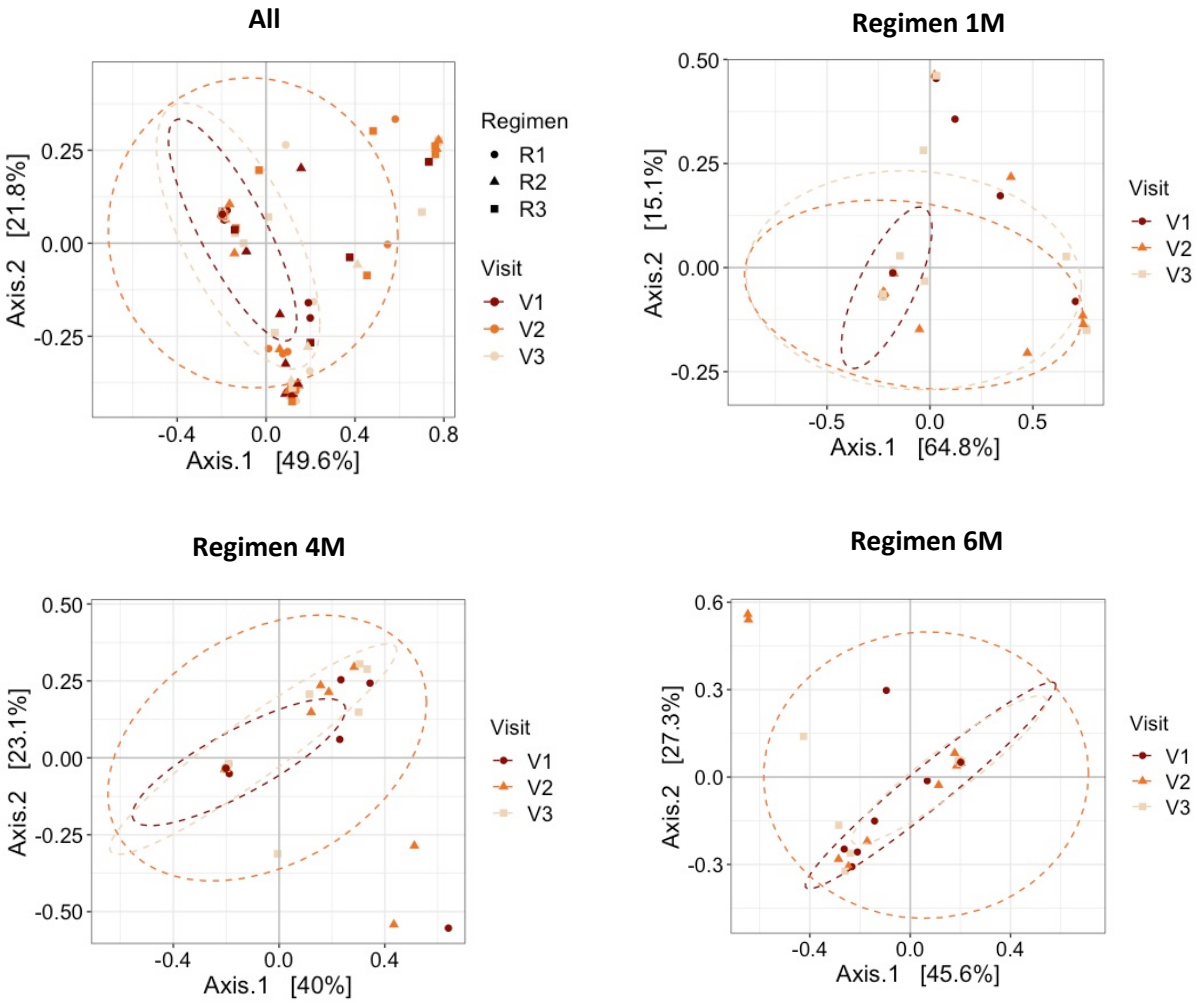


Figure 12. Beta diversity analysis of fungal communities. Principal coordinate analysis plots of weighted UniFrac distances based on the overall structure of the supragingival plaque mycobiome. Each data point represents a sample, coloured according to visit. The ellipses represent a 95% confidence level. No significant separation of samples was noted between regimens or visits.

Taxonomic assignment showed that *Candida, Blumeria*, and *Malassezia* were the most abundant genera (Figure 13). Figure 14 shows the relative abundances of the top 25 most abundant fungal taxa and it shows that *Candida albicans* was highly abundant in all groups regardless of number of visits or regimens (Figure 14).

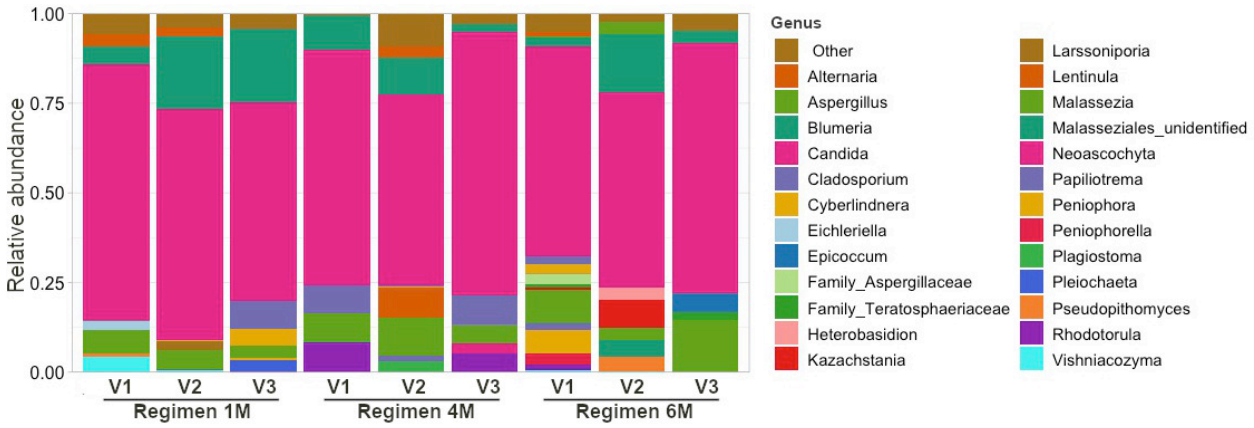


Figure 13. Taxonomic profiles of dental plaque according to regimen and visit at fungal genus level. Colours were assigned to the top 25 most abundant taxa.

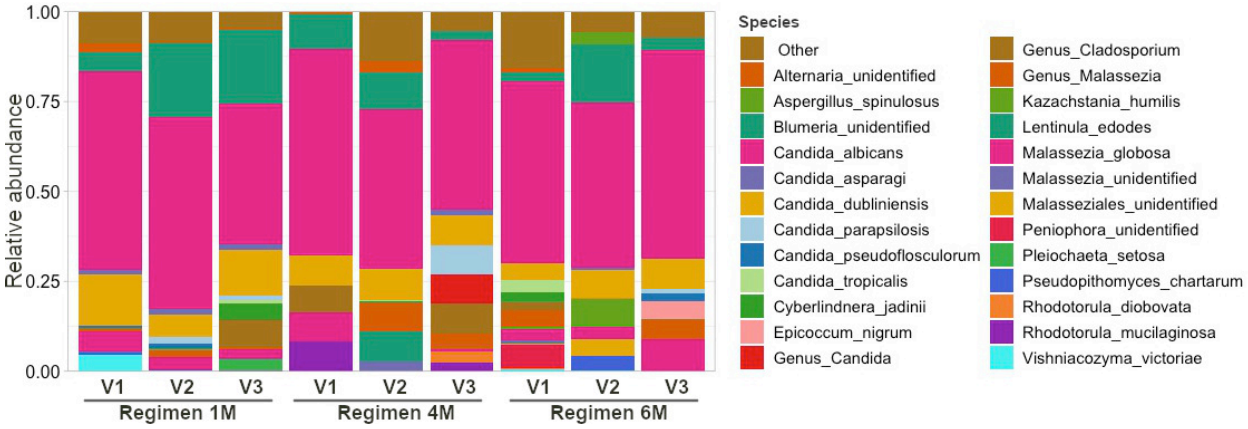


Figure 14. Taxonomic profiles of dental plaque according to regimen and visit at fungal species level. Colours were assigned to the top 25 most abundant taxa.

The differential abundance analysis showed many fungal species that were significantly enriched or depleted between visits 1 and 2 or between visits 1 and 3 (Figures 15-17). Taken together, these results suggest that SDF treatment may have an effect on the abundance of specific fungi but it does not modify the overall microbial structure of the supragingival plaque.

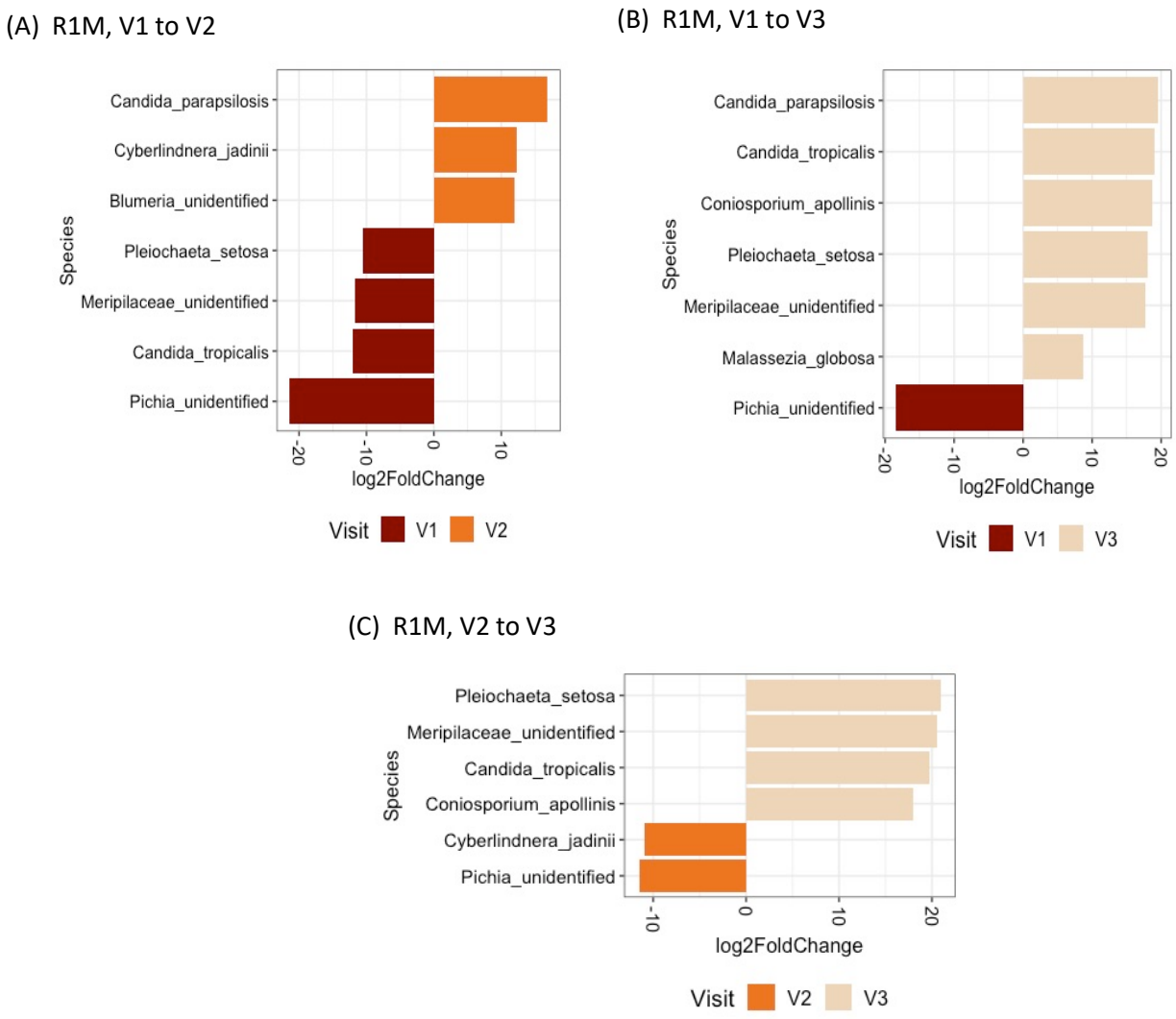


Figure 15: Differential abundance analysis for fungal species in Regimen 1M. The figure shows the relative fold change in fungal abundance between (A) visits 1 and 2, (B) visits 1 and 3 and (C) visits 2 and 3. Only fungal taxa that had FDR adjusted $P < 0.05$ are shown.

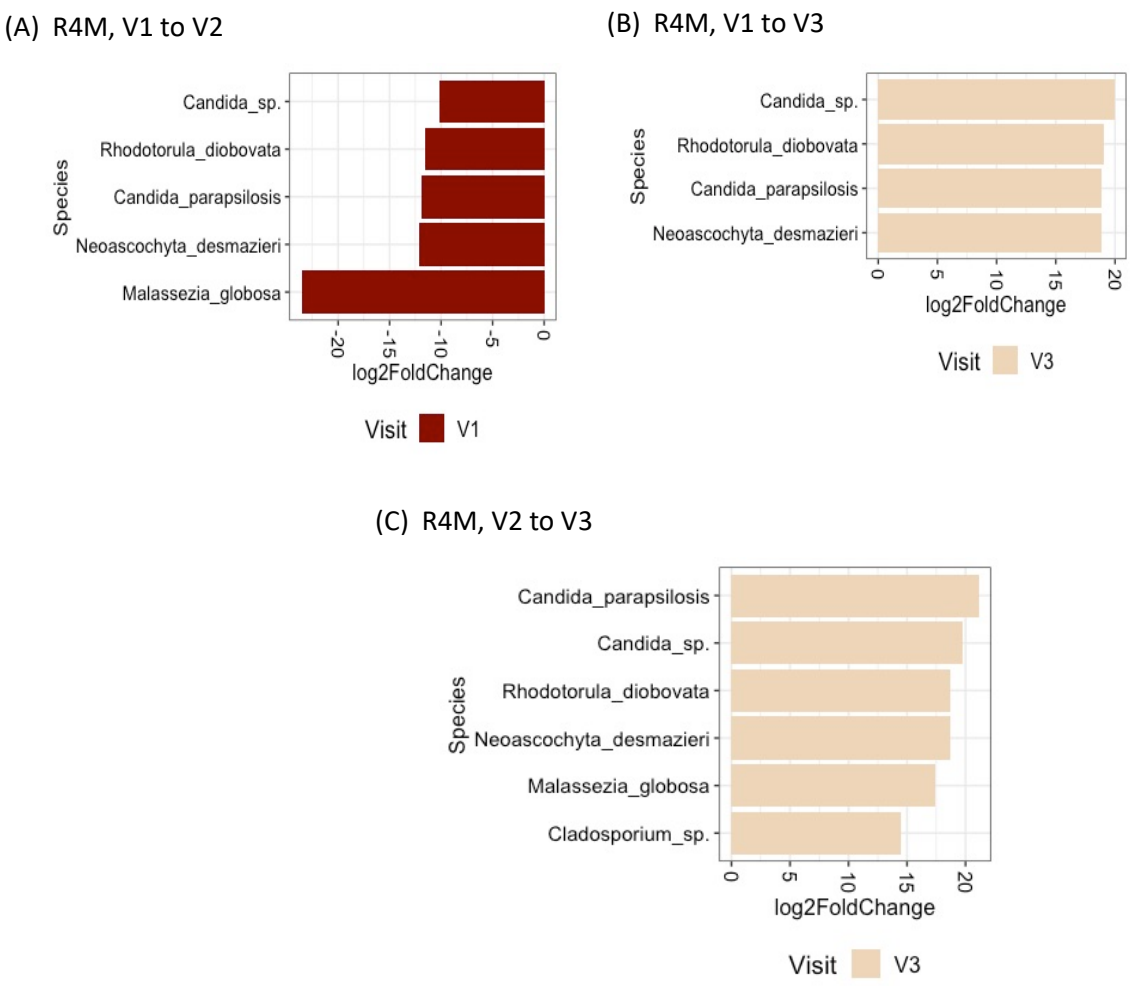
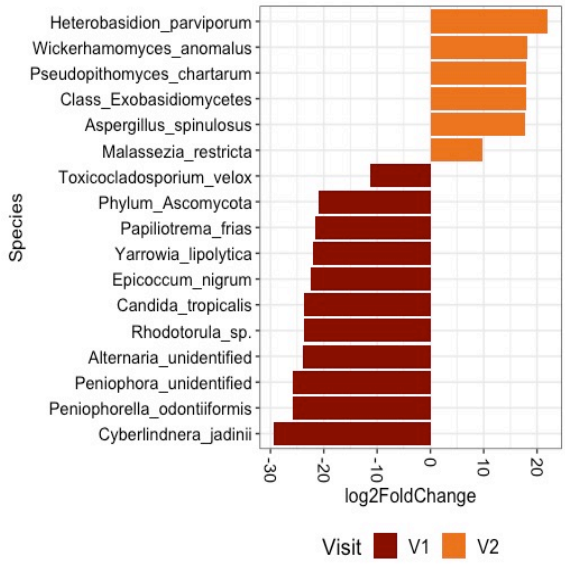
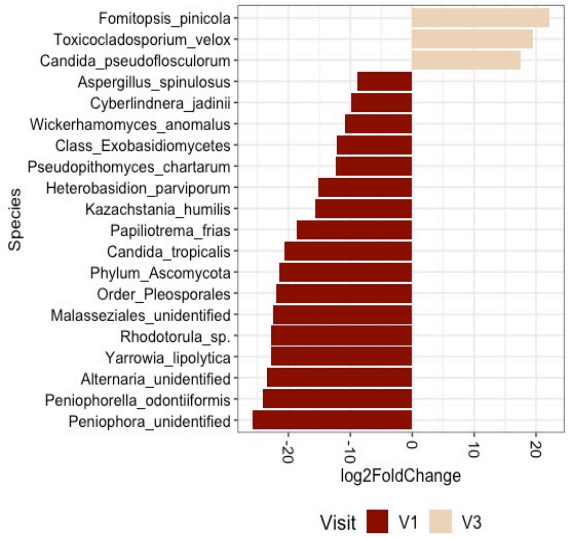


Figure 16. Differential abundance analysis for fungal species in Regimen 4M. The figure shows the relative fold change in fungal abundance between (A) visits 1 and 2, (B) visits 1 and 3 and (C) visits 2 and 3. Only fungal taxa that had FDR adjusted $P < 0.05$ are shown.

(A) R6M, V1 to V2



(B) R6M, V1 to V3



(C) R6M, V2 to V3

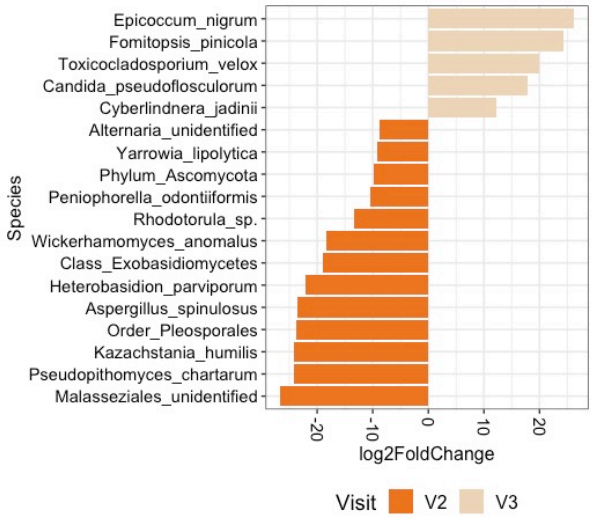
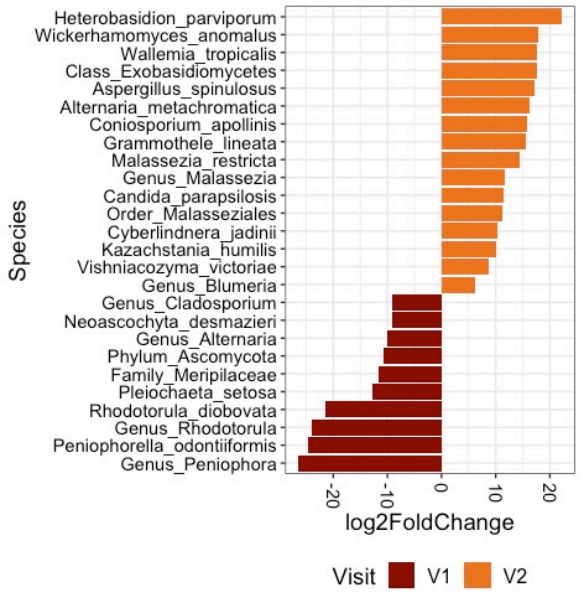
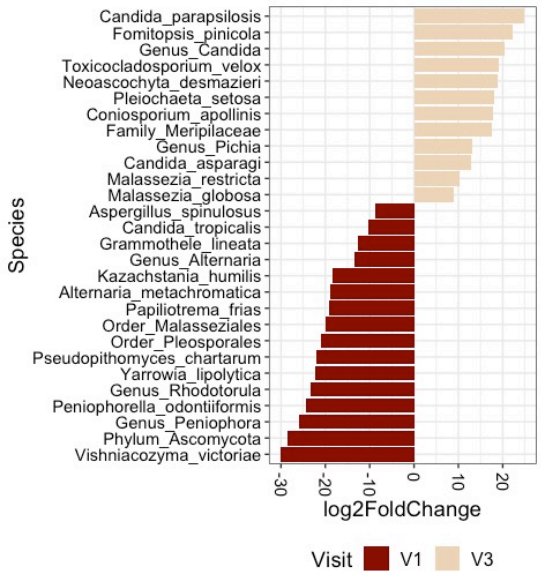


Figure 17: Differential abundance analysis for fungal species in Regimen 6M. The figure shows the relative fold change in fungal abundance between (A) visits 1 and 2, (B) visits 1 and 3 and (C) visits 2 and 3. Only fungal taxa that had FDR adjusted $P < 0.05$ are shown.

(A) All Regimens, V1 to V2



(B) All Regimens, V1 to V3



(C) All Regimens, V2 to V3

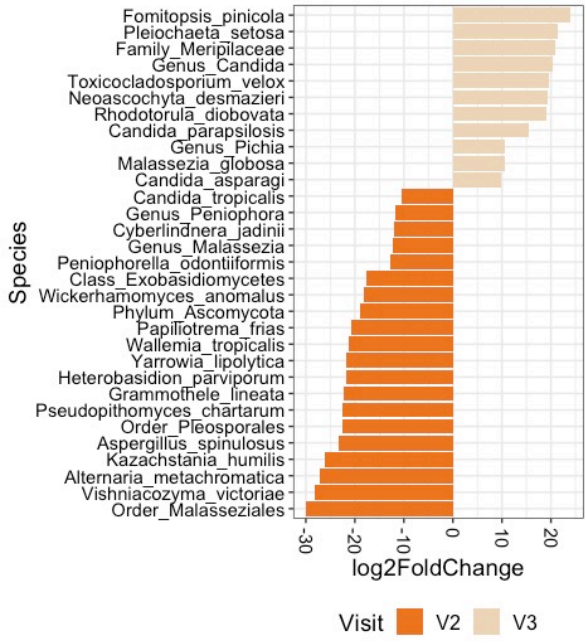


Figure 18: Differential abundance analysis for fungal species in all regimens. The figure shows the relative fold change in fungal abundance between (A) visits 1 and 2, (B) visits 1 and 3 and (C) visits 2 and 3. Only fungal taxa that had FDR adjusted $P < 0.05$ are shown.

6.0 DISCUSSION

6.1 Lesion-Level Analysis

The current AAPD Clinical Practice Guideline recommends monitoring carious lesions for arrest at 2-4 weeks following application of SDF with reapplication as necessary to achieve arrest of all targeted lesions and re-care monitoring based on disease activity and caries risk level at three, four or six months (44). SDF is known to have a reservoir effect with sustained antimicrobial effects over time (38). Bacteria killed by silver ions have biocidal effects on viable populations of the same bacterium (66). When bacteria that have been killed by silver ions are then added to living bacteria, the silver is re-activated resulting in the death of the living bacteria through a "zombie effect" (38). Application to carious lesions is as effective in preventing caries in other teeth and surfaces as applying SDF directly (38). This is consistent with our findings for Regimen 4M and Regimen 1M where average dmft remained the same over all three visits. Although average arrest rates were higher for all lesions after two applications of SDF compared to one application of SDF, arrest rates were higher with application frequencies of 4 months and 1 month compared to 6 months.

Regimen 6M showed lower arrest rates compared to Regimen 4M and Regimen 1M and also showed increased dmft between Visit 1 and Visit 2/Visit 3. The literature suggests that increasing the frequency of SDF application can increase caries arrest rates (44). The effectiveness of lesion arrest by 38% SDF decreases over time (44, 67). Re-activation of treated lesions has been observed in a 2-year study where a single application of 38% SDF was sufficient to prevent only 50% of the arrested surfaces at six months from reverting to active lesions again over 24 months (67). Six months between applications may not be optimal for arrest of caries in high-risk children. Other factors which may be influencing arrest rates and dmft with six months application frequency include the number of erupted teeth, diet high in fermentable carbohydrates, plaque index, amount of plaque present in lesions, medical status and use of antibiotics.

6.2 Microbiology

Two other studies have looked at microbial changes following SDF treatment in pediatric patients. Milgrom et al (2018) found no consistent changes in relative abundance of caries-

associated microbes or microbiota diversity in their small sample when microbial composition was assessed by RNA sequencing from two lesions and one unaffected surface before treatment and at follow up (14-21 days) for 3 children from each group (38% SDF vs placebo) (39). Mei et al (2020) found no overall microbiome changes in 5-year-old children immediately before, 2 weeks after, and 12 weeks after, one application of 38% SDF (68). Microbiota showed a temporal shift in the positive direction after two weeks but returned to original status after twelve weeks. The authors noted that *S. mutans* tended to decline in arrested carious lesions while *Neisseria* species and *Actinomyces naeslundii* tended to increase in arrested carious lesions. Carious lesions that remained active after SDF treatment showed a trend of increased abundance of *S. mutans, S. sobrinus*, and *Lactobacillus sp*. compared to pre-SDF treatment levels though not all were statistically significant.

Agnello et al (2017) studied the plaque microbiome (16S rRNA) associated with severe caries in Canadian First Nations children and found that plaque samples from caries-free and S-ECC subjects did not differ in terms of species richness or phylogenetic diversity when alpha (within-sample) diversity was analyzed (48). In our study, microbiological analyses of supragingival plaque samples did not show any statistically significant differences in alpha diversity or beta diversity for bacteria and for fungi for all regimens and visits. Principal component analysis showed high distribution with low attribution to any component suggesting that overall bacteriome and mycobiome composition remained stable. Although supragingival plaque samples showed no statistically significant differences for alpha diversity, there were some clear trends. Bacterial alpha diversity increased from Visit 1 to Visit 3 in Regimen 4M and remained stable from Visit 1 to Visit 3 in Regimen 6M and Regimen 1M. Fungal alpha diversity remained stable from Visit 1 to Visit 3 in Regimen 4M, decreased from Visit 1 to Visit 3 in Regimen 6M and increased from Visit 1 to Visit 3 in Regimen 1M. Despite this, the slight trend towards increasing bacterial and fungal alpha diversity observed in some regimens suggests a transition to a healthier plaque microbiome. It is interesting to note that we did not see any statistically significant loss of diversity following the use of SDF for all regimens and visits. This supports the findings of Milgrom et al (2018) that use of SDF is safe with minimal risks of disruption of the dental plaque microbiome unlike other antimicrobials such as traditional antibiotics which may enable the growth of opportunistic infections such as *Clostridium difficile* (39).

The supragingival plaque bacteriome showed a decrease in relative abundance from Visit 1 to Visit 3 for *S. mutans* for all regimens, however, this trend was not found to be statistically significant (Figure 5). Takahashi et al (2021) have shown that 38% SDF can decrease the number of *S. mutans*, the amount of water-insoluble glucan, and the thickness of the formed biofilm *in vitro* (69). They suggest that this may be partially due to an inhibitory effect of silver ions on glucosyltransferase activity, which is used by *S. mutans* to synthesize extracellular polysaccharides from sucrose, and the rupture of the bacterial cells.

Differential abundance analysis showed that the supragingival plaque of children at baseline (Visit 1) was enriched with cariogenic bacteria such as *Lactobacillus spp.* and/or *Bifidobacterium spp.,* when compared to Visit 2 and 3. When all regimens were taken together (Figure 10), *L. salivarius* was enriched in Visit 1 compared to Visit 2 and Visit 3, signifying a decrease in abundance from Visit 1 to Visit 3. *L. salivarius* is known to be a dominant species commonly isolated from the dentition of adults and children with caries (70). An interesting finding was that *L. salivarius* was higher at Visit 2 and Visit 3 compared to Visit 1 for Regimen 6M. Regimen 6M also showed higher dmft scores and lower arrest rates at Visits 2 and 3 when compared to the other regimens. An *in vitro* analysis using microscopy techniques showed that a probiotic of inactivated *L. salivarius* inhibited the cariogenic biofilm formation of *C. albicans* and *S. mutans* (71). Under the influence of *L. salivarius,* the biofilm mass and the number of colonies in the biofilm was decreased and fungi did not form hyphae or germ tubes, weakening its pathogenic potential. These results suggested that *L. salivarius* can secrete intermediates capable of producing anti-cariogenic effects. Therefore, further research is required to understand the long-term effects of SDF treatment in the overall supragingival plaque microbiome.

Until recently, *Candida* species were believed to be the predominant fungal species of clinical significance in the oral cavity (72). Ghannoum et al. (2010) described the “basal oral mycobiome” of healthy individuals as containing 74 culturable and 11 nonculturable fungal genera (73). They found a total of 101 different fungal species in the oral samples from 20 healthy individuals. The most common fungal genera included *Candida* species (*Candida albicans, Candida parapsilosis, Candida tropicalis, Candida khmerensis* and *Candida metapsilosis*), *Cladosporium, Aureobasidium, Saccharomycetales, Aspergillus, Fusarium* and

Cryptococcus. Dupuy et al. (2014) studied the "human core mycobiome" and found five new genera of fungi: *Malassezia, Irpex, Cytospora/Valsa, Lenzites/Trametes* and *Sporobolomyces/Sporidiobolus* in the saliva samples of all six individual participants (74). Many of these fungal species had not previously been found in the oral cavity through culture and microscopy methods. Similarly, our study found a vast diversity of fungal species (Figures 13-18).

High levels of *Candida albicans* are frequently detected in the plaque biofilms of children with ECC (75-77). Other *Candida* species such as *Candida tropicalis*, *Candida krusei* and *Candida glabrata* are also detected in plaque biofilms of children with ECC, though not in the numbers of *C. albicans* (75). In their study on the characterization of supragingival plaque in children with S-ECC, de Jesus et al (2021) found that *C. dubliniensis* and *C. tropicalis* were more abundant in oral swab samples of children with S-ECC compared to caries-free controls (49). In contrast, healthy, ECC-free children have plaque biofilms where *C. albicans* is absent or sporadically detected (75-77). In our study, taxonomic assignment showed that *Candida, Blumeria*, and *Malassezia* were the most abundant genera. *Malassezia* is known to be a prominent commensal in the oral cavity (74). *Candida albicans* was found to be highly abundant in all groups regardless of the number of visits or regimens. Our differential abundance analysis showed many fungal species that were significantly enriched or depleted between visits 1 and 2 or between visits 1 and 3 suggesting that SDF treatment may have an effect on the abundance of specific fungi. A recent study by Fakhruddin et al has shown that SDF has antifungal effects against some Candida species including *C. albicans, C. krusei, C. tropicalis* and *C. glabrata* (45). In our study, we noted decreased abundance of *C. tropicalis* in Visit 3 compared to baseline (V1) in Regimen 6M and when all regimens were analyzed together (Figure 18).

In 2015, Li and Tanner completed a systematic review identifying 41 clinical studies that incorporated microbiological evaluations of ECC treatments and other interventions including effects of fluoride applications, chlorhexidine varnish, povidone iodine, full-mouth restorative treatment under general anesthesia, children's xylitol trials, maternal xylitol trials, silver compounds and ECC on oral microbial community diversity. The studies investigating antibacterial efficacy of silver compounds could not be selected for meta-analysis. However, other antimicrobial interventions showed an initial decrease in *Mutans Streptococci* species

colonization followed by bacterial regrowth once the intervention had ceased. Full-mouth comprehensive treatment under general anesthesia, with and without single or combined antimicrobial therapies (chlorhexidine, povidone iodine and fluoride varnish), also showed significant reduction of *Mutans Streptococci* species and *Lactobacillus* species immediately after treatment. However, bacterial levels in saliva and plaque increased significantly 6-12 months after treatment and 20%-60% of children developed new carious lesions following treatment under general anesthesia. The most consistently effective intervention was found to be anti-cariogenic microbial interventions in mothers, which significantly reduced *Mutans Streptococci* species acquisition in children. Since dental caries is a multifactorial disease caused by a combination of factors, further studies with a larger sample size are needed to confirm whether SDF causes any overall changes in the microbiome over 6-12 months following treatment, while considering any risk factors for dental caries and the initial acquisition of the oral microbiome.

6.3 Limitations and Further Studies

Bacterial and fungal community composition significantly differs between different oral sampling sources (49). Our patient population ranged from 21 months to 71 months in age with a subset that were pre-cooperative and required additional or advancement behavior guidance techniques. It is possible that supragingival plaque samples were contaminated by saliva or subgingival plaque, each of which would have unique microbial profiles.

In our study, plaque samples were not site-specific and so data on the microbiome could not be localized to the site of the carious lesion. Further studies with different sampling techniques (supragingival plaque samples, unstimulated whole saliva samples, supragingival dental plaque mixed with unstimulated whole saliva, site-specific sample from carious lesions using slow-speed handpiece and No. 6 round bur, site-specific sample from dentition of caries-free children, consistency of timing of sample collection following food consumption or oral hygiene) comparing overall microbiome changes with site-specific changes that are lesion-specific would help determine whether SDF has an effect only on the treated area or has an effect on the overall oral microbiome. It is also important to further investigate whether SDF is indiscriminately inhibiting or killing all bacteria and fungi within the lesion or selecting cariogenic bacteria or fungal species specifically.

Fifteen children were recruited for each regimen in our study. Most oral fungi are present at low biomass and may be difficult to detect in oral samples (79). In our study, five samples had low quality of sequence reads and a total of eighteen samples had to be removed to allow for paired analysis of the remaining 114 plaque samples. This further decreased the sample size for mycobiome analysis.

The small sample size for each regimen in our study may explain why we were able to see some trends in the bacteriome and mycobiome but not any statistically significant changes in alpha diversity and beta diversity. Increasing the sample size and using more sensitive techniques such as shotgun metagenomic sequencing may help to better evaluate diversity and abundance of the supragingival plaque microbiome in future studies. Along with a larger sample size, increasing sampling of supragingival plaque over the 6–12 month period following treatment, while including any possible risk factors for dental caries in the statistical analysis, would help to confirm whether SDF causes any overall changes in the microbiome.

7.0 CONCLUSIONS

SDF was an effective modality for arresting dental caries with higher arrest rates for all lesions after two applications of SDF. The overall arrest rate at Visit 2 was 77.69% and the overall arrest rate at Visit 3 was 93.61%. There was no significant difference between all supragingival plaque samples with both alpha diversity analysis and beta diversity analysis. Taxonomic assignment showed that *Streptococcus*, *Corynebacterium* and *Actinomyces* were the most abundant genera overall for bacteria while *Candida, Blumeria*, and *Malassezia* were the most abundant genera overall for fungi. The differential abundance analysis showed significant changes in both bacterial and fungal species, particularly *Lactobacillus spp.*, *Bifidobacterium spp. and Candida spp.* Further studies with a larger sample size are needed to confirm whether the presence or absence of various bacterial and fungal species are the result of SDF application at various frequencies or simply the result of a disrupted microbiome.

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