An extensive description of the microbiological effects of silver diamine fluoride using an oral *in situ* model

Kittipit Klanliang

Ph.D. Dissertation (2018–2022)

Osaka University Graduate School of Dentistry

Course for Oral Science

Department of Restorative Dentistry and Endodontology

Supervisor: Professor Mikako Hayashi

Acknowledgments

First and foremost, I would like to express my sincere gratitude to Dr. Yoko Asahi, my thesis advisor and mentor, for her kind assistance and great support at every stage of the research project. Her encouragement on this project gave me a wonderful academic learning experience.

My research would have been impossible without the aid and support of Professor Mikako Hayashi, the head of the Department of Restorative Dentistry and Endodontology. I am profoundly grateful to her treasured support, which was influential in shaping the overview of my whole study.

I would like to extend my sincere thanks to Dr. Hazuki Maezono, my clinical instructor and my first laboratory teacher here. I greatly admire her for sharing valuable experiences and encouragement for not only academic research but also daily life. I also greatly appreciate all the help provided by Dr. Katsuaki Naito, who kindly taught me how to use many laboratory devices and was always patient with all my technical mistakes.

I would also like to thank all the members of the biofilm research group for their kind support. I also wish to extend my thanks to all my friends and department members. It is their kind help and advice that have made my study and life in Japan memorable.

Additionally, I am deeply thankful to the Thai Government and Chiang Mai University for giving me this invaluable opportunity through financial support for my education and living expenses. To all my senior colleagues in the Division of Endodontics, Department of Restorative Dentistry, Chiang Mai University, thank you for choosing this ordinary student to be part of your team and for always believing in me.

Lastly, I must express my very profound gratitude to my mother and my brother for providing me with unwavering support and always believing in me. Without their mental support over the past few years, it would have been more difficult for me to complete my study.

INDEX

Results

Tables and figures

Abbreviations

- SDF: Silver diamine fluoride/silver diammine fluoride
- 16S rRNA: 16S ribosomal ribonucleic acid
- DMFT: Decayed/missing/filled teeth
- CPI: Community periodontal index
- CFU: Colony forming unit
- PCR: Polymerase chain reaction
- ATCC: American type culture collection
- SEM: Scanning electron microscope
- CLSM: Confocal laser scanning microscopy
- HA: Hydroxyapatite
- NGS: Next generation sequencing
- PERMANOVA: Permutational multivariate analysis of variance

Chemical formulas

- $Ag(NH_3)_2F$: Silver diamine fluoride
- CaF_2: Calcium fluoride
- Ag_3PO_4: Silver phosphate
- AgCl: Silver chloride

Introduction

1. Introduction

Biofilms are polymicrobial communities adhered onto surfaces, including surfaces of the human body, consisting of multiple species of microorganisms embedded within a matrix containing extracellular polysaccharides. They are spatially and functionally organized by the intimate interactions between their components and with host tissues in many parts of the body such as the oral cavity. It is generally accepted that the interactions within and among dental biofilms, as well as with the host, are potentially responsible for oral health and disease states[1–3]. Periodontal diseases and dental caries are prime examples of oral infectious diseases that originate from the consequences of the interactions between microorganisms, hosts, and environment conditions[1]. The ecological plaque hypothesis proposed by Marsh describes the shift in microbial communities during caries development to a predominance of acidogenic and aciduric species due to changes in local environments driven by dietary factors[4]. This breaks the dynamic balance within a symbiotic biofilm and eventually leads to an imbalance between demineralization and remineralization of tooth structures[5,6].

Human life expectancy has increased, leading to an increasing awareness of dental health, including advanced oral therapeutic modalities. The number of remaining teeth with gingival recession has become significantly greater in the

elderly population. Once root surface/dentin is exposed to the oral environment, it is likely to be susceptible to demineralization due to the acidic environment created by acid-producing bacteria in the supragingival biofilm, leading to the initiation of root caries[7,8].

The advancement in current culture-independent molecular approaches such as high-throughput gene sequencing has facilitated a broader understanding of the microorganisms in dental biofilms, not only in terms of species identification and quantification, but also in terms of their roles in regulating oral status[9,10]. This consequently opens a new era of microbiome study in which genes of microbiota living in a community can be investigated[11]. The human oral microbiome project has discovered more than 700 species of prevalent taxa[12]. Although previous studies were unable to define the core microbiome of healthy supragingival biofilms, several genera were frequently found to be associated with health, including *Streptococcus*, *Neisseria*, *Actinomyces*, *Rothia*, *Veillonella*, *Fusobacterium*, and *Granulicatella*[7,13].

Among the multiple taxa that predominate in dental biofilms, oral streptococci make up two-thirds of the total commensal population[14]. Typical commensal *Streptococcus* species, normally recognized as initial colonizers, are mitis group streptococci, including *Streptococcus gordonii*, *Streptococcus oralis*, and *Streptococcus sanguinis*[15]. Substantial colonization of these species at the early stage of biofilm development is likely due to their ability to express adhesin molecules that can effectively bind to receptors in acquired pellicle-covered tooth

surfaces[16]. In addition, these streptococci can also interfere with caries-related pathogens by secreting alkali compound, bacteriocins, and hydrogen peroxide[6,17,18]. However, when there is a shift within the biofilm community resulting from an environmental disturbance, such as a particularly high cariogenic carbohydrate intake, some acidogenic and aciduric streptococci, such as *Streptococcus mutans*, become more abundant. *S. mutans* has long been recognized as a critical causative pathogen of dental caries[6,19,20]; they were detected at a higher abundance in both childhood caries[21,22] and root caries[23,24] using sequencing techniques. Critical pathogenic factors of *S. mutans*, rather than their acidogenicity and acid tolerance, include avid glucan synthesis via glycosyltransferase, which protects biofilms from antimicrobial agent penetration[25–27].

Various clinical approaches for caries management have been introduced[28,29]. Silver diamine fluoride (SDF) is one of these approaches that has gained considerable attention as a caries-preventive agent of choice in the past decade[30,31]. SDF is a clear alkali solution containing 24%–27% silver, 7.5%–11.0% ammonia, 5%–6% fluoride, and ≤62.5% water[32,33] and its application is known to be safe, easy-to-use, and cost-effective[34]. The use of SDF was first introduced in Japan in the 1970s, but it has not been popular because it blackens carious lesions, causing esthetic problems. Nonetheless, the Food and Drug Administration (FDA) finally granted breakthrough therapy designation to SDF

for use in arresting dental caries in children and adults in 2016[35]; SDF has received more attention since then.

The means of SDF application according to the manufacturer's guidelines start with soaking a piece of cotton pellet in a few drops (0.15 to 0.20 mL) of the solution and then applied to the teeth. The dose may be adjusted according to the number of affected teeth and symptoms. Subsequently, the teeth surface is rinsed thoroughly using water or a dilute saline solution. The procedure should be performed three to four times with intervals of several days[36].

A number of clinical trials have pointed out that the application of SDF arrested caries progress in primary and permanent dentitions[37], and previous randomized controlled trials indicated better preventive efficacy of carious lesions with SDF than without treatment[38] or treatment with NaF[39]. SDF was also shown to be capable of preventing and arresting root carious lesions in the elderly[40–42].

Although the specific mechanism of SDF on carious lesions remains unclear, previous laboratory studies have shown that the mineral density of enamel caries and microhardness increased after SDF application[43,44]. SDF protects the collagen fibers from being exposed[45] and preserves them from degradation[46], facilitating remineralization. In addition, SDF possesses potent antibacterial activity against various biofilm models[47]. Previous *in vitro* studies have shown that SDF significantly inhibits the growth of *S. mutans* and *Actinomyces naeslundii* mono-species biofilms on dentin blocks[48].

Correspondingly, the growth of a dual-species cariogenic biofilm consisting of *S. mutans* and *Lactobacillus acidophilus* was reduced in demineralized dentin blocks treated with SDF[49]. Mei *et al*. investigated the effect of SDF on multi-species biofilms containing *S. mutans*, *Streptococcus sobrinus*, *L. acidophilus*, *Lactobacillus rhamnosus*, and *A. naeslundii*; the results demonstrated that the growth of all bacteria was inhibited by SDF[50].

The change in the microbiome at the site of carious lesions after SDF treatment was also investigated in recent studies utilizing a gene-based sequencing approach. An RNA sequencing analysis using plaque samples from preschool children with dental caries revealed no significant differences in microbial diversity and no consistent changes in the relative abundance of caries-related microbes between the cavities treated with SDF and those treated with water[51]. The findings from Mei *et al*. also showed no differences in the overall microbiota diversity of arrested and active caries 12 weeks after SDF treatment. Nonetheless, the relative abundance of *S. mutans* decreased in arrested lesions yet increased in active lesions[52]. This is in line with another study on root carious lesions, which also observed a decrease in several acid-producing species, such as *S. mutans*, post-SDF application[53].

According to the studies mentioned earlier, the use of *in vitro* biofilm models has been shown to affirm an inhibitory effect of SDF on various microbes. However, due to many disadvantages, *in vitro* models cannot mimic natural biofilm ecosystems in actual oral environments[54]; therefore, the development of

intraoral *in situ* models has recently become an issue. A systematic review demonstrated several types of *in situ* oral devices used for cultivating biofilms for various specific purposes and reported that buccal devices were the most common in current biofilm studies[55]. Our research group used a substrate-holding *in situ* device to enable the growth of dental biofilm and observe temporal changes in biofilm mass, including bacterial composition, using a gene sequencing approach[56]. Based on current evidence, there is no study utilizing such an *in situ* model to investigate the microbiological changes in terms of biomass and microbial composition of dental biofilms grown on SDF-treated substrates.

2. Hypothesis

The null hypotheses of this study were that (I) the application of SDF on demineralized dentin does not inhibit the growth and viability of biofilms, and (II) the microbiome structure of the SDF biofilm community is different from that of the control biofilm.

3. Objectives

The objective of this study is to investigate the microbiological effects of applying 38% SDF solution on demineralized dentin on dental biofilm grown *in situ.*

4. Significance of this study

This study extends the microbiological perspective of using SDF as a topical medication for arresting and preventing dental caries by comprehensively assessing microbial biofilms in terms of both biomass and community, analyzed using 16S rRNA sequencing. Based on *in situ* conditions, this is the first study to cultivate dental biofilms on SDF-treated substrates that closely mimic the actual oral situation. These findings may broaden the understanding of the nature of the antibacterial effect of SDF.

Materials and methods

1. Materials

The 38% SDF solution used in this study was manufactured by Toyo Seiyaku Kasei Company (Osaka, Japan) under the trademark of SAFORIDE®. The Japanese standard product classification number is 87279. The SAFORIDE® product contained 380 mg of $Ag(NH_3)_2F$ per milliliter (38% w/v) and had a pH of 10.

2. Methods

2.1. Volunteer recruitment

The study was approved by the Ethics Committee of the Osaka University Graduate School of Dentistry (approval No. H29-E17-2). Volunteers who participated in this study were recruited from the students and staff of the Osaka University Graduate School of Dentistry. General medical information was obtained from each volunteer and they were each given an oral health examination; in particular, the DMFT index and community periodontal index (CPI) of each volunteer were determined. The inclusion criteria for the participants were as follows:

1) The volunteer did not present with clinical signs of pulpal and radicular diseases and/or periodontitis.
2) The volunteer had not taken antibiotic within 3 months of the experimental period.
3) The volunteer had no history of smoking.
4) The volunteer had not been receiving orthodontic treatment or wearing a denture.

All volunteers who met the inclusion criteria were thoroughly informed about the study protocols and were asked to sign an informed consent form.

2.2. *In situ* model

A customized intraoral *in situ* device made from a thermoplastic sheet was used in this study. It was a modification of the biofilm model developed in previous studies[56,57]. Devices were separated into left and right pieces, which each had eight rectangular slots for positioning the dentin slabs. These were used as a substrate for biofilm cultivation in this study (**Fig. 1**). The slots were perforated to create a 1 × 3 mm opening at the buccal surface using a round diamond bur (1 mm in diameter). The device trial and adjustment, such as border smoothing, were performed on all volunteers prior to the experimental period.

2.3. Dentin slab preparation

According to **Fig. 2**, the root dentin of the bovine anterior incisors was cut into rectangular slabs. First, the bovine roots were cut along the first plane (thickness) using an Isomet cutting machine (Buehler Ltd., Lake Bluff, IL, USA). Then, the pieces of the cut dentin were cut along the second plane (width and length) to the designated dimension (3 × 5 × 1 mm) using a diamond wire saw (Well Diamond Wire Saws, Inc., GA, USA). The prepared dentin slabs were sonicated in an aqueous solution of 2.5% sodium hypochlorite for 60 min to remove the tissue (the solution was replenished every 10 min), and the slabs were washed with distilled water for 30 s. To mimic the demineralized zone of superficial carious lesions, the dentin slabs were immersed in a 20% citric acid solution for 30 min with ultrasonication. The slabs were cleaned again with distilled water. Half of the dentin slabs were assigned to the control with no treatment and the other half were treated with 38% SDF solution (SAFORIDE®, Toyo Seiyaku Kasei Co., Osaka, Japan) for 4 min before washing with distilled water for 30 s (SDF group). All slabs were subsequently fitted into the intraoral devices and sterilized using ethylene oxide gas.

2.4. Experimental design

The study volunteers were asked to wear the left and right pieces of the devices during the same experimental period. Each piece contained eight dentin

slabs for 96 h to enable biofilm growth. Thus, one period of the experiment provided 16 specimens for following analyses. Four dentin slabs with biofilms were randomly extracted from the device at 24, 48, 72, and 96 h from both groups and were assigned to different biofilm analyses. Each time duration were specified as day 1 to day 4 respectively in the results. The first slab was used for viable cell counts, the second slab underwent DNA extraction, the third slab was used for CLSM analysis, and the last slab was prepared for SEM observation (**Fig. 3**). All sample collection procedures for each volunteer were performed during the same period throughout the experiment. Volunteers were only allowed to remove the devices during meals and when drinking. Tooth brushing was allowed, but without toothpaste.

2.5. Viable cell count

After extraction, the dentin slabs were gently washed twice, vortexed for 30 s, and ultrasonicated in distilled water at 4 °C for 15 min to detach the biofilms from the dentin surface. The resulting bacterial suspension was serially 10-fold diluted. Droplets from each dilution were cultured on Columbia blood agar plates with 5% sheep blood (Nippon Becton Dickinson, Tokyo, Japan) and incubated under aerobic and anaerobic conditions (using an AnaeroPack®, Mitsubishi Gas Chemical Co., Tokyo, Japan) for 48 h. After incubation, the colonies were counted and calculated as $\log_{10}$ CFU/cm^3.

2.6. Quantification of biofilm-forming cells by real-time PCR

To quantify the number of biofilm-forming cells, real-time PCR was performed as previously described by Sotozono *et al*.[57]. Bacterial genomic DNA was extracted from each biofilm sample using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at −80 °C until further analysis. The assays (20 μL) were prepared by mixing 1 μL of extracted DNA, 10 μL of SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA, USA), and 0.5 μL of each bacterial universal primer as shown in **Table 1**. The Applied Biosystems 7500 Fast real-time PCR system (Thermo Fisher Scientific Co., Ltd., Tokyo, Japan) was used, and standard curves were generated using *S. mutans* ATCC 25175 genomic DNA to amplify serial dilutions. The experiments were repeated thrice per sample. Data were acquired and analyzed using the Applied Biosystems 7500 System SDS software (version 2.0.2, Applied Biosystems).

To investigate the abundance of specific *Streptococcus* species associated with health and caries, commensal streptococci, namely *S. gordonii* and *S. oralis*, were chosen, while *S. mutans* represented caries-associated species. All the procedures for real-time PCR quantification of these species were the same as mentioned above using species-specific primers, as shown in **Table 1**.

2.7. Confocal microscopy

The dentin slabs with biofilms were washed in distilled water before being labeled with a LIVE/DEAD BacLight Bacterial Viability Kit L7012 (Invitrogen, Carlsbad, CA, USA) for 30 min in the dark at room temperature. The slabs were observed using a confocal laser scanning microscope (LSM700; Carl Zeiss, Oberkochem, Germany), and five areas on the slabs were randomly selected and captured. Digital reconstruction was performed on the obtained images using image analysis software (Imaris 9.2.1; Bitplane AG, Zurich, Switzerland), and the thickness of the biofilm as well as the volume of the dead and live bacteria were assessed.

2.8. SEM observation

Biofilm samples were prepared according to a previously described protocol[58]. The samples were immersed in 50% Karnovsky's solution for 30 min before being dehydrated in a sequence of aqueous ethanol solutions (50%, 70%, 80%, 90%, 95%, and 100%) and transferred into t-butyl alcohol. The specimens were then freeze-dried (JFD-320; JEOL, Tokyo, Japan) and sputter-coated with platinum (Sputter Coater SC7620, Quorum Technologies, East Sussex, UK) before observation under an SEM (JSM-6390LV, JEOL, Tokyo, Japan) using the secondary electron emission mode at an accelerating voltage of 20 kV. Magnifications of 5,000 and 7,500 were used.

2.9. 16S rRNA gene amplicon sequencing

PCR amplification targeting the V1–V2 hypervariable region of the bacterial 16S rRNA was performed separately using the primer set 27F (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-AGRGTTTG ATYMTGGCTCAG-3′) and 338R (5′-GTGACTGGAGTTCAGACGTGTG CTCTTCCGATCT-NNNNN-TGCTGCCTCCCGTAGGAGT-3′). Paired-end sequencing (2 × 300 bp) of the amplicons was performed using an Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to standard protocols. Raw sequences obtained from all samples were de-multiplexed using the Fastq barcode splitter tool from the Fastx toolkit (version 0.0.13). Only sequences that exactly matched the start of the reading sequence or the primers used were extracted. After removing the primer sequence, the QIIME pipeline (version 2.0) was used to denoise and cluster quality-checked sequences into operational taxonomic units (OTUs) according to a similarity cut-off of 97% using UCLUST (version 1.2.22q). Representative sequences for each OTU were aligned using the Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu/) against the Greengenes Database, and each sequence was assigned to the genus level. The relative abundance of the taxa and alpha and beta diversities were computed using the QIIME script (version 2.0).

2.10. Statistical analysis

Statistical analyses were computed in R using RStudio (version 1.2.1335) for the Mac OS X development environment. All sets of data were tested for normal distribution using the Shapiro–Wilk test. A repeated measures ANOVA test was used to compare the mean CFU counts in both aerobic and anaerobic conditions, including real-time PCR bacterial loads between groups and by days, followed by the Wilcoxon signed-rank test. Repeated measures ANOVA and multiple pairwise comparisons with Bonferroni's correction were used to compare the means of biofilm thickness in both groups and over different days. Dead and live cell volumes were calculated as percentage before testing across group in each day via the Mann–Whitney U Test. Based on the 16S rRNA sequencing data, Phyloseq and DESeq2 packages were used to perform bacterial community analysis[59,60]. For alpha diversity, the Chao1 and Shannon indices, indicating taxa richness and taxa abundance plus evenness, respectively, were determined using rarefied OTU tables. The Wilcoxon rank sum test was used to compare the alpha diversity indices of the control and SDF groups on each day. The Bray–Curtis dissimilarity, as a beta diversity index, was measured from raw sequences that had been normalized based on the DESeq2 calculation. Using SHAMAN, a web application for metagenomic analysis[61], a principal coordinates analysis (PCoA) plot was generated, and a PERMANOVA test, which demonstrates the compositional difference across the groups of data, was performed. All relative abundance comparisons were tested via the Kruskal–

Wallis test with adjusted p-values using the Benjamin–Hochberg method. For all tests, a p-value < 0.05 was considered statistically significant.

Results

1. Recruited volunteers

Ten healthy volunteers (five males and five females) aged 26–34 years who met the inclusion criteria were recruited in this study. According to dental and periodontal evaluation, the mean DMFT and CPI of volunteers were 3.1 ± 2.0 and 2.2 ± 1.5, respectively (**Table 2**).

2. Viable cell count

Cultivable bacterial cell counts are shown in **Fig. 4.** The number of viable cells in the control group increased substantially on the first day of the experiment in both conditions. Growth continued to increase until day 3 and dropped slightly under aerobic conditions, while the growth reduced after day 2 for anaerobic cultivation. Viable cell growth showed the same pattern in the SDF group under both cultivation conditions; the biofilm grew rapidly in the middle of the experimental period (from day 2 to day 3). However, a lower count was observed under aerobic conditions. In comparison, there were significantly lower log CFU counts in the SDF-treated biofilms than in the control group under both aerobic and anaerobic conditions throughout the experiment (repeated measure ANOVA, $p < 0.01$).

3. Quantification of biofilm-forming cells by real-time PCR

The number of biofilm-forming cells estimated from the standard curve in the control groups increased steadily from day 1 to day 3, then more slowly from day 3 to day 4. However, there was only a slight increase in day 3 and 4 of the experiment in the SDF group. Similar to the viable cell count results, there were significantly fewer biofilm-forming cells in the SDF group than in the control group on all days of the experiment (repeated measure ANOVA, $p < 0.001$) (**Fig. 5**).

4. Confocal microscopy

4.1. CLSM image

Representative images from CLSM (**Fig. 6A**) demonstrated a noticeable increase in biofilm mass with the number of abundant live cells in the control group over time. In contrast, the biofilms grown on SDF-treated dentin slabs were found to be sparse, and red-fluorescent dead cells were apparently enclosed within microcolonies.

4.2. Biofilm thickness

The biofilm thickness results were in line with the CLSM images; the biofilm thicknesses in control and SDF groups increased until the end of the experimental period. However, SDF biofilms had significantly lower thickness than the control at all time points of the experiment (repeated measure ANOVA with multiple pairwise comparisons using Bonferroni's correction) (**Fig. 6B**).

4.3. Dead and live cell volume

As shown in **Fig. 6C** and **Table 3**, the total biofilm volume in the SDF group was clearly lower than that in the control group throughout the experimental period. Furthermore, the proportions of dead cells present in SDF biofilms were greater than those in biofilms in the control group (Friedman test with multiple pairwise comparisons using Bonferroni's correction).

5. SEM observation

To investigate the ultrastructure characteristics of *in situ* biofilms obtained from both groups, SEM images at high magnification were taken. **Fig. 7** shows co-aggregations of rod-shaped and columnar microcolonies, which penetrated the dentinal tubules of bovine root dentin in the control group on the first day. The biofilm structure became more complex because of the presence of substantial filamentous and coccal bacteria on subsequent days. In contrast, the

microcolonies in the SDF group were sparsely detectable on the dentin surface and were less complex. In addition, small particles of silver precipitated on the dentin surface could be observed in some SDF group specimens.

6. 16S rRNA gene amplicon sequencing

6.1. Community diversity analysis

The Chao1 index was significantly different between the control and the SDF groups, regardless of the day of the experiment (control, 183.3 ± 46.8; SDF, 235.0 ± 65.1; adjusted $p < 0.001$). Similarly, there was a significant difference in the Shannon index between the groups (control, 4.0 ± 0.3; SDF, 4.4 ± 0.4; adjusted $p < 0.001$) (**Fig. 8A**). However, when each day was analyzed separately, there were no significant differences in either indices, except on day 2 when the Shannon index significantly differed between groups. Moreover, both the Chao1 and Shannon indices were similar in each group on day 4 (**Fig. 8B**).

The PERMANOVA test showed significant differences in beta diversity based on the Bray–Curtis values of the biofilm communities when the groups were compared (adjusted $p < 0.001$), but there were no differences between the groups when each day was analyzed separately. Similarly, the PCoA plots showed no clear clustering for any of the factors considered (**Fig. 9A, B**).

6.2. Bacterial profile assessment

Fourteen phyla, 21 classes, 33 orders, 58 families, and 90 genera were detected across all samples (40 samples from each group) using 16S rRNA Illumina sequencing. Based on the relative abundance of the taxa, the most abundant phyla were Proteobacteria (31.5%) and Firmicutes (37.2%) in the control and SDF groups, respectively (**Fig. 10A**). At the genus level, the three most abundant phyla were *Neisseria* (18.8%), *Streptococcus* (15.3%), and *Porphyromonas* (13.2%) in the control group, and *Streptococcus* (22.2%), *Haemophilus* (11.0%), and *Neisseria* (10.9%) in the SDF group (**Fig. 10B**).

Further analyses only included the 20 most dominant genera. Of these, only two genera, *Streptococcus* and *Actinomyces*, significantly differed between the groups. Both genera had higher relative abundances in the SDF group compared with the control group at almost every time point (except for *Streptococcus* on day 1) (**Fig. 11**). Despite the significant difference noted, the relative abundance of the genus *Actinomyces* was observed to be less than 3% (mean ± SD = 2.2 ± 0.6%) in the overall population.

7. Quantification of biofilm-forming cells on specific streptococci by real-time PCR

The quantity of three streptococcal species consisting of two commensals (*S. oralis* and *S. gordonii*) and one caries-associated species (*S. mutans*) were

subsequently examined. All three *Streptococci* followed a similar pattern; their cell numbers remained similar until the end of the experiment in the SDF biofilm, whereas the cell numbers tended to increase steadily in the control group (**Fig. 12A**). In particular, on days 3 and 4, all three *Streptococci* had lower cell counts in the SDF group than in the control group. When data were transformed to percentages, there were no significant differences for all comparisons (except on day 2 for *S. gordonii*) (**Fig. 12B**).

Discussion

In this study, an extensive assessment of the effect of SDF treatment, from a microbiological perspective, was conducted, combining both conventional cultivation and microbial community profile analyses using a gene sequencing method. Recently, the emergence of high-throughput sequencing techniques, such as next generation sequencing (NGS), has drastically transformed the way researchers study and characterize oral microbiota for a better understanding of the changes in microbial ecology associated with states of health or disease. The discovery of new uncultivated species has provided an extensive view of the oral microbiome in terms of microbiota composition, function, and metabolites[62,63].

A newly designed oral device was utilized in this study as it is capable of fitting dentin slabs to enable the growth of dental biofilms under natural oral conditions. Despite its lower hardness than human root dentin[64], bovine root dentin has been suggested as a substrate for investigating anticariogenic agents[65]. Several advantageous rationales for using bovine dentin instead of a human counterpart were proposed, including availability, ease of manipulation, and a more uniform mineral composition that minimizes the discrepancies in the experiment[66].

Dental caries is the most common oral disease affecting humans of all ages and all parts of the tooth structure, including smooth root surfaces. Similarly, root

caries in elderly populations has become a challenging problem for preventive and restorative dental science. The etiology of root caries is comparable to that of coronal caries to some extent; the acidic environment created by acidogenic and aciduric bacteria in the biofilm leads to an imbalance between demineralization and remineralization on the tooth surface[8,67]. There are several factors that cause root caries, such as the structural composition of root and dentin surfaces[68] or the presence of gingival crevicular fluid[8] that may alter the composition of microbiota living in a biofilm. Therefore, the current study investigated carious lesions located on the root surface, and the root dentin part was selected as a representative substrate of human root dentin to investigate changes in dental biofilm *in situ*.

SDF has been used for decades to prevent and arrest carious lesions in primary teeth[39,69] and in the root surface, particularly in the elderly[70]. Although the specific mechanism of SDF in arresting carious lesions has not been completely elucidated, a number of *in vitro* studies have been used to investigate multiple aspects of the efficiency of SDF on dental caries[47]. Apart from its efficacy in preventing the degradation of dentin collagen[46] and increasing microhardness and mineral contents after application[45,48], SDF has been demonstrated to exert various effects on cariogenic bacteria. The viable cell count results from the current study corroborate previous laboratory studies[48–50] by demonstrating decreased CFUs in the SDF group under both aerobic and

anaerobic culture conditions. Similarly, real-time PCR quantification revealed a significantly lower total number of bacterial cells in the SDF biofilms than in the control group, indicating that SDF effectively reduced the number of viable cells in the biofilms formed on dentin slabs. SDF is a complex compound. The silver and fluoride ions have separate activities. Silver compounds have been recognized as potent antimicrobial agents in both medicine and dentistry[30]. Owing to their trimodal action on a wide range of microorganism[71], silver ions can bind strongly to sulfhydryl groups and proteins on bacterial cell membranes, eventually causing bacterial cell death and inhibiting biofilm formation[72]. In addition, they inactivate intracellular enzymes by interacting with thiol groups. Silver ions interfere with bacterial DNA synthesis and inhibit bacterial cell division[73,74]. Furthermore, the number of viable cells in the control group, from both analyses, exhibited a similar trend to that observed in our previous *in situ* study using hydroxyapatite (HA) disks[56], suggesting the potential of HA disks to be used as a practical substrate alternative to bovine root dentin for cultivating dental biofilms.

Regarding the biomass of the biofilm, CLSM images exhibited noticeably less biofilm formation on the SDF-treated dentin slabs than in the control with dead cells. These findings are in accordance with previous *in vitro* studies[48–50], although the sequence of SDF application was different and our experimental period was shorter. Consistently, the lower thickness of the SDF biofilms and the lower viability of bacterial cells, as demonstrated by the dead to live cell ratio,

indicates the anti-biofilm efficacy of SDF after application on demineralized root dentin. The CLSM results were in accordance with the ultrastructural characteristics of the biofilms observed by SEM. The actual maturation of biofilms was observed in the control group as previously demonstrated *in vivo*[75] and in an *in situ* study using HA disks[56], which showed the co-aggregation of various bacteria and the presence of matrix-like structures after 48 h of the experiment. Biofilms were scarce on the dentin surface treated with SDF, and the bacterial composition was less complex. In addition, silver particles precipitated on the SDF-treated dentin surface were also detected, consistent with earlier *in vitro* studies[48,50]. It has also been suggested that silver and fluoride ions in SDF interact with HA in the tooth and subsequently form calcium fluoride (CaF_2) and silver phosphate (Ag_3PO_4)[30]. CaF_2 is a crucial fluoride reservoir that can eventually react with HA and convert into fluoroapatite, which has been considered a favorable acid-resistant crystalline that reduces susceptibility to tooth surface demineralization[76–78]. Ag_3PO_4 reacts with alkali chloride compounds to form silver chloride (AgCl), which is the major precipitate found on SDF-treated surfaces[46,77]. Due to its low solubility, AgCl has been confirmed to generate a slow-releasing effect of silver ions, which can sustain antibacterial properties[79]. In addition, the pronounced antibacterial effect of SDF was thought to be derived from the metallic silver nanoparticles, which were also the product of the reaction of SDF and hydroxyapatite[46]. These particles are inert, but after contact with moisture in the oral cavity, silver ions are released[80]. SDF-coated

root dentin was recently shown to significantly decrease lactate production in *S. mutans*[81]. For these reasons, SDF is considered an efficient agent for arresting caries.

The 16S rRNA-based approach could be used as an advanced tool to expand our understanding of the impact of SDF-treated substrates on the structure of the microbial community of dental biofilms. The current findings about biodiversity reject the null hypothesis, as SDF did not significantly affect either alpha or beta diversities between groups when analyzed on each day. However, when data from each day were pooled together, the alpha diversity indices of the SDF group were significantly greater than those of the control, suggesting that the bacterial taxa detected in the SDF group were richer and more equally distributed than the control group. Nonetheless, most of the comparisons showed no significant differences between the groups. These results are consistent with previous studies of gene sequencing in plaques, investigating the effect of SDF application on bacterial profiles using plaque samples[52,53].

In agreement with alpha diversity, the PERMANOVA test based on Bray–Curtis distances showed no significant differences in beta diversity when comparing biofilm composition at each time point. Bray–Curtis dissimilarity is a non-Euclidean distance that is frequently used to quantify the compositional dissimilarity between two different sites in environmental sciences[82]. This measure is usually applied to raw abundance data, but it can also be applied to relative abundances[83]. Thus, Bray–Curtis dissimilarity was used as a beta

diversity index to quantify the difference between samples of biofilm communities in this study. However, when pooled data were analyzed, the PERMANOVA test showed that the community structure of the SDF biofilms was significantly different from that of the control, which is in contrast with a previous study[53]. This is probably due to the differences in the experimental design, the method for normalization including the statistical analysis used[84], and delayed colonization, which may influence the number of bacterial populations in the community analysis result and the type of bacterial taxa in the initial biofilm.

The taxonomic results at the phylum level were in line with previous findings; Firmicutes and Actinobacteria were predominant in the supragingival biofilm[24,85]. At the genus level, *Streptococcus* and *Neisseria* were in the top three genera detected across all samples. Oral streptococci have long been known as one of the primary colonizers of tooth structures, as their cell-surface adhesins facilitate them in binding avidly with surface-coated pellicle-covered tooth structures[16]. Commensal *Streptococcus* species include *S. mitis*, *S. gordonii*, and *S. oralis*[86,87]. Similarly, *Neisseria* has been detected at a high frequency in the human oral microbiome and is regarded as an essential early colonizer[85,88]. They have also been recognized as part of the healthy core oral microbiome[89]. This indicates that the biofilms grown on dentin slabs under *in situ* conditions in this study mainly consisted of healthy oral microbiota. Furthermore, when comparisons of the relative abundance of frequently detected bacteria were

conducted, there were only two genera, *Streptococcus* and *Actinomyces*, whose relative abundances were noticeably higher in SDF group biofilms. *Actinomyce*s, a group of gram-positive, mainly facultative anaerobic bacilli, are classified as a fundamental component of early colonizers of tooth structures[90,91] and are also involved in root caries etiology[91,92]. The current results regarding the relative abundance of *Actinomyces* are somewhat different from a study by Mitwalli *et al*., who investigated changes in bacterial profiles in plaque samples collected from root carious lesions. In their study, they observed a decreasing trend in several acid-producing species, such as *Scardovia* and root caries-related *Actinomyces*[53]. However, the relative abundance of *Actinomyces* in the present study was still considered low (overall relative abundance less than 3%). Hence, this genus was not examined further in species-specific quantification analyses.

According to the subsequent quantification of specific streptococci, neither health- nor caries-related species were affected by treatment with SDF (**Fig. 12A,B**). The relative quantity of *S. mutans* was not reduced by SDF treatment in a recent study conducted in preschool children using plaque samples from SDF-applied carious lesions[52]. Moreover, the relative abundance of *S. mutans* decreased after SDF treatment in samples collected from arrested lesions. However, the findings of the current study may differ with those of previous studies due to differences in SDF application method, experiment duration, and gene sequencing protocol. Therefore, interpretation should be performed with care. All volunteers participated in this study aged under 35 years with relatively

good oral health status. Hence, the biofilms used in current analyses were still unable to be entirely mimicked the condition of root caries that tends to occur in the elderly population. Another limitation of this study is the short experimental period. The cultivation of biofilms was performed within four consecutive days of the start of the study because of the practical difficulties associated with volunteer availability to wear the device for this *in situ* study, including concerns about oral hygiene. In addition, the difference among individuals' microbiome in terms of composition and diversity is another concerning factor in the result interpretation.

According to the present results, the anti-biofilm efficacy of SDF seems shorter than expected. To date, there is still no clear evidence indicating the longevity of SDF treatment in arresting and preventing dental caries. A clinical study showed that SDF lost its efficacy over time in the elderly if only one application was performed[93]. Increasing the application frequency to twice a year enhanced the arrest rate of dentin caries rather than annual application[94]. Based on clinical studies, 1–2 applications per year are recommended[35,95]. Future work with standardized methods and longer duration is necessary to affirm previous findings regarding the effect of SDF on the microbiome and to establish more evidence on the longevity of SDF. This will contribute to a reliable protocol for SDF application for dental caries management.

Conclusions

In conclusion, the application of SDF to demineralized dentin decreased the growth of dental biofilms and increased the proportion of dead bacterial cells. SDF did not appear to affect the diversity of the microbiome growing on demineralized dentin at the end of the experimental period, nor did it alter the relative abundance of certain streptococci that were or were not associated with root caries. The noticeable decrease in the biomass of biofilms grown on SDF-treated dentin may potentially influence the efficacy of SDF in preventing and arresting caries on root dentin over a result of the change in bacterial composition.

References

1. Marsh PD, Moter A, Devine DA. Dental plaque biofilms: communities, conflict and control. *Periodontol 2000*. 2011;55(1):16-35. doi:10.1111/J.1600-0757.2009.00339.X
2. Mosaddad SA, Tahmasebi E, Yazdanian A, et al. Oral microbial biofilms: an update. *Eur J Clin Microbiol Infect Dis*. 2019;38(11):2005-2019. doi:10.1007/s10096-019-03641-9
3. Mira A, Simon-Soro A, Curtis MA. Role of microbial communities in the pathogenesis of periodontal diseases and caries. *J Clin Periodontol*. 2017; 44 Suppl 1:S23-S38. doi:10.1111/jcpe.12671
4. Marsh PD. Are dental diseases examples of ecological catastrophes? *Microbiology*. 2003;149(Pt 2):279-294. doi:10.1099/mic.0.26082-0
5. Marsh PD. Dental plaque as a biofilm and a microbial community - Implications for health and disease. *BMC Oral Health*. 2006;6(SUPPL. 1):1-7. doi:10.1186/1472-6831-6-S1-S14
6. Bowen WH, Burne RA, Wu H, Koo H. Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends Microbiol*. 2018;26(3):229-242. doi:10.1016/j.tim.2017.09.008
7. Sanz M, Beighton D, Curtis MA, et al. Role of microbial biofilms in the maintenance of oral health and in the development of dental caries and

periodontal diseases. Consensus report of group 1 of the Joint EFP/ORCA workshop on the boundaries between caries and periodontal disease. *J Clin Periodontol*. 2017;44:S5-S11. doi:10.1111/jcpe.12682

8. Takahashi N, Nyvad B. Ecological hypothesis of dentin and root caries. *Caries Res*. 2016;50(4):422-431. doi:10.1159/000447309
9. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol*. 2005;43(11):5721-5732. doi:10.1128/JCM.43.11.5721-5732.2005
10. Dewhirst FE, Chen T, Izard J, et al. The human oral microbiome. *J Bacteriol*. 2010;192(19):5002-5017. doi:10.1128/JB.00542-10
11. Ursell LK, Metcalf JL, Parfrey LW, Knight R. Defining the human microbiome. *Nutr Rev*. 2012;70(suppl_1):S38-S44. doi:10.1111/j.1753-4887.2012.00493.x
12. Escapa IF, Chen T, Huang Y, Gajare P, Dewhirst FE, Lemon KP. New insights into human nostril microbiome from the expanded human oral microbiome database (eHOMD): a resource for the microbiome of the human aerodigestive tract. *Msystems*. 2018;3(6):e00187-18.
13. Wirth R, Maróti G, Lipták L, et al. Microbiomes in supragingival biofilms and saliva of adolescents with gingivitis and gingival health. *Oral Dis*. 2021;00:1-15. doi:10.1111/odi.13883

14. Nobbs AH, Jenkinson HF, Jakubovics NS. Stick to your gums: mechanisms of oral microbial adherence. *J Dent Res*. 2011;90(11):1271-1278. doi:10.1177/0022034511399096

15. Rosan B, Lamont RJ. Dental plaque formation. *Microbes Infect*. 2000;2(13):1599-1607. doi:10.1016/S1286-4579(00)01316-2

16. Nobbs AH, Lamont RJ, Jenkinson HF. Streptococcus adherence and colonization. *Microbiol Mol Biol Rev*. 2009;73(3):407-450. doi:10.1128/MMBR.00014-09

17. Liu Y-L, Nascimento M, Burne RA. Progress toward understanding the contribution of alkali generation in dental biofilms to inhibition of dental caries. *Int J Oral Sci*. 2012;4(3):135-140. doi:10.1038/ijos.2012.54;

18. Qi F, Kreth J. Methods to study antagonistic activities among oral bacteria. In: *Oral Biology*. Springer; 2017:203-218. doi:10.1007/978-1-4939-6685-1_12

19. Klein MI, Hwang G, Santos PHS, Campanella OH, Koo H. Streptococcus mutans-derived extracellular matrix in cariogenic oral biofilms. *Front Cell Infect Microbiol*. 2015;5:10. doi:10.3389/fcimb.2015.00010

20. Tanner ACR, Kressirer CA, Rothmiller S, Johansson I, Chalmers NI. The caries microbiome: implications for reversing dysbiosis. *Adv Dent Res*. 2018;29(1):78-85. doi:10.1177/0022034517736496

21. Johansson I, Witkowska E, Kaveh B, Lif Holgerson P, Tanner ACR. The microbiome in populations with a low and high prevalence of caries. *J Dent Res*. 2016;95(1):80-86. doi:10.1177/0022034515609554

22. Xiao J, Grier A, Faustoferri RC, et al. Association between oral Candida and bacteriome in children with severe ECC. *J Dent Res*. 2018;97(13): 1468-1476. doi:10.1177/0022034518790941

23. Chen L, Qin B, Du M, et al. Extensive description and comparison of human supra-gingival microbiome in root caries and health. *PLoS One*. 2015;10(2):1-15. doi:10.1371/journal.pone.0117064

24. Preza D, Olsen I, Aas JA, Willumsen T, Grinde B, Paster BJ. Bacterial profiles of root caries in elderly patients. *J Clin Microbiol*. 2008;46(6):2015-2021. doi:10.1128/JCM.02411-07

25. Bowen WH. Do we need to be concerned about dental caries in the coming millennium? *Crit Rev Oral Biol Med*. 2002;13(2):126-131. doi:10.1177/ 154411130201300203

26. Beighton D. The complex oral microflora of high-risk individuals and groups and its role in the caries process. *Community Dent Oral Epidemiol*. 2005;33(4):248-255. doi:10.1111/j.1600-0528.2005.00232.x

27. Pratten J, Wilson M. Antimicrobial susceptibility and composition of microcosm dental plaques supplemented with sucrose. *Antimicrob Agents Chemother*. 1999;43(7):1595-1599. doi:10.1128/AAC.43.7.1595

28. Bader JD, Shugars DA, Bonito AJ. A systematic review of selected caries prevention and management methods. *Community Dent Oral Epidemiol Syst Rev*. 2001;29(6):399-411. doi:10.1034/j.1600-0528.2001.290601.x

29. Tan HP, Lo ECM, Dyson JE, Luo Y, Corbet EF. A randomized trial on root caries prevention in elders. *J Dent Res*. 2010;89(10):1086-1090. doi:10.1177/0022034510375825

30. Rosenblatt A, Stamford TCM, Niederman R. Silver diamine fluoride: A caries "silver-fluoride bullet." *J Dent Res*. 2009;88(2):116-125. doi:10.1177/0022034508329406

31. Gluzman R, Katz R V, Frey BJ, McGowan R. Prevention of root caries: a literature review of primary and secondary preventive agents. *Spec Care Dent*. 2013;33(3):133-140. doi:10.1111/j.1754-4505.2012.00318.x

32. Tinanoff N. Silver Diamine Fluoride (SDF) Fact Sheet. *Astdd Bod*. 2017;14(3):1. doi:10.1186/1472-6831

33. Crystal YO, Niederman R. Evidence-based dentistry update on silver diamine fluoride. *Dent Clin North Am*. 2019;63(1):45-68. doi:10.1016/j.cden.2018.08.011

34. Crystal YO, Niederman R. Silver diamine fluoride treatment considerations in children's caries management. *Pediatr Dent*. 2016; 38(7):466-471.

35. Horst JA. Silver fluoride as a treatment for dental caries. *Adv Dent Res*. 2018;29(1):135-140. doi:10.1177/0022034517743750

36. Fluoride SD. サホライド ® 液歯科用 38 %. Published online 2009. https://bee.co.jp/wp/wp-content/uploads/2019/05/treatment02_interview.pdf

37. Nishino M, Yoshida S, Sobue S, Kato J, Nishida M. Effect of topically applied ammoniacal silver fluoride on dental caries in children. *J Osaka Univ Dent Sch*. 1969;9:149-155.

38. Llodra JC, Rodriguez A, Ferrer B, Menardia V, Ramos T, Morato M. Efficacy of silver diamine fluoride for caries reduction in primary teeth and first permanent molars of schoolchildren: 36-month clinical trial. *J Dent Res*. 2005;84(8):721-724. doi:10.1177/154405910508400807

39. Chu CH, Lo ECM, Lin HC. Effectiveness of silver diamine fluoride and sodium fluoride varnish in arresting dentin caries in Chinese pre-school children. *J Dent Res*. 2002;81(11):767-770. doi:10.1177/0810767

40. Tan HP, Lo ECM, Dyson JE, Luo Y, Corbet EF. A randomized trial on root caries prevention in elders. *J Dent Res*. 2010;89(10):1086-1090. doi:10.1177/0022034510375825

41. Zhang W, McGrath C, Lo ECM, Li JY. Silver diamine fluoride and education to prevent and arrest root caries among community-dwelling elders. *Caries Res*. 2013;47(4):284-290. doi:10.1159/000346620

42. Li R, Lo ECM, Liu BY, Wong MCM, Chu CH. Randomized clinical trial on arresting dental root caries through silver diammine fluoride applications in community-dwelling elders. *J Dent*. 2016;51:15-20. doi:10.1016/j.jdent.2016.05.005

43. Liu BY, Lo ECM, Li CMT. Effect of silver and fluoride ions on enamel demineralization: a quantitative study using micro-computed tomography. *Aust Dent J*. 2012;57(1):65-70. doi:10.1111/j.1834-7819.2011.01641.x

44. Chu CH, Lo ECM. Microhardness of dentine in primary teeth after topical fluoride applications. *J Dent*. 2008;36(6):387-391. doi:10.1016/j.jdent.2008.02.013

45. Mei ML, Ito L, Cao Y, Lo ECM, Li QL, Chu CH. An ex vivo study of arrested primary teeth caries with silver diamine fluoride therapy. *J Dent*. 2014;42(4):395-402. doi:10.1016/j.jdent.2013.12.007

46. Mei ML, Ito L, Cao Y, Li QL, Lo ECM, Chu CH. Inhibitory effect of silver diamine fluoride on dentine demineralisation and collagen degradation. *J Dent*. 2013;41(9):809-817. doi:10.1016/j.jdent.2013.06.009

47. Zhao IS, Gao SS, Hiraishi N, et al. Mechanisms of silver diamine fluoride on arresting caries: a literature review. *Int Dent J*. 2018;68(2):67-76. doi:10.1111/idj.12320

48. Chu CH, Mei LEI, Seneviratne CJ, Lo ECM. Effects of silver diamine fluoride on dentine carious lesions induced by Streptococcus mutans and Actinomyces naeslundii biofilms. *Int J Paediatr Dent*. 2012;22(1):2-10. doi:10.1111/j.1365-263X.2011.01149.x

49. Mei ML, Chu CH, Low KH, Che CM, Lo ECM. 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*. 2013;18(6):e824. doi:10.4317/medoral.18831

50. Mei ML, Li QL, Chu CH, Lo ECM, Samaranayake LP. Antibacterial effects of silver diamine fluoride on multi-species cariogenic biofilm on caries. *Ann Clin Microbiol Antimicrob*. 2013;12(1):1. doi:10.1186/1476-0711-12-4

51. Milgrom P, Horst JA, Ludwig S, et al. 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*. 2018;68:72-78. doi:10.1016/j.jdent.2017.08.015

52. Mei ML, Yan Z, Duangthip D, et al. Effect of silver diamine fluoride on plaque microbiome in children. *J Dent*. 2020;102:103479. doi:10.1016/j.jdent.2020.103479

53. Mitwalli H, Mourao MDA, Dennison J, Yaman P, Paster BJ, Fontana M. Effect of silver diamine fluoride treatment on microbial profiles of plaque biofilms from root/cervical caries lesions. *Caries Res*. 2019; 53(5):555-566. doi:10.1159/000499578

54. Wood SR, Kirkham J, Shore RC, Brookes SJ, Robinson C. Changes in the structure and density of oral plaque biofilms with increasing plaque age. *FEMS Microbiol Ecol*. 2002;39(3):239-244. doi:10.1111/j.1574-6941. 2002.tb00926.x

55. Prada-López I, Quintas V, Vilaboa C, Suárez-Quintanilla D, Tomás I. Devices for in situ development of non-disturbed oral Biofilm. A systematic review. *Front Microbiol*. 2016;7:1055. doi:10.3389/fmicb. 2016.01055

56. Wake N, Asahi Y, Noiri Y, et al. Temporal dynamics of bacterial microbiota in the human oral cavity determined using an in situ model of dental biofilms. *npj Biofilms Microbiomes*. 2016;2. doi:10.1038/npj biofilms.2016.18

57. Sotozono M, Kuriki N, Asahi Y, et al. Impacts of sleep on the characteristics of dental biofilm. *Sci Rep*. 2021;11(1):1-12. doi:10.1038/ s41598-020-80541-5

58. Asahi Y, Noiri Y, Igarashi J, Suga H, Azakami H, Ebisu S. Synergistic effects of antibiotics and an N-acyl homoserine lactone analog on Porphyromonas gingivalis biofilms. *J Appl Microbiol*. 2012;112(2):404-411. doi:10.1111/j.1365-2672.2011.05194.x

59. McMurdie PJ, Holmes S. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*. 2013;8(4). doi:10.1371/journal.pone.0061217

60. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550. doi:10.1186/s13059-014-0550-8

61. Volant S, Lechat P, Woringer P, et al. SHAMAN: A user-friendly website for metataxonomic analysis from raw reads to statistical analysis. *bioRxiv*. Published online 2019:1-15. doi:10.1101/2019.12.18.880773

62. Yamashita Y, Takeshita T. The oral microbiome and human health. *J Oral Sci*. 2017;59(2):201-206. doi:10.2334/josnusd.16-0856

63. Sampaio-Maia B, Caldas IM, Pereira ML, Pérez-Mongiovi D, Araujo R. Chapter Four - The oral microbiome in health and its implication in oral and systemic diseases. In: Sariaslani S, Michael Gadd G, eds. Vol 97. Advances in Applied Microbiology. Academic Press; 2016:171-210. doi:https://doi.org/10.1016/bs.aambs.2016.08.002

64. Turssi CP, Messias DF, Corona SM, Serra MC. Viability of using enamel and dentin from bovine origin as a substitute for human counterparts in an intraoral erosion model. *Braz Dent J*. 2010;21(4):332-336. doi:10.1590/s0103-64402010000400008

65. Hara AT, Queiroz CS, Paes Leme AF, Serra MC, Cury JA. Caries progression and inhibition in human and bovine root dentine in situ. *Caries*

Res. 2003;37(5):339-344. doi:10.1159/000072165

66. Mellberg JR. Hard-tissue substrates for evaluation of cariogenic and anti-cariogenic activity in situ. *J Dent Res*. 1992;71 Spec No:913-919. doi:10.1177/002203459207100s25

67. McLean JS. Advancements toward a systems level understanding of the human oral microbiome. *Front Cell Infect Microbiol*. 2014;4:1-13. doi:10.3389/fcimb.2014.00098

68. Nyvad B, Ten Cate JM, Fejerskov O. Arrest of root surface caries in situ. *J Dent Res*. 1997;76(12):1845-1853. doi:10.1177/00220345970760120701

69. Mei ML, Lo EC, Chu C-H. Clinical use of silver diamine fluoride in dental treatment. *Compend Contin Educ Dent*. 2016;37(2):93-98.

70. Hendre AD, Taylor GW, Chávez EM, Hyde S. A systematic review of silver diamine fluoride: Effectiveness and application in older adults. *Gerodontology*. 2017;34(4):411-419. doi:10.1111/ger.12294

71. Morones JR, Elechiguerra JL, Camacho A, et al. The bactericidal effect of silver nanoparticles. *Nanotechnology*. 2005;16(10):2346. doi:10.1088/0957-4484/16/10/059

72. Wu M-Y, Suryanarayanan K, Van Ooij WJ, Oerther DB. Using microbial genomics to evaluate the effectiveness of silver to prevent biofilm formation. *Water Sci Technol*. 2007;55(8-9):413-419. doi:10.2166/wst.2007.285

73. Lansdown ABG. Silver in Health Care : Antimicrobial Effects and Safety in Use. 2006;33:17-34. doi:10.1159/000093928

74. Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO. A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. *J Biomed Mater Res*. 2000;52(4):662-668. doi:10.1002/1097-4636(20001215)52:4<662::AID-JBM10>3.0.CO;2-3

75. Theilade E, Wright WH, Jensen SB, Löe H. Experimental gingivitis in man: II. A longitudinal clinical and bacteriological investigation. *J Periodontal Res*. 1966;1(1):1-13. doi:10.1111/j.1600-0765.1966.tb01842.x

76. Yamaga R, Nishino M, Yoshida S, Yokomizo I. Diammine silver fluoride and its clinical application. *J Osaka Univ Dent Sch*. 1972;12:1-20.

77. Mei ML, Nudelman F, Marzec B, et al. Formation of Fluorohydroxyapatite with Silver Diamine Fluoride. *J Dent Res*. 2017;96(10):1122-1128. doi:10.1177/0022034517709738

78. Ten Cate JM, Featherstone JDB. Mechanistic aspects of the interactions between fluoride and dental enamel. *Crit Rev Oral Biol Med*. 1991;2(3):283-296. doi:10.1177/10454411910020030101

79. Li X, Zuo W, Luo M, Shi Z, Cui Z, Zhu S. Silver chloride loaded hollow mesoporous aluminosilica spheres and their application in antibacterial coatings. *Mater Lett*. 2013;105:159-161. doi:10.1016/j.matlet.2013.04.077

80. Peng JJY, Botelho MG, Matinlinna JP. Silver compounds used in dentistry for caries management: A review. *J Dent*. 2012;40(7):531-541. doi:10.

1016/j.jdent.2012.03.009

81. Ishiguro T, Mayanagi G, Azumi M, et al. Sodium fluoride and silver diamine fluoride-coated tooth surfaces inhibit bacterial acid production at the bacteria/tooth interface. *J Dent*. 2019;84:30-35. doi:10.1016/j.jdent. 2018.12.017

82. Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. *Ecol Monogr*. 1957;27(4):326-349.

83. Ricotta C, Podani J. On some properties of the Bray-Curtis dissimilarity and their ecological meaning. *Ecol Complex*. 2017;31:201-205. doi:10.1016/ J.ECOCOM.2017.07.003

84. Lin H, Peddada S Das. Analysis of microbial compositions: a review of normalization and differential abundance analysis. *npj Biofilms Microbiomes*. 2020;6(1). doi:10.1038/s41522-020-00160-w

85. Keijser BJF, Zaura E, Huse SM, et al. Pyrosequencinq analysis of the oral microflora of healthy adults. *J Dent Res*. 2008;87(11):1016-1020. doi:10. 1177/154405910808701104

86. Chen H, Jiang W. Application of high-throughput sequencing in understanding human oral microbiome related with health and disease. *Front Microbiol*. 2014;5:1-6. doi:10.3389/fmicb.2014.00508

87. Sulyanto RM, Thompson ZA, Beall CJ, Leys EJ, Griffen AL. The predominant oral microbiota is acquired early in an organized pattern. *Sci Rep*. 2019;9(1):10550. doi:10.1038/s41598-019-46923-0

88. Liu G, Tang CM, Exley RM. Non-pathogenic neisseria: Members of an abundant, multi-habitat, diverse genus. *Microbiol (United Kingdom)*. 2015;161(7):1297-1312. doi:10.1099/mic.0.000086

89. Zaura E, Keijser BJ, Huse SM, Crielaard W. Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiol*. 2009;9:1-12. doi:10.1186/1471-2180-9-259

90. Könönen E, Kanervo A, Takala A, Asikainen S, Jousimies-Somer H. Establishment of oral anaerobes during the first year of life. *J Dent Res*. 1999;78(10):1634-1639. doi:10.1177/00220345990780100801

91. Nanna S, Eija K, Paula S, Mauno K, Hannele J-S. Phenotypic identification of actinomycesand related species isolated from human sources. *J Clin Microbiol*. 2001;39(11):3955-3961. doi:10.1128/JCM.39.11.3955-3961.2001

92. Papone VY. Actinomyces. Its relation to dental plaque--root caries--actinomycosis. *Odontoestomatologia*. 1989;2(2):47-53.

93. Yee R, Holmgren C, Mulder J, Lama D, Walker D, van Palenstein Helderman W. Efficacy of silver diamine fluoride for arresting caries treatment. *J Dent Res*. 2009;88(7):644-647. doi:10.1177/0022034509338671

94. Zhi QH, Lo ECM, Lin HC. Randomized clinical trial on effectiveness of silver diamine fluoride and glass ionomer in arresting dentine caries in preschool children. *J Dent*. 2012;40(11):962-967. doi:10.1016/j.jdent.

2012.08.002

95. Horst JA, Ellenikiotis H, Milgrom PL. UCSF protocol for caries arrest using silver diamine fluoride: rationale, indications and consent. *J Calif Dent Assoc*. 2016;44(1):16-28.

96. Lane DJ. *16S/23S RNA Sequencing*. Stackebrandt E, Goodfellow M, eds; 1991; 115-175

97. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*. 1991;173(2):697-703. doi:10.1128/jb.173.2.697-703.1991

98. Suzuki MT, Giovannoni SJ. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol*. 1996;62(2):625-630. doi:10.1128/aem.62.2.625-630.1996

99. Hoshino T, Fujiwara T, Kilian M. Use of phylogenetic and phenotypic analyses to identify nonhemolytic streptococci isolated from bacteremic patients. *J Clin Microbiol*. 2005;43(12):6073-6085. doi:10.1128/JCM.43.12.6073-6085.2005

100. Xu X, Zhou XD, Wu CD. Tea catechin epigallocatechin gallate inhibits Streptococcus mutans biofilm formation by suppressing gtf genes. *Arch Oral Biol*. 2012;57(6):678-683. doi:10.1016/j.archoralbio.2011.10.021

Tables and figures

Table 1. Primers used in real-time PCR quantification

Target	Primer sequence	Ref. no.
Universal bacterial primer	F: 5′ AGRGTTTGATCMTGGCTCAG 3′ R: 5′ TGC TGCCTCCCGTAGGAGT 3′	96–98
S. gordonii 16S rRNA	F: 5′ AAACGGAATGCACGATGGAGTCTTG 3′ R: 5′ CTCATTAATATTCACAGAGTTTCGG 3′	99
S. oralis 16S rRNA	F: 5′ AGGATAAGGAACTGCACATTGGTC 3′ R: 5′ TGCATTACTTGGTGATCTCTCACC 3′	99
S. mutans 16S rRNA	F: 5′ AGCGTTGTCCGGATTTATTG 3′ R: 5′ CTACGCATTTCACCGCTACA 3′	100

Table 2. Characteristics of the volunteers

Volunteer no.	Gender	Age	DMFT	CPI
1	M	31	2	2
2	F	31	2	0
3	M	29	2	3
4	M	34	5	4
5	M	31	3	4
6	F	28	3	0
7	M	30	6	3
8	F	33	2	3
9	F	27	6	2
10	F	26	0	1
	M:F = 1:1	Median = 30.5	Mean = 3.1 ± 2.0	Mean = 2.2 ± 1.5

Table 3. Live and dead cell volume determined by CLSM

Day	Control			SDF		
	Live (%)	Dead (%)	SD*	Live (%)	Dead (%)	SD*
1	99.2	0.8[a]	2.3	91.0	9.0[a]	10.8
2	99.9	0.1[b]	0.2	88.6	11.4[b]	12.4
3	98.3	1.7[c]	4.3	87.3	12.7[c]	8.3
4	98.2	1.8[d]	4.1	85.4	14.6[d]	20.5

SD* refers to the standard deviation, which is the same value for both live and dead cells of each group.
Letters indicate significant differences in the volume of dead cells (%) between groups at each time point (Mann–Whitney U Test; $p < 0.001$).

Figures

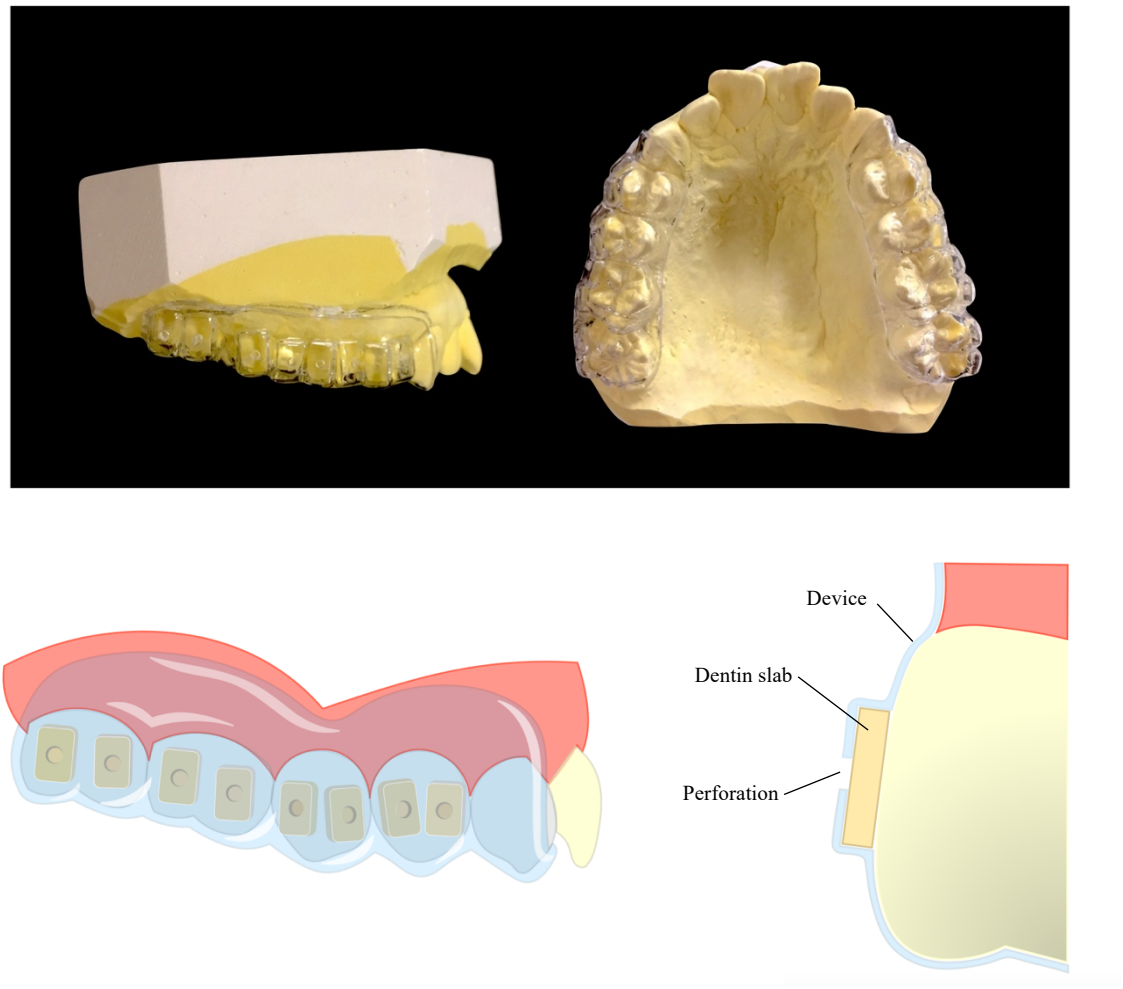

Fig 1. *In situ* device: An intraoral *in situ* device made from a thermoplastic sheet was designed to have eight rectangular slots on the left and eight on the right to fit the dentin slabs. Each slot was created as a circular perforation, enabling the growth of the biofilm.

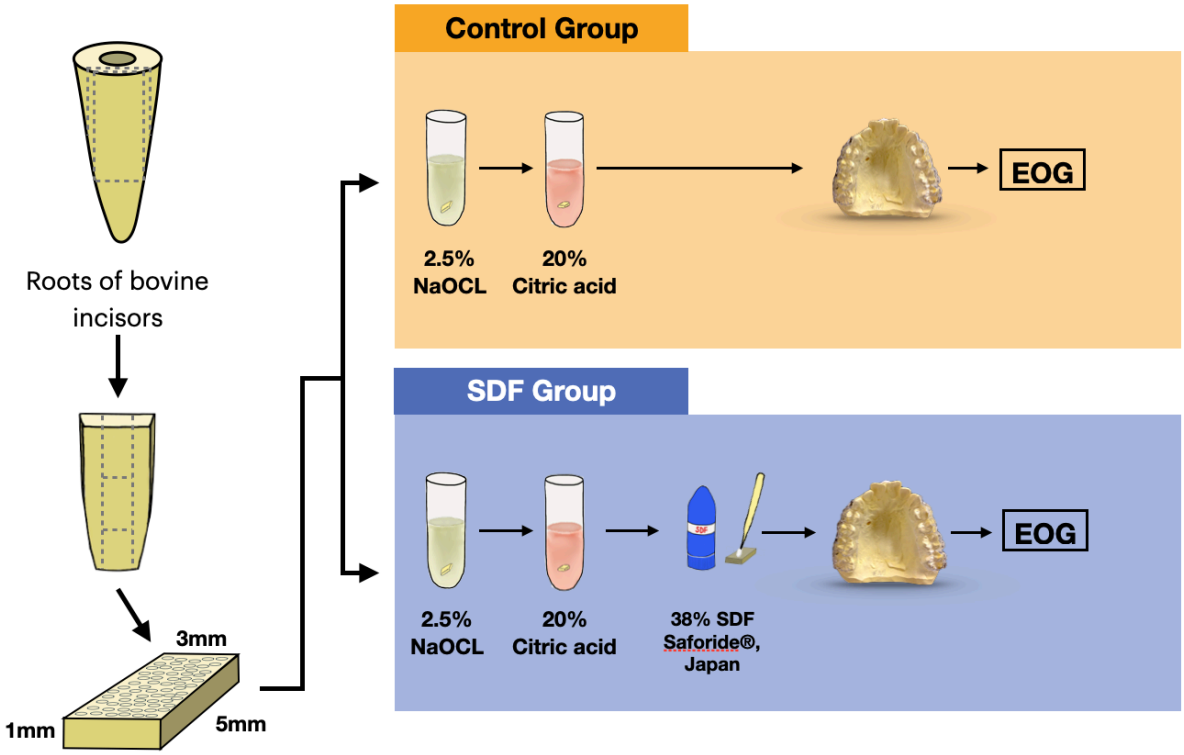

Fig 2. Dentin slab preparation: Bovine root incisors were cut into designated dimensions (3 × 5 × 1 mm). Then the slabs were disinfected with 2.5% NaOCl and were demineralized the surface with 20% citric acid. Half were assigned to the control group, while the other half were assigned to the SDF group. Control dentin slabs were fitted to the device after all pre-treatment procedure. The dentin slabs of the SDF group were treated with 38% SDF solution before being placed in the device. The devices were sterilized using ethylene oxide gas before the experiments.

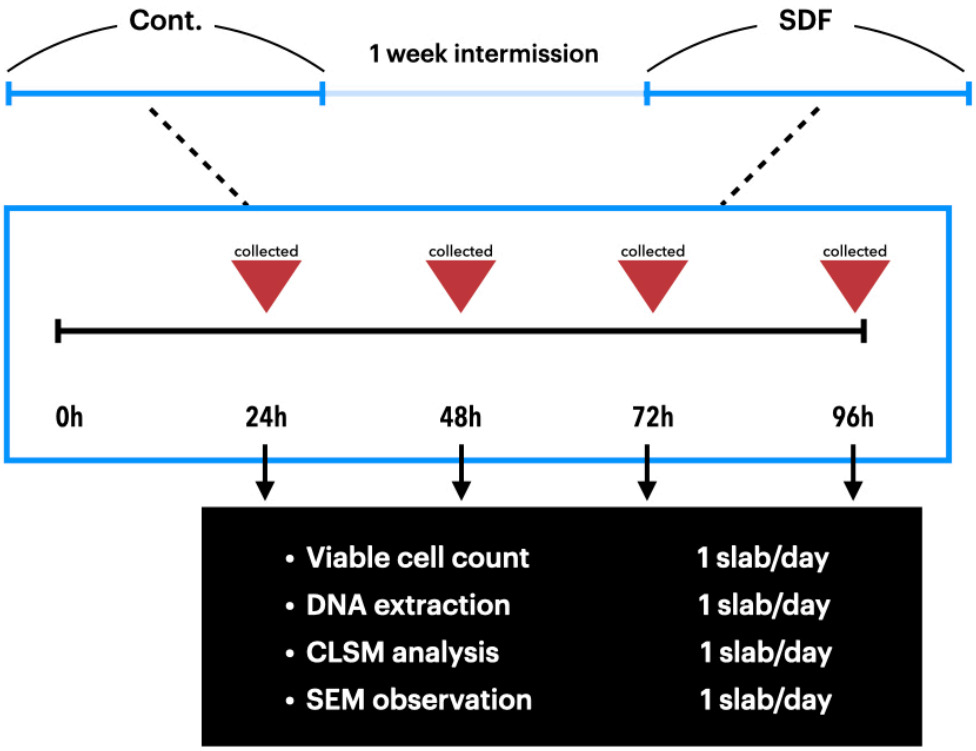

Fig 3. Experimental design: The volunteers were asked to wear the left and right pieces of the devices for 96 h to enable the growth of the biofilm. Four dentin slabs with biofilms were randomly extracted from the device at 24, 48, 72, and 96 h for biofilm analyses.

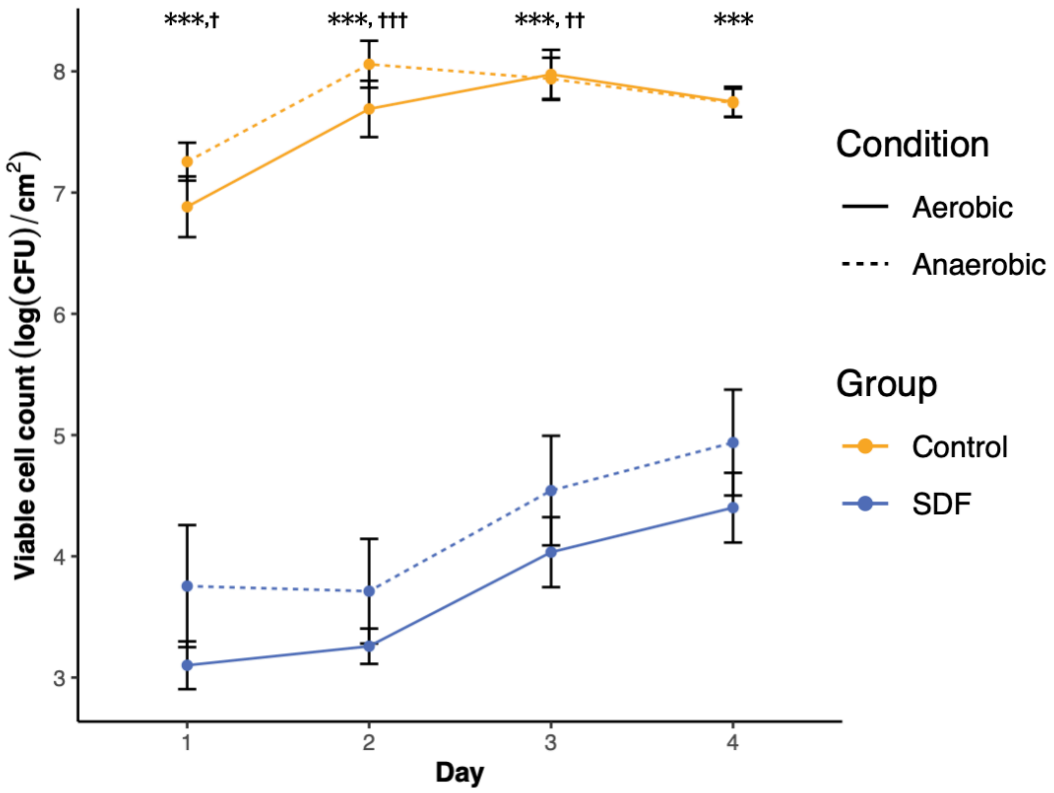

Fig 4. Viable cell count: Log CFU counts in aerobic and anaerobic conditions; data are presented as the mean ± SE (standard error) (repeated measure ANOVA, ***$p < 0.001$ for the aerobic condition; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ for the anaerobic condition).

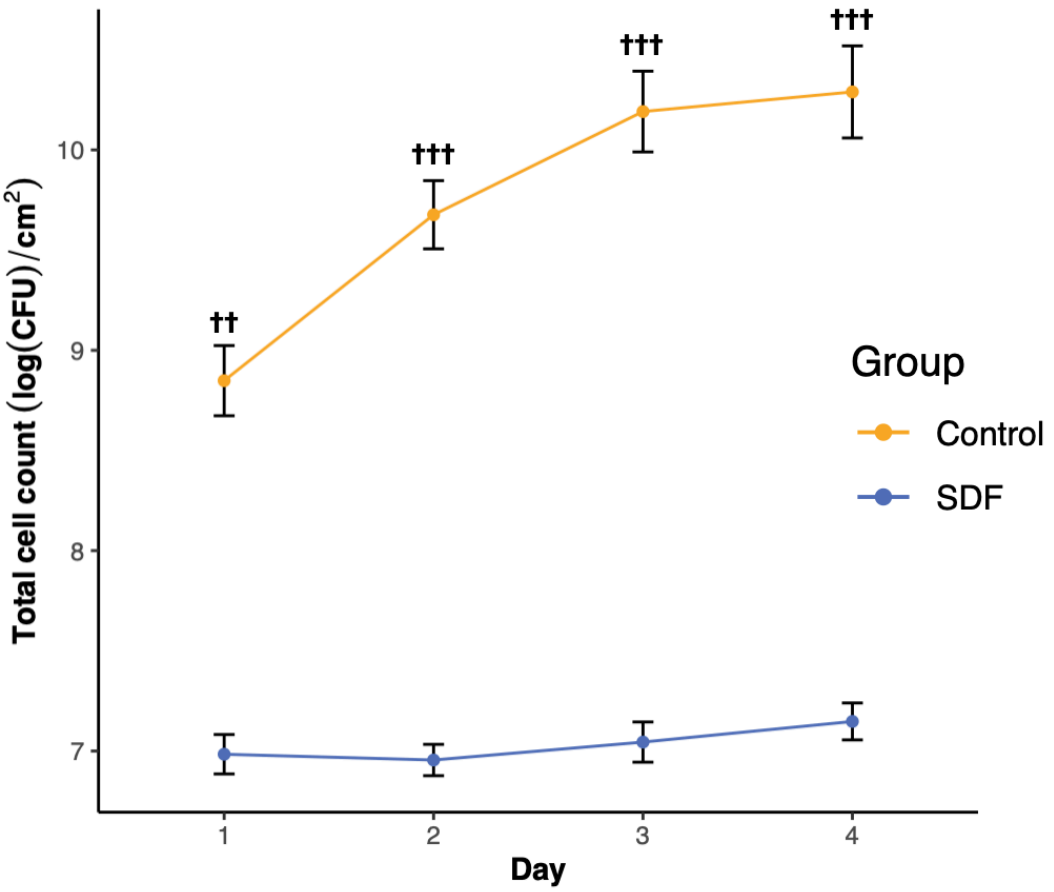

Fig 5. Quantification of biofilm-forming cells by real-time PCR: Number of total cell were assessed as log CFU per unit data are shown as the mean ± SE (repeated measure ANOVA; $^{††}p < 0.01$, $^{†††}p < 0.001$).

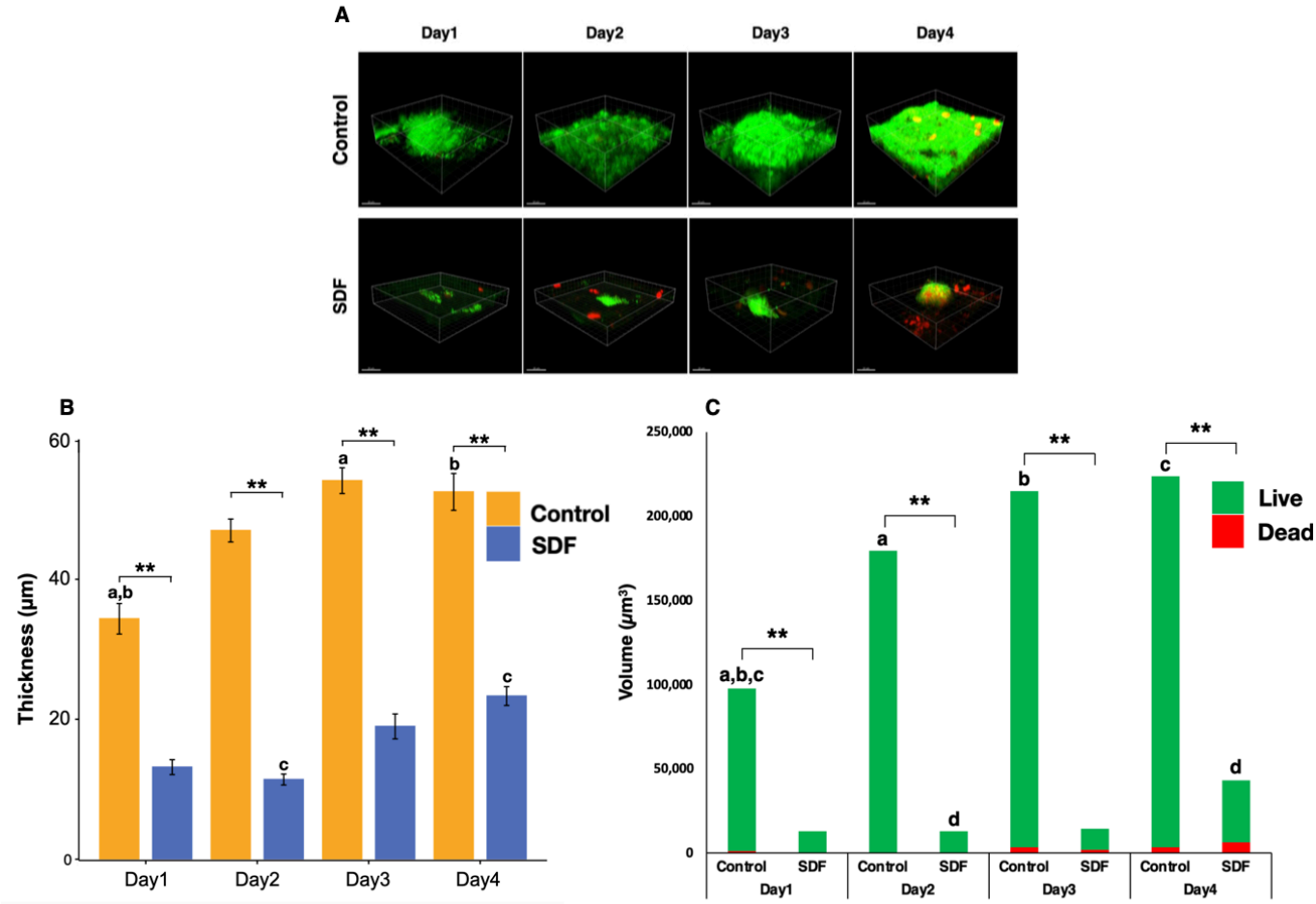

Fig 6. Confocal microscopy: CLSM analysis of biofilms grown on dentin slabs in the control and the SDF groups using Imaris imaging software. (A) Representative LIVE/DEAD staining images comparing biofilms in each group over the days. Live cells were labelled green and dead cells were labelled red (scale bar, 20 µm). (B) Thickness of the biofilm. (mean ± SD, repeated measure ANOVA with multiple pairwise comparisons using Bonferroni's correction, ** denotes significant difference between groups, $p < 0.01$; the same letters indicate significant differences among days within the same group, $p < 0.05$). **(C)** live to dead cell proportion of biofilms (Friedman test with multiple pairwise comparisons using Bonferroni's correction, ** indicates significant difference between groups, $p < 0.01$; the same letters indicate significant differences among days within the same group, $p < 0.05$).

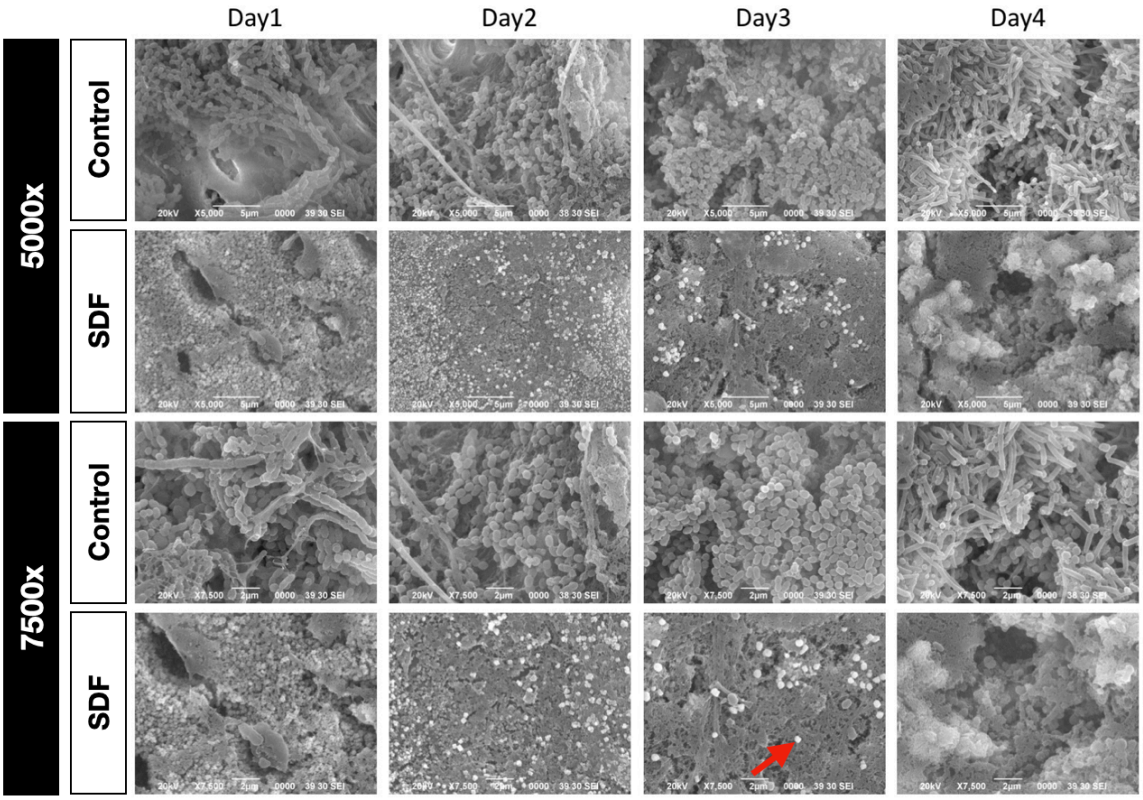

Fig 7. SEM observation: Representative SEM images of the biofilms in both groups by days. Red arrow indicates an example of the metallic-like particles precipitated on dentin slabs.

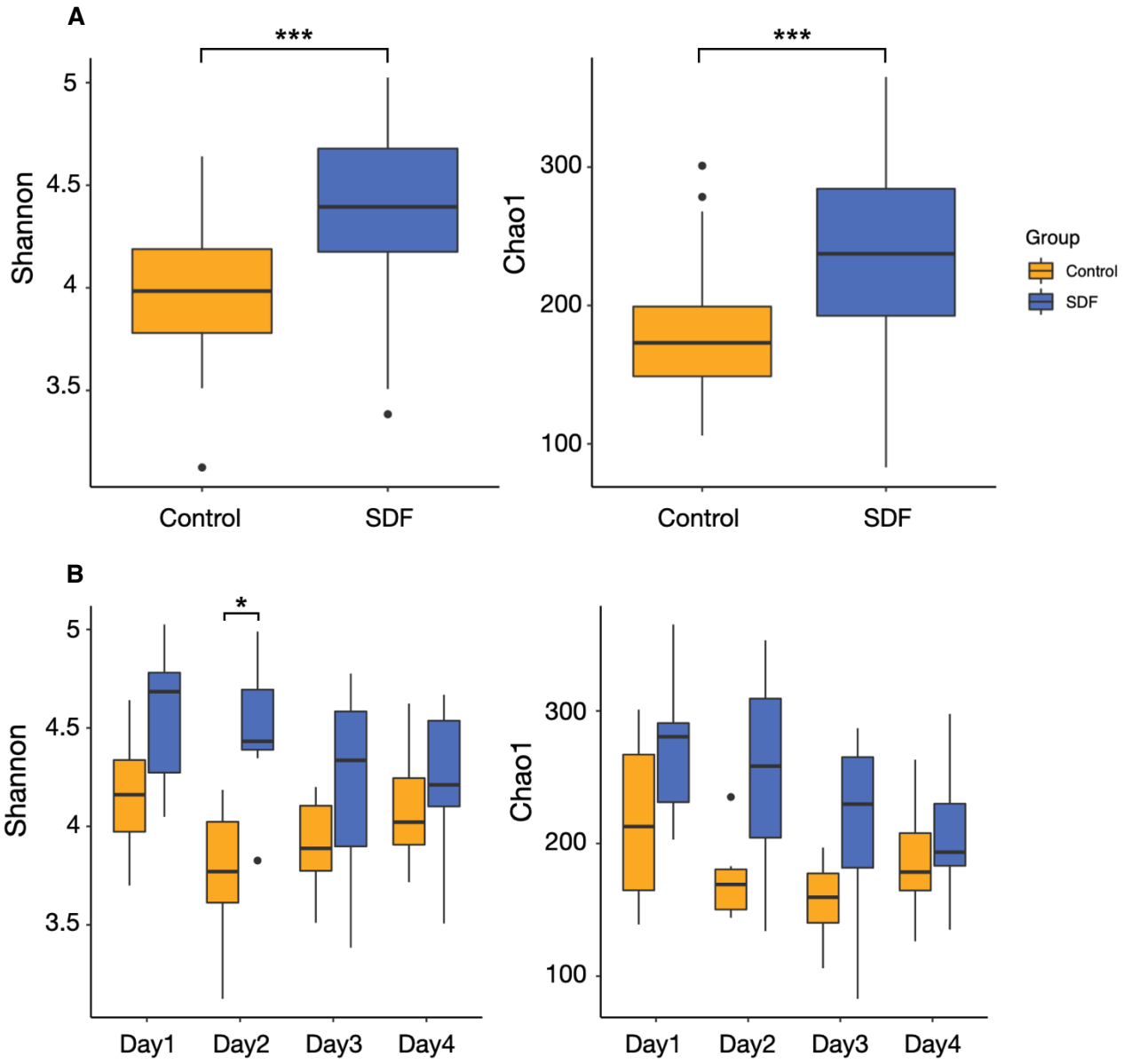

Fig 8. Alpha diversity: Shannon and Chao1 indices demonstrates the difference between groups. (A) Pooled data of biofilm from each group regardless of days. (B) Groups and days were taken into account (mean ± SD; A, Wilcoxon rank sum test; B, Kruskal–Wallis test; *$p < 0.05$, ***$p < 0.001$).

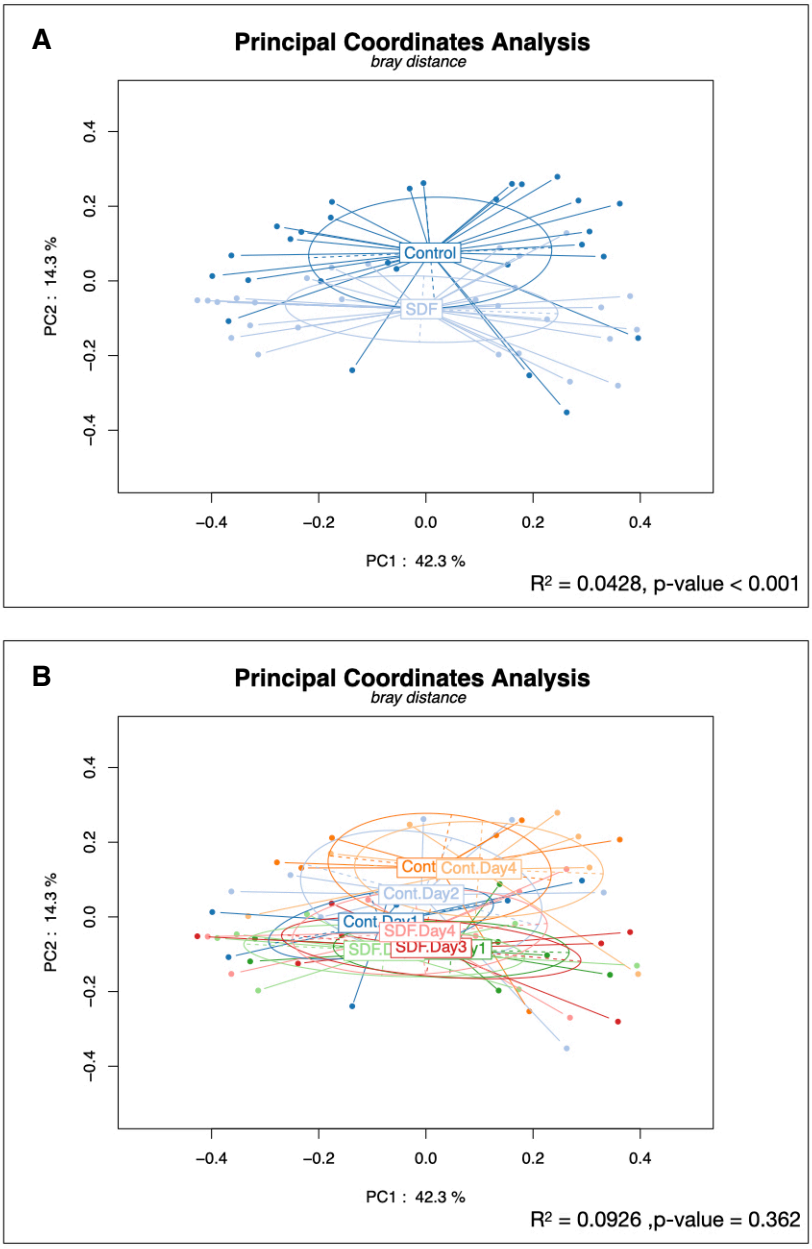

Fig 9. Beta diversity: Principal coordinates analysis (PCoA) plot of pooled biofilm samples from the control and the SDF groups based on Bray-Curtis dissimilarities**.** (A) Pooled data of biofilm from each group regardless of days. (B) Both group and day factors were assessed. Statistical comparisons were performed using PERMANOVA test.

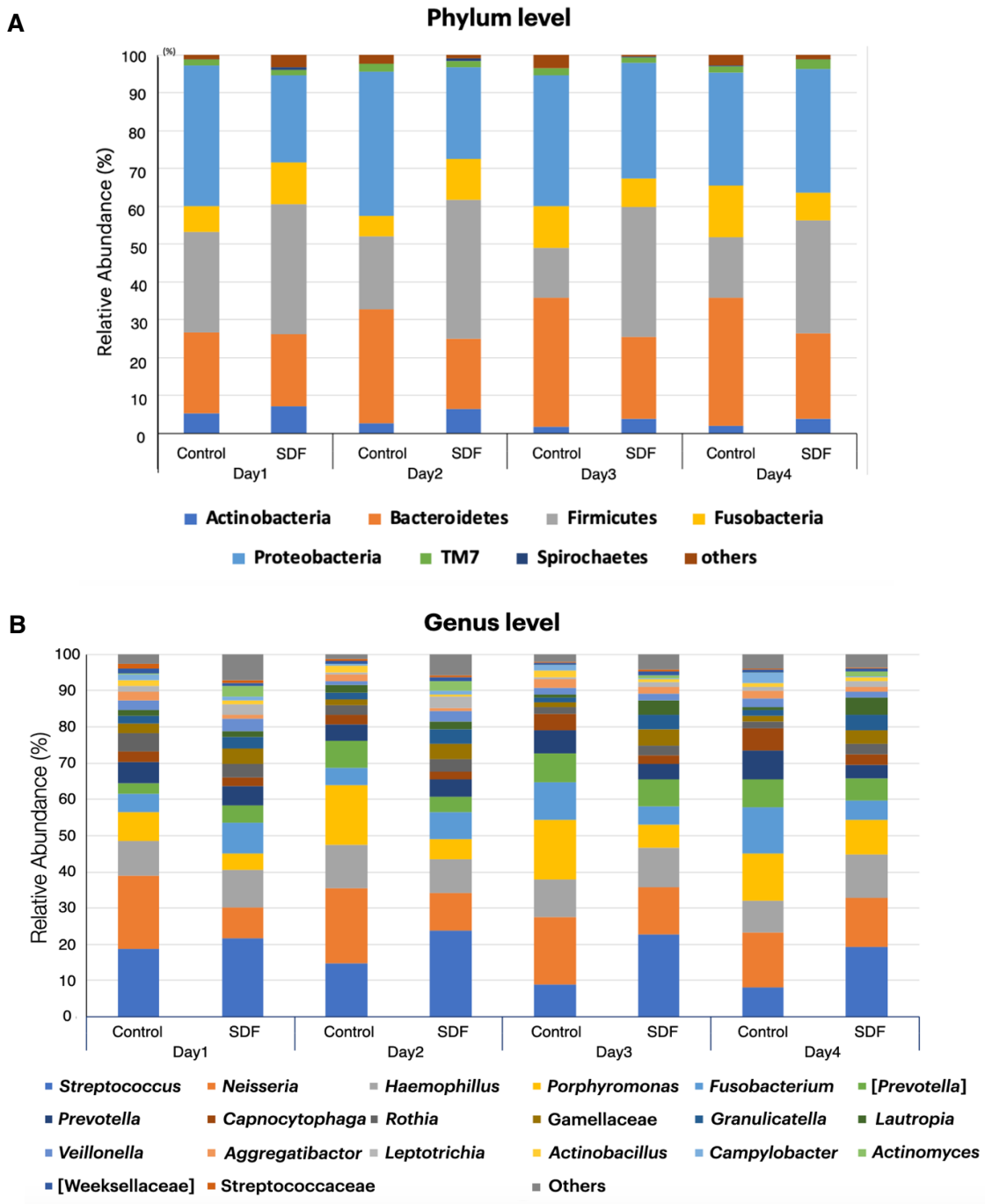

Fig 10. Relative abundance of taxa: (A) At the phylum level. (B) At the genus level.

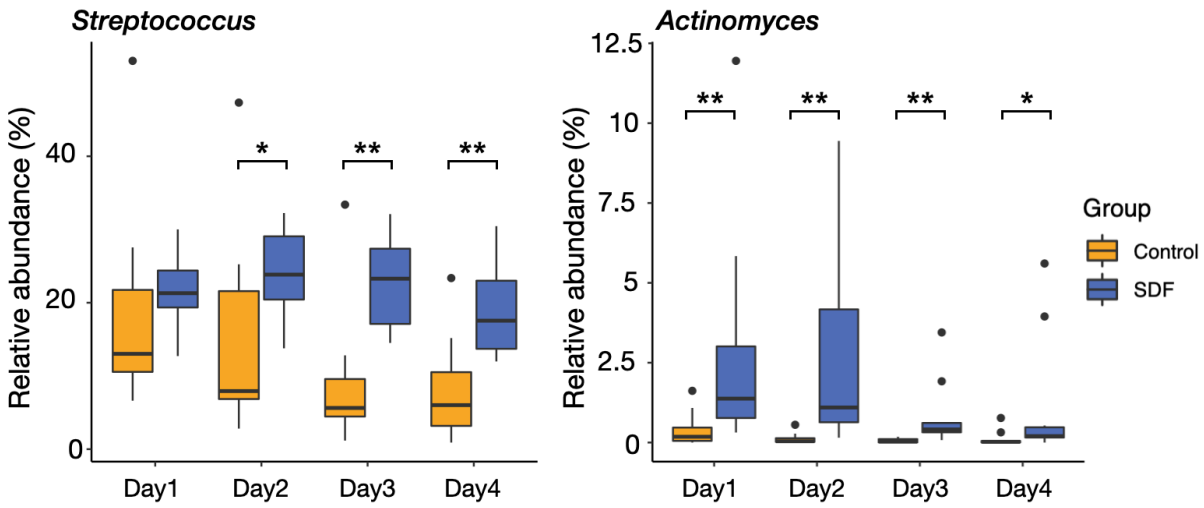

Fig 11. Comparison of microbial relative abundance: The relative abundances of the 20 most dominant genera were further analyzed. The statistical analysis revealed some significant differences between biofilm groups in *Streptococcus* and *Actinomyces* (mean ± SD; Kruskal–Wallis test, *p < 0.05, **p < 0.01, black circles refer to outliers of the data).

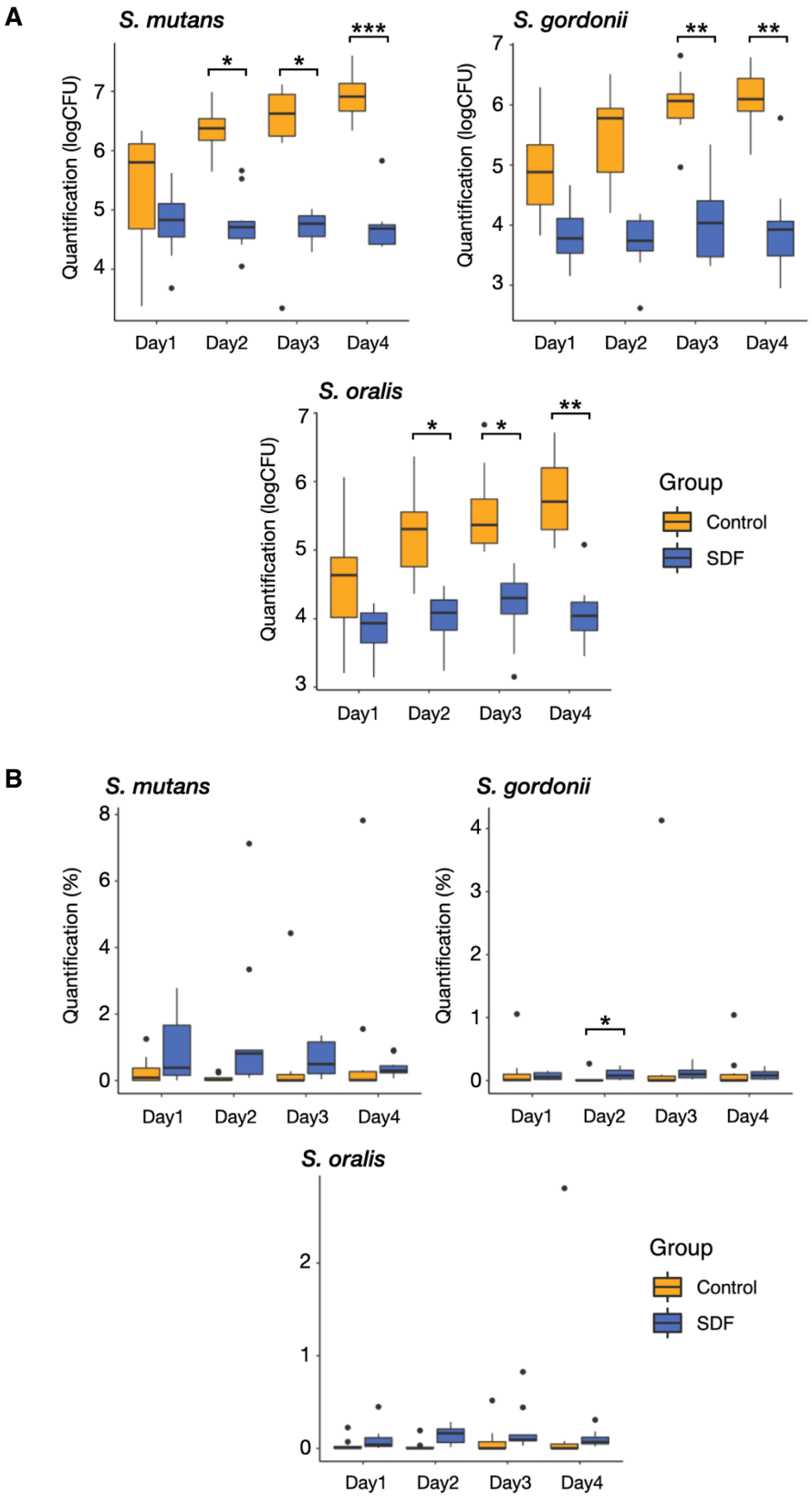

Fig 12. Quantification of biofilm-forming cells of specific streptococci by real-time PCR: (A) Cell numbers quantified by log CFU. (B) Percent-transformed cell numbers (mean ± SD, Kruskal–Wallis test, *p < 0.05, **p < 0.01, ***p < 0.001, black circles refer to the outlier of the data).