2018

A comparison of the antimicrobial efficacy of silver diamine fluoride and silver nitrate: an in vitro study

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A comparison of the antimicrobial efficacy of silver diamine fluoride and silver nitrate: an *in vitro* study

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

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Acknowledgements

The author wishes to thank several people. First, I would like to thank everyone whom made this project possible, especially Drs. Dahlke, Wunsch, Carrico, and Lewis. I would like to thank Ross, who was instrumental to the project. Lastly, I would like to thank my wonderful wife Sara who makes all things in life worth doing.

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Abstract

A COMPARISON OF THE ANTIMICROBIAL EFFICACY OF SILVER DIAMINE FLUORIDE AND SILVER NITRATE: AN *IN VITRO* STUDY

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

Virginia Commonwealth University, May 2018

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Purpose: To determine the antimicrobial efficacy of SDF and SN/NaF.

Methods: Three bacterial species were combined to create an *in vitro* biofilm. Treatment was completed with SN, SN/NaF, SDF, SDF½ or untreated (control).

Results: The untreated group demonstrated significantly higher growth than all other treatment groups across the study. On the BHI-plates (1-day), there were significant differences between all treatments except SDF and SDF½. On the BHI-plates (3-days), SN/NaF was not significantly different from SDF or SDF½. On the L-MRS-plates (1-day), both SN treatment groups yielded significantly higher growth than the SDF groups. On the L-MRS-plates (3-days), SN yielded significantly higher growth than SN/NaF, SDF, and SDF½.

Conclusion: SDF is more effective than SN/NaF, with the exception of BHI-plates (3-days) only and SN/NaF is more effective than SN on primarily *S. mutans* and *L. acidophilus*. There is evidence of a possible antimicrobial tolerance of oral bacteria to silver.

Introduction

Silver diamine fluoride (SDF) and silver nitrate (SN) have become increasingly popular in the United States. Pediatric dentists are utilizing these antimicrobial agents to help address the growing epidemic of dental caries. Dental caries affects 28% of all toddlers and preschool aged children and 23% percent of carious primary teeth remain untreated.[1] Dental caries is now the most common chronic disease experienced in children and is five times more common than asthma. The burden of this chronic disease experience falls on children of low socioeconomic status. Poor children's caries experience is twice that of affluent children and their disease is more likely to go untreated[1,2]. The effects of caries can be wide reaching. It has been demonstrated that caries results in the loss of 51 million school hours per year, with poor children experiencing nearly twelve times more restricted activity days than children from more affluent families.[2]

The first reported use of silver for its antimicrobial properties appears to have occurred as early as 1000 BC when water was stored in silver vessels as a means of rendering it potable**.**[3] In 1891, Stebbins reported on his use of argenti nitras to treat dental caries. Stebbins reasoned the known effects of nitrate of silver could be used to treat decay in the oral cavity and that led him to argenti nitras. Stebbins demonstrated a 61% inhibition of caries progression in primary teeth at three years following application.[4] In 1917, Howe found the application of silver nitrate to be

"effective in the sterilization of disintegrated dentin overlying pulps, as in the large cavities of carious first molars".[5] In 2014, Silver diamine fluoride was first approved for use in the United States and first became available for use in 2015.[6]

The following excerpt from Stebbins' 1891 paper accurately reflects the mindset regarding SDF and SN/NaF today:

"*Some patients would object to have it applied to any tooth on account of the color. Others would be pleased to have it used where the cavity would not be seen. But there are thousands who must have some treatment, or become edentulous. Children who are too sensitive to have teeth filled, or whose parents have not the means to pay for filling, must have some relief, or suffer untold misery, and lose their temporary teeth too soon, thereby involving themselves in life long troubles."*[4]

A recent survey of pediatric dentistry residency program directors demonstrated 25.7% of responding programs used SDF. Of the 74 directors, 68.9% expected that number to increase. The survey also demonstrated 9.5% of responding programs used SN/NaF. Of the 74 directors, 91.8% reported the most frequent barrier to use of SDF was parental acceptance, with the most cited concern being esthetics at over 80%.[7] A recent randomized clinical trial demonstrated parent satisfaction with the appearance of SDF treated teeth between 61.5% and 70.8%.[8]

The mechanisms of action for both SDF and SN are very similar, yet very complex. The silver particles interact with the sulfhydryl groups of proteins, which results in inhibition of respiratory processes, cell wall synthesis, and cell division. This also alters hydrogen bonding and results in DNA unwinding.[9–13] Bacterial metabolism is interfered with by silver interacting with the sulfhydryl groups and bacterial DNA.[14] These effects manifest as bacterial death and inhibition of biofilm formation.[15] Silver salts and silver phosphate form on dentin creating a

resistant layer and blocking dentin tubules respectively. The fluoride in SDF interacts with hydoxyapatite and calcium to create fluorapatite. This results in an increased acid tolerance of enamel and dentin, along with an increase in mineral density.[14] What we do not yet understand is how the antimicrobial effects of both silver and fluoride work in combination.

Although the mechanisms of action for SDF and SN/NaF are different they do result in similar outcomes. Both SDF and SN/NaF treatments result in the formation of dense granular structures in the inter-tubular areas of dentin. Silver nitrate with sodium fluoride varnish has an added benefit over SDF because it is believed the varnish prevents the SN from being washed away by saliva.[16]

Numerous *in vitro* studies have examined the effects of SDF applications on oral bacteria. Specifically, these studies have noted SDF application to inhibit *Streptococcus mutans* growth, *Lactobacillus acidophilus, and Actinomyces Naeslundii*.[17,18] The main pathologic species for dental caries have been identified and include *Streptococcus mutans, S. sobrinus, S. salivarius, S. vestibularis, S. parasanguinis, Lactobacillus acidophilus, L. gasseri, L. johnsonii, L. casei, L. paracasei, Veillonella atypical, V. dispar, and V. parvula.*[18–23] Most SDF and SN studies haven't studied the effects the medicaments have on the aforementioned species. These studies have also demonstrated SDF to inhibit biofilm formation, the metabolic activity of dental plaque organisms, and caries lesion depth progression.[15] One study has demonstrated the antimicrobial effects of SN *in vivo.* They concluded that the tested oral bacteria were most susceptible to SN.[24]

Safety of application of SDF and SN has and should be a concern. Silver toxicity is one of the concerns with SDF and SN/NaF. In one drop of 38% SDF, 25 microliters, there is 9.5 mg of silver. One drop of SDF is sufficient to treat at least five carious teeth. This has been shown to be 400 times lower than the lethal dose for a 10 kg child.[6] A recent study investigating the

adverse effects of SDF in preschool children reported no acute systemic illness or major adverse effects.[8] The most common adverse effects noted were tooth or gum pain at 6.6%.[8] SDF may have effects on the pulp as well. A new study demonstrated higher pulpal cell death as the dentin thickness between SDF and the pulp decreases.[25] In one drop of 25% SN, 25 microliters, there is 6.25 mg of silver. The amount at which a dose could be lethal is 2 grams**.** A dose of 6.25 mg of SN would then be 320 times less than the lethal dose.[26] With parental acceptance and incidence of oral sedation and general anesthesia for dental treatment increasing, it is important to note the risks of these procedures. Forty-four children died from dental treatment with oral sedation or general anesthesia between 1980 and 2011, while there has never been a reported death from SDF or SN/NaF treatment.[27]

Savas et al studied the effects of different antibacterial agents on enamel in biofilm caries model (mono species, *S. mutans*). The study compared the effects of SDF, acidulated phosphate fluoride (APF), ammonium hexafluorosilicate (AHF), AHF+ cetylpyridinium chloride (AHF+CPC), and 0.2% chlorhexidine (CHX) to distilled water. Their study demonstrated that SDF showed the highest antimicrobial activity ($p<0.05$).[28]

Klein et al studied the effects of four anti-caries agents on lesion depth progression. They compared the effectiveness of SN, SDF, CHX, and silver fluoride/stannous fluoride (AgF/SnF_2) to isotonic saline. Their study demonstrated 29% and 19% less lesion progression with AgF/SnF_2 and SN respectively.[29] This is one of the only studies to compare SN and SDF directly.

Chu CH et al studied the effects of SDF on dentine caries induced by *S. mutans* and *A. naeslundii*. Their study demonstrated that SDF significantly reduced the CFU counts of both *S. mutans and A. naeslundii* and the viable bacterial counts in biofilms of both were reduced to

zero. Using confocal laser scanning microscopy (CLSM) they determined the dead-to-live ratios were significantly higher in the test groups compared to groups receiving water treatment. Secondarily, the study demonstrated that SDF significantly reduced the demineralization of caries lesions in dentin.[30]

Mei ML et al studied the antimicrobial effects of SDF on dentine carious lesions with *S. mutans* and *L. acidophilus* co-cultured biofilms. Their study demonstrated log CFU counts were significantly lower in the SDF than those in the control group ($p<0.01$). They also demonstrated the dead-to-live ratio of the SDF group was significantly higher than the control group ($p=0.03$). Using CLSM, they demonstrated the majority of the bacteria in the SDF group were dead.[31] They also found an antimicrobial tolerance of the dual species biofilm, compared to a previous study which used a single species biofilm.[30,31] They concluded that under a more complex environment the bacteria species seem to survive better than compared to a mono species biofilm.[31] These findings supported the previously stated findings of multiple studies. One study demonstrated a biofilm composed of *S. mutans* and *V. parvula* was less susceptible to chlorhexidine than a mono species biofilm.[32] Another study reported a commensal relationship in dual species biofilms.[33] The mechanisms for this antimicrobial tolerance is not yet well understood.[34,35]

Mei ML and Li QL et al studied the antimicrobial effects of SDF on multi-species cariogenic biofilms. The bacteria included in their study were *S. mutans, S. sobrinus, L. acidophilus, L. rhamnosus, and A.* naeslundii. Their study demonstrated SDF inhibited the growth of all five species on carious dentine at the end of day 7, 14, and 21 ($p<0.01$). They also demonstrated the log CFU were lowest at day 7 and increased over time, though still significantly lower than in the control group. The study also found the biofilm in the test group to

be a mono-layer compared to the confluent biofilm in the control group.[36] For this study the investigators used 38% SDF because it was found to be the most effective concentration.[17,18,37,38]

There are many implications that would result from the proposed study. First, it would be the first time an *in vitro/ex vivo* study has studied the effects of SDF and SN/NaF in the same study. This would not only contribute to filling the gap in the current understanding of both products, but it could indicate whether more discussion is necessary regarding which product to use, based on antimicrobial efficacy. Secondly, it would be one of the few studies to examine the effects each product has on multiple oral microbial species. This is clinically relevant as clinical caries is not the result of one organism, but an entire oral microbiome.

Currently, there is a greater understanding of SDF than SN. More research on SN/NaF is needed and necessary if dentistry wants to be seen and held to the same standards as medicine. This study could be useful in guiding further research in this field and help the dental community design and implement further *in vitro*/*ex* vivo/in *vivo* studies to fill the gap in knowledge and contribute to our use of evidence based dentistry.

Specific Aim 1: To develop a reliable model to replicate an *in vivo* biofilm.
Specific Aim 2: To develop the protocol for a future *ex vivo* component of this project.
Specific Aim 3: To compare the antimicrobial efficacy of SDF and SN/NaF.
Specific Aim 4: To compare the antimicrobial efficacy of SN/NaF and SN.

Working hypothesis: SN/NaF will demonstrate a comparable antimicrobial efficacy to SDF. SN/NaF will demonstrate a greater antimicrobial efficacy than SN alone.

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Methods

Growth of Bacterial Strains

Three cariogenic bacteria were used for this study: *Streptococcus mutans* (*S. mutans,* strain ATCC 25175), *Lactobacillus acidophilus* (*L. acidophilus*, strain ATCC 4356), and *Veillonella atypica* (*V. atypica*, strain ATCC 17744). The bacteria were grown anaerobically in an atmosphere consisting of 85% N_2 10% CO_2 and 5% H_2 at 37 ºC in an anaerobic chamber (Coy Manufacturing). Liquid cultures of *S. mutans* were grown in brain heart infusion (BHI) broth and strains were maintained on BHI agar plates. Liquid cultures of *L. acidophilus* were grown in Lactobacillus MRS (L-MRS) liquid culture and maintained on L-MRS agar plates. Liquid cultures of *V. atypica* were grown in reinforced clostridial media (RCM) supplemented with 1% lactate and maintained on RCM + 1% lactate agar plates.

Creation of *in vitro* cariogenic biofilm and treatment

Overnight cultures of the cariogenic oral bacteria *S. mutans, L. acidophilus,* and *V. atypica* were grown in their respective media. Each bacterium was combined into a sample with a final OD_{660} of 0.1. The combined bacterial samples were grown in biofilm media consisting of 1 part BHI broth, 1 part L-MRS broth, and 1% sucrose. To form biofilms for treatment, a 24 well plate was coated with saliva to allow the bacteria to attach to the salivary proteins. To the coated wells, 500µL of the combined bacterial sample was added. The 24 well plates were incubated

for 24 hours under anaerobic conditions at 37°C, after which old media was aspirated off and fresh media added and grown for 24 more hours. After 48 hours of growth the biofilms were treated with 25% silver nitrate (SN), 12.5% silver nitrate + 2.5% sodium fluoride (SN/NaF), 38% silver diamine fluoride (SDF), 19% silver diamine fluoride (SDF½) or left untreated (control).

For treatment, excess media was aspirated off and the biofilms washed with saline. Each chemical was added directly to the biofilm for 1 minute at varying concentrations. Approximately 250 μL of each chemical will be added to the well, enough to cover the bottom of the well. After 1 minute of exposure, treatment will be removed and the biofilm will be washed 3 times with fresh media. After treatment, fresh media will be added to the biofilms and the biofilms will be grown for 1 or 3 days post-treatment. Treated biofilms will be removed from the wells and stored at -20°C until ready for qPCR analysis.

Survival of Cariogenic bacteria in Biofilm

To quantify the survival of the biofilm after exposure to the silver medicaments, treated and untreated samples were washed 3 times with PBS after 1 or 3 days incubation post-treatment. The biofilms were re-suspended via aspiration and scraping. The suspended biofilms were then diluted and plated on L-MRS and BHI plates. Colonies appeared on the plates after 36-48 hours of anaerobic growth. Colonies were counted and CFU per mL of calculated.

Quantitative PCR analysis of Biofilms

To quantify the composition of the untreated biofilm, real-time quantitative PCR (qPCR) was performed using a syber green based detection system on an Applied Biosystems 7500 fast real-time PCR system. Primers used in this study are listed in Table 1. Genomic DNA from biofilms was harvested using the Wizard Genomic DNA Purification Kit (Promega) following manufactures protocol. The qPCR reaction was performed using a SYBR green qPCR mix

(Applied Biosystems). The equivalent of 10 ng of gDNA was added to each reaction and experimental samples were tested in triplicate.

Crystal violet biomass quantification

After incubation and treatment of biofilms, supernatants were removed and the biofilm was washed once with PBS. 250 μL of a 0.1% crystal violet solution was added to each well of the 24 well plate and incubated for 15 minutes at room temperature after which excess crystal violet solution was removed. The plate was rinsed 3-4 times with water by submerging the plate. The plate was shaken and blotted vigorously to remove un-attached cells and excess dye. After washing the 24 well plate was set out to dry overnight at room temperature.

To quantify the biofilm, 500 μL of 30% acetic acid was added to each well to solubilize the crystal violet. After a 15-minute incubation, the crystal violet-acetic acid solution was removed from the well and placed in cuvettes where it was diluted with 500 μL of water. The absorbance was measured at 550 nm on a Biomate 3S UV-Vis spectrophotometer (Thermo-Fisher Scientific).

Statistical Methods

Repeated measures ANOVA was used to estimate qPCR data and demonstrated each bacteria's presence in the overall samples. Poisson regression was used to estimate bacteria colony counts as a function of treatment group, grow time, and the interaction between grow time and treatment group. A random effect for experiment date was used to adjust for a possible plate effect. Post hoc pairwise comparisons were adjusted for using Tukey's HSD. Biomass after one and three days growth were compared using repeated measures ANOVA to determine if longer grow time resulted in greater biomass, while adjusting for a random plate effect.

A significance level of 0.05 was used for all analyses. All analyses were performed with SAS EG v.6.1.

Results

Results from the qPCR data demonstrate a significant presence of all 3 of the expected bacteria (Table 2). Since lower Ct values indicate more bacteria present, the highest concentrations of bacteria were of *L. acidophilus* followed by *V. atypica* and lastly, *S. mutans*.

Due to excessive growth in the untreated group, the sample was diluted significantly more than the treated groups. After accounting for the dilution factor, the raw colony count created issues with modeling due to the amount of variability introduced. Therefore, the analysis was performed on the unadjusted colony counts (i.e. assumes dilution was same across all samples). Therefore, any differences demonstrated in the data are even more severe after accounting for the additional dilution (1:100 vs. 1:100,000).

In the analysis with diluted untreated group on BHI plates, the effects of the treatment differed across the treatments and grow times (p-value<0.0001). The estimated *S. mutans* colony counts are given in Figure 1 and Table 3. Post hoc pairwise comparisons were performed with Tukey's adjustment for multiple comparisons. After one day of growth, there were significant differences between all treatments except SDF and SDF ½ (p-value=0.9895, Table 3). Even without adjusting for the additional dilution applied to the untreated group, the untreated group still had significantly more growth at one day than any of the treatment groups. The second highest growth was seen in SN, followed by SN/NaF. The lowest growth was seen in both SDF

and SDF ½. After three days of growth, the untreated group still had significantly greater growth than all the other groups (Table 4). In terms of the treated groups, the highest growth was in SN group. The SN/NaF group was not significantly different from SDF or SDF ½ (p-value=0.6795, 0.2781 respectively). SDF demonstrated higher growth than SDF ½ (p-value=0.0195).

In the analysis with diluted untreated group on L-MRS plates, the effects of the treatment differed across the treatments and grow times (p-value<0.0001). The estimated *L. acidophilus* colony counts are given in Figure 2 and Table 5. Again, there was significantly higher growth in the untreated group than any of the treated groups even without adjusting for the additional dilution in the untreated group (p-value<0.0001 for all comparisons, Table 5). For one-day growth, the differences between SN and SN/NaF were not statistically significant (p-value=0.8482). Both silver nitrate treatment groups yielded significantly higher growth than the two SDF groups (Table 6). The difference in growth was also not significantly different for SDF and SDF ½ (p-value=1.00). After three days of growth, SN yielded significantly higher growth than SN/NaF (p-value<0.0001), SDF (p-value<0.0001), and SDF½ (p-value<0.0001). SDF yielded significantly lower three-day growth than SN/NaF (p-value=0.0007) and SDF ½ (p-value=0.0077). The difference in three-day growth for SN/NaF and SDF ½ was not statistically significant (p-value=0.9711).

Results from biomass analysis yielded no difference in biomass based on the treatment group (p-value=0.4227) or number of grow days (p-value=0.8722).

Discussion

The first aim of this study was to develop a reliable model to replicate an *in vivo* biofilm. Due to a lack of data or information on multispecies biofilm formations, the authors had to develop a new multispecies biofilm. Initially, the authors attempted to establish single species biofilms consisting of *S. mutans*, *L. acidophilus*, or *V. atypica*. These three bacteria were selected to represent the multiple species that can be found in a cariogenic biofilm. This attempt was unsuccessful as only *S. mutans* formed a stable biofilm. This demonstrated that *L. acidophilus* and *V. atypica* would not form a stable biofilm alone. Previous studies have demonstrated that *L. acidophilus* does not grow well alone, but growth will be promoted by the addition of *S.* mutans.[39,40] One paper documented significantly less growth of *Veillonella parvula* in a single species biofilm than in combination with *S. mutans* in a dual species biofilm.[32]

The authors next attempt to form a biofilm was much more successful. The authors implemented and followed the aforementioned protocol (Methods - Creation of *in vitro* cariogenic biofilm). This procedure produced biofilms that were stable enough to undergo treatment without destroying the biofilm. This will be the first documented procedure to successfully combine these three different bacteria into a successful multispecies biofilm. To quantify the composition of the biofilms qPCR was completed. The highest concentrations of bacteria were of *L. acidophilus* followed by *V. atypica* and lastly, *S. mutans*. This result was surprising, but demonstrated that *L. acidophilus* and *V. atypica* grow much better when *S. mutans* is present.

This finding supports the literature on growth of *L. acidophilus* and represents the first study to demonstrate improved growth of *V. atypica* in the presence of *S. mutans*.

To quantify the biomass of the biofilms, crystal violet assay was performed. This, however, proved to be unsuccessful due to the silver component of SDF and SN. The silver interfered with the crystal violet assay, due to the precipitation and interactions of silver. As stated earlier, silver particles interact with the sulfhydryl groups of proteins, which results in inhibition of respiratory processes, cell wall synthesis, and cell division. This also alters hydrogen bonding and results in DNA unwinding.[9–13] It is generally understood that single stranded DNA is much less stable than double stranded DNA. Also, other "junk" particles and precipitants interfered with the crystal violet assay. As a result, it was not feasible to quantify the biomass of the biofilms alone. This is one limitation of this study.

After one day of growth on the BHI plates (primarily *S. mutans*), it appears SDF was more effective than SN/NaF, with the exception of three days of growth, and SN/NaF was more effective than SN (Table 4). The variability in growth between one day of growth and three days of growth, in groups SN/NaF and SDF ½, may be due to laboratory error.

Similarly, after one day of growth on the L-MRS plates (primarily *L. acidophilus*), both SN/NaF and SN groups yielded significantly higher growth than SDF and SDF ½ groups (Table 6). Therefore, SDF may be more effective than SN/NaF and SN/NaF is more effective than SN.

Results from this study also suggest evidence of a possible antimicrobial tolerance of oral bacteria to silver medicaments (SDF and SN/NaF), as previously reported.[30–36] Still, there is not a great understanding about the resistance of oral bacteria to silver medicaments. This is an important finding for many reasons. One, it expands our knowledge and understanding of how these silver products work on oral bacteria. Two, it may reinforce the need for multiple clinical

applications of either SDF or SN/NaF. Lastly, this is the first study to demonstrate evidence of a possible antimicrobial tolerance with SN/NaF.

There were additional limitations to the study. Due to the poor growth of *V. atypica*, the authors were unable to estimate the effect of the treatment groups on this portion of the biofilm. An aqueous NaF was used for this study to prevent destruction of the biofilms. This was a limitation as well because the NaF in the SN/NaF protocol utilized by dentists is a varnish. Future studies should also include NaF only as a treatment group.

Future studies should investigate the antimicrobial efficacy of SDF and SN/NaF on an *ex vivo* biofilm. A study of this nature could determine the specific bacterial species in healthy children and children who suffer from early childhood caries and establish an *ex vivo* biofilm model for which the antimicrobial efficacy of SDF and SN/NaF can be measured. Additionally, the evidence of a possible antimicrobial tolerance to SDF and SN/NaF should be evaluated.

Conclusion

- This is the first study to develop a reliable multispecies biofilm containing *S. mutans, L. acidophilus,* and *V. atypica*.
- SDF is more effective than SN/NaF, with the exception of three days of growth, and SN/NaF is more effective than SN on primarily *S. mutans*.
- SDF is more effective than SN/NaF and SN/NaF is more effective than SN on primarily *L. acidophilus*.
- There is evidence of a possible antimicrobial tolerance of oral bacteria to silver medicaments, SDF and SN/NaF, and this is the first study to report evidence of a possible antimicrobial tolerance to SN/NaF.

References

1. Dye B a, Tan S, Smith V, et al. Trends in oral health status: United States, 1988-1994 and 1999-2004. *Vital Health Stat 11*. 2007;(248):1-92. doi:10.1080/713604647.

2. Services USD of H and H. *Oral Health in America: A Report of the Surgeon General*. Rockville, MD; 2000.

3. Russell A, Hugo W. Antimicrobiological Activity and Action of Silver. *Prog Med Chem*. 1994;31:351-370. doi:10.1016/j.pcad.2015.11.006.

4. Stebbins EA. What value has argenti nitras as a therapeutic agent in dentistry? *Int Dent J*. 1981;12:661-670. doi:10.1016/j.pcad.2015.11.006.

5. Howe P. A method of sterilizing and at the same time impregnating with a metal affected dentinal tissue. *Dent Cosm*. 1917;59:891-904. doi:10.1016/j.pcad.2015.11.006.

6. 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. doi:10.1007/978-1-4614-5915-6.

7. Nelson T, Scott JM, Crystal YO, Berg JH, Milgrom P. Silver Diamine Fluoride in Pediatric Dentistry Training Programs: Survey of Graduate Program Directors. *Pediatr Dent*. 2016;38(3):212-217. http://www.ncbi.nlm.nih.gov/pubmed/27306245.

8. Duangthip D, Fung MHT, Wong MCM, Chu CH, Lo ECM. Adverse Effects of Silver Diamine Fluoride Treatment among Preschool Children. *J Dent Res*. 2017:2203451774667. doi:10.1177/0022034517746678.

9. OPPERMANN RV, JOHANSEN JR. Effect of fluoride and non-fluoride salts of copper, silver and tin on the acidogenicity of dental plaque in vivo. *Eur J Oral Sci*. 1980;88(6):476-480. doi:10.1111/j.1600-0722.1980.tb01257.x.

10. OPPERMANN RV, ROLLA G, JOHANSEN JR, ASSEV S. Thiol groups and reduced acidogenicity of dental plaque in the presence of metal ions in vivo. *Eur J Oral Sci*. 1980;88(5):389-396. doi:10.1111/j.1600-0722.1980.tb01244.x.

11. OPPERMANN, RUI VICENTE, Rolla G. Effect of Some Polyvalent Cations on the Acidogenicity of Dental Plaque in vivo. *Caries Res*. 1980;14:422-427.

12. Lansdown AB. Siver in healthcare: antimicrobial effects and safety in use. *Curr Probl Dermatol*. 2006;33:17-34.

13. Lansdown AB. Silver I: its antibacterial properties and mechanism of action. *J Wound Care*. 2002;11(4):125-130.

14. Chibinski AC, Wambier LM, Feltrin J, Loguercio AD, Wambier DS, Reis A. Silver Diamine Fluoride Has Efficacy in Controlling Caries Progression in Primary Teeth: A Systematic Review and Meta-Analysis. *Caries Res*. 2017;51(5):527-541. doi:10.1159/000478668.

15. Wu MY, 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.

16. Zhao IS, Mei ML, Li QL, Lo ECM, Chu CH. Arresting simulated dentine caries with adjunctive application of silver nitrate solution and sodium fluoride varnish: an in vitro study. *Int Dent J*. 2017;67(4):206-214. doi:10.1111/idj.12291.

17. 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.

18. 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;40:962-967. http://dx.doi.org/10.1016/j.jebdp.2010.11.011.

19. Gross EL, Leys EJ, Gasparovich SR, et al. Bacterial 16S sequence analysis of severe caries in young permanent teeth. *J Clin Microbiol*. 2010;48(11):4121-4128. doi:10.1128/JCM.01232-10.

20. Gross EL, Beall CJ, Kutsch SR, Firestone ND, Leys EJ, Griffen AL. Beyond Streptococcus mutans: Dental Caries Onset Linked to Multiple Species by 16S rRNA Community Analysis. *PLoS One*. 2012;7(10). doi:10.1371/journal.pone.0047722.

21. Marsh PD, Moter A, Devine DA. Dental plaque biofilms : communities , conflict and control. *Periodontology*. 2011;55(90):16-35.

22. Marsh PD. Are dental diseases examples of ecological catastrophes? *Microbiology*. 2003;149(2):279-294. doi:10.1099/mic.0.26082-0.

23. Quivey RG J. Caries. *Oral Microbiol Immunol*. 2006:233-252. doi:10.1016/j.pcad.2015.11.006.

24. Youravong N, Carlen A, Teanpaisan R, Dahlén G. Metal-ion susceptibility of oral bacterial species. *Lett Appl Microbiol*. 2011;53(3):324-328. doi:10.1111/j.1472-765X.2011.03110.x.

25. Burgess JO, Vaghela PM. Silver Diamine Fluoride: A Successful Anticarious Solution with Limits. *Adv Dent Res*. 2018;29(1):131-134. doi:10.1177/0022034517740123.

26. Gao SS, Zhao IS, Duffin S, Duangthip D, Lo ECM, Chu CH. Revitalising silver nitrate for caries management. *Int J Environ Res Public Health*. 2018;15(1).

doi:10.3390/ijerph15010080.

27. Horst JA. Silver Fluoride as a Treatment for Dental Caries. *Adv Dent Res*. 2018;29(1):135-140. doi:10.1177/0022034517743750.

28. Savas S, Kucukyilmaz E, Celik EU, Ates M. Effects of different antibacterial agents on enamel in a biofilm caries model. *J Oral Sci*. 2015;57(4):367-372. doi:10.2334/josnusd.57.367.

29. Klein U, Kanellis M, Drake D. Effects of four anticaries agents on lesion depth progression in an in vitro caries model. *Pediatr Dent*. 1999;21(3):176-180. doi:10.1016/j.pcad.2015.11.006.

30. Chu CH, Mei L, 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.

31. 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):2-9. doi:10.4317/medoral.18831.

32. Kara D, Luppens SBI, Cate JM. Differences between single- and dual-species biofilms of Streptococcus mutans and Veillonella parvula in growth, acidogenicity, and susceptibility to chlorhexidine. *Eur J Oral Sci*. 2006;114:58-63. doi:10.1172/JCI109382.

33. Cowan SE, Gilbert E, Liepmann D, Keasling JD. Commensal interactions in a dual-species biofilm exposed to mixed organic compounds. *Appl Environ Microbiol*. 2000;66(10):4481-4485. doi:10.1128/AEM.66.10.4481-4485.2000.

34. Burmølle M, Webb JS, Rao D, Hansen LH, Sørensen SJ, Kjelleberg S. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Appl Environ Microbiol*. 2006;72(6):3916-3923. doi:10.1128/AEM.03022-05.

35. Behnke S, Camper AK. Chlorine dioxide disinfection of single and dual species biofilms, detached biofilm and planktonic cells. *Biofouling*. 2012;28(6):635-647. doi:10.1080/08927014.2012.700705.

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

37. Fung MHT, Duangthip D, Wong MCM, Lo ECM, Chu CH. Arresting Dentine Caries with Different Concentration and Periodicity of Silver Diamine Fluoride. *J Dent Res Clin Transl Res*. 2016;1(2):143-152. doi:10.1177/2380084416649150.

38. Fung MHT, Duangthip D, Wong MCM, Lo ECM, Chu CH. Randomized Clinical Trial of 12% and 38% Silver Diamine Fluoride Treatment. *J Dent Res*. 2017:2203451772849. doi:10.1177/0022034517728496.

39. Filoche SK, Anderson SA, Sissons CH. Biofilm growth of Lactobacillus species is promoted by Actinomyces species and Streptococcus mutans. *Oral Microbiol Immunol*. 2004;19(5):322-326. doi:10.1111/j.1399-302x.2004.00164.x.

40. Merritt J, Xu P, Kreth J, et al. Streptococcus mutans Displays Altered Stress Responses While Enhancing Biofilm Formation by Lactobacillus casei in Mixed-Species Consortium. *Artic Front Cell Infect Microbiol*. 2017;7(7):5243389-5243524. doi:10.3389/fcimb.2017.00524.

Tables

Table 1: Primers Used in this Study

16s Primers	Sequence
L. acidophilus-Forward	5'-GGATAGAGGTAGTAACTGGCCTTTATT-3'
L. acidophilus-Reverse	5'-CAGTTTCCGATGCAGTTCCTCG-3'
S. mutans-Forward	5'-GCACACCGTGTTTTCTTGAGTCG-3'
S. mutans-Reverse	5'-CGGCTATGTATCGTCGCCTT-3'
V. atypica-Forward	5'-CGGCTACTGATCATCGCCTT-3'
V. atypica-Reverse	5'-ATCTTAGTGGCGAACGGGTG-3'

Table 2: Results from qPCR Analysis of Constructed Samples

Detector	Estimated Mean Ct Value	SE	
L. acidophilus	15.75	0.394	a
S. mutans	21.41	0.394	b
V. atypica	18.60	0.394	c

*Lower Ct values indicate greater presence in samples; Items with different letters are statistically significantly different

Table 3: Mean Colony Counts by Treatment Group and Grow Time for *S. mutans* on BHI Plates

Treatment Group	Grow Time	Mean Colony Count*	SE Mean	
SN	1 Day	27.4	8.05	a
	3 Days	84.4	30.55	A
SN/NaF	1 Day	15.0	4.48	b
	3 Days	5.4	2.27	B, C
SDF	1 Day	6.5	2.11	c
	3 Days	9.1	3.62	C
SDF ½	1 Day	5.1	1.70	c
	3 Days	1.6	0.87	D
Untreated (Diluted)	1 Day	160.0	44.64	d
	3 Days	216.5	77.83	E

*Results from Poisson Regression model; lower case letters for 1-day growth; capital for 3 day growth; those with same letters are not significantly different

Table 4: Pairwise Comparisons for *S. mutans* Growth on BHI Plates

Comparison		Difference (LogCFUs)	Adjusted P-value
1-day Growth: BHI			
SN	SN/NaF	0.6	0.0147
SN	SDF	1.4	<.0001
SN	SDF ½	1.7	<.0001
SN	Untreated	-1.8	<.0001
SN/NaF	SDF	0.8	0.0106
SN/NaF	SDF 1/2	1.1	0.0017
SN/NaF	Untreated	-2.4	<.0001
SDF	SDF ½	0.3	0.9895
SDF	Untreated	-3.2	<.0001
SDF ½	Untreated	-3.5	<.0001
3-day Growth: BHI			
SN	SN/NaF	2.8	<.0001
SN	SDF	2.2	<.0001
SN	SDF ½	4.0	<.0001
SN	Untreated	-0.9	<.0001
SN/NaF	SDF	-0.5	0.6795
SN/NaF	SDF ½	1.2	0.2781
SN/NaF	Untreated	-3.7	<.0001
SDF	SDF ½	1.7	0.0195
SDF	Untreated	-3.2	<.0001
SDF ½	Untreated	-4.9	<.0001

*Tukey's adjusted p-values

Table 5: Mean Colony Counts by Treatment Group and Grow Time for *L. acidophilus* on L-MRS Plates

Treatment Group	Grow Time	Mean Colony Count	SE Mean	
SN	1 Day	18.4	6.2	a
	3 Days	148.5	61.9	A
SN/NaF	1 Day	14.6	4.9	a
	3 Days	33.8	14.4	B
SDF	1 Day	6.0	2.2	b
	3 Days	13.2	5.8	C
SDF ½	1 Day	6.5	2.3	b
	3 Days	28.8	12.3	B
Untreated	1 Day	313.5	100.9	c
	3 Days	540.2	224.1	D

*Results from Poisson Regression model; lower case letters for 1-day growth; capital for 3-day growth; those with same letters are not significantly different

Table 6: Pairwise Comparisons for *L. acidophilus* Growth on L-MRS Plates

Comparison		Difference (LogCFUs)	Adjusted P-value*
1-day Growth: L-MRS			
SN	SN/NaF	0.2	0.8482
SN	SDF	1.1	0.0003
SN	SDF ½	1.0	0.0005
SN	Untreated	-2.8	<.0001
SN/NaF	SDF	0.9	0.0045
SN/NaF	SDF ½	0.8	0.0088
SN/NaF	Untreated	-3.1	<.0001
SDF	SDF ½	-0.1	1.0000
SDF	Untreated	-4.0	<.0001
SDF ½	Untreated	-3.9	<.0001
3-day Growth: L-MRS			
SN	SN/NaF	1.5	<.0001
SN	SDF	2.4	<.0001
SN	SDF ½	1.6	<.0001
SN	Untreated	-1.3	<.0001
SN/NaF	SDF	0.9	0.0007
SN/NaF	SDF ½	0.2	0.9711
SN/NaF	Untreated	-2.8	<.0001
SDF	SDF ½	-0.8	0.0077
SDF	Untreated	-3.7	<.0001
SDF ½	Untreated	-2.9	<.0001

*Tukey's adjusted p-values

Figures

Figure 1: Mean *S. mutans* Colony Count by Treatment and Grow Time

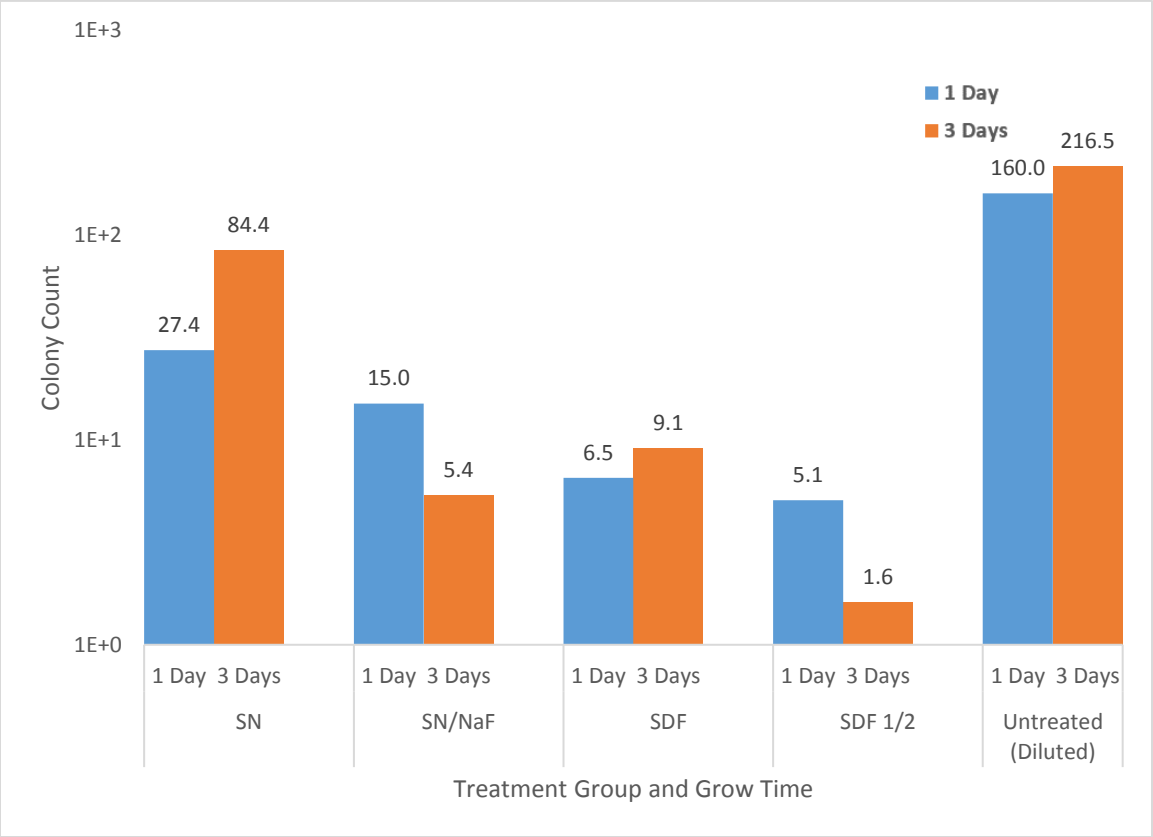

Figure 2: Mean *L. acidophilus* Colony Count by Treatment and Grow Time