Title
The Effects of White Spot Lesion Therapy on Different Salivary Parameters After Removal of Fixed Orthodontic Appliances: A Pilot Randomized Clinical Trial

Author
Setoudeh Maram, Shahin

Publication Date
2023

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

The Effects of White Spot Lesion Therapy on Different Salivary Parameters After Removal of Fixed Orthodontic Appliances: A Pilot Randomized Clinical Trial

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Oral Biology

by

Shahin Setoudehmaram

2023

ABSTRACT OF THE THESIS

The Effects of White Spot Lesion Therapy on Different Salivary Parameters After Removal of Fixed Orthodontic Appliances: A Pilot Randomized Clinical Trial

by

Shahin Setoudehmaram

Master of Science in Oral Biology

University of California, Los Angeles, 2023

Professor Renate Lux, Chair

Although orthodontic treatment does not directly damage the teeth, it causes a change in the oral environment leading to an increased risk of periodontal disease and white spot lesions (WSLs), which are visible areas of demineralized enamel. This study aims to investigate the efficacy of different commercially available fluoride varnishes to treat WSLs after the completion of orthodontic treatment and evaluate their effect on the salivary parameters and the oral microbiome. The study followed a split-mouth design, with each of the subjects receiving different treatment on the left and right sides of their mouth. Subjects were assigned to one of three groups, with each group receiving two of the following three treatment options: placebo varnish (petroleum jelly), 5% sodium fluoride varnish (Henry Schein Acclean), and a light-cured fluoride varnish (3M XT Extended Contact Varnish). There was a total of 4 visits for this study: T0 (within 30 days of removing braces), T1 (day 30), T2 (day

90), and T3 (day 180). At each visit, subjects completed an Oral Health Questionnaire, and the severity of the WSLs was assessed using DIAGNOdent laser fluorescence pen. The scores were used to assess the efficacy of the different interventions at re-mineralizing WSLs. To test the resting flow rate of saliva and salivary consistency, lower lip labial secretion and the resting salivary consistency in the oral cavity were visually assessed, respectively. Collected saliva was kept for measuring flow rate, pH, and buffering capacity.

Although the study is still ongoing and therefore had a small sample size of 17 subjects enrolled at the time of data analysis, some notable trends were observed. DIAGNOdent measurements showed an interesting trend with a decrease of 20% in scores for WSLs treated with the placebo varnish and 5% NaF groups over the observation period. In contrast, the LCFV group showed a small albeit not significant increase in DIAGNOdent scores during the first 6 months after orthodontic treatment. The differences in pH between the treatments were relatively small and not clinically significant. When examining the colony-forming units (CFUs), an interesting pattern emerged in terms of the proportions of *S. mutans* and *C. albicans* relative to the total microbial load. The non-significant 9.75% increase in total microbial load, was accompanied by an 81.66% decrease in *C. albicans* load, and an 81.35% decrease in *S. mutans* load. This resulted in an overall decreasing trend in the proportions of *S. mutans* and *C. albicans* relative to the total bacterial load. However, to obtain more reliable and conclusive results it is necessary to increase the sample size.

The thesis of Shahin Setoudehmaram is approved.

Nini Chaichanasakul Tran

Yong Kim

Renate Lux, Committee Chair

University of California, Los Angeles

2023

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Renate Lux, my research mentor throughout this project. Dr. Lux has provided unwavering support and guidance throughout my education, offering valuable opportunities for personal and academic growth. I am deeply appreciative of her commitment to my development as both a student and a researcher.

I would also like to extend my heartfelt thanks to Dr. Nini Tran, who, alongside Dr. Lux, served as a mentor during the development and implementation of this ambitious and significant project. I am immensely grateful for their guidance and unwavering support throughout the entire process.

I extend my appreciation to the other members of my committee for their valuable time and effort in reviewing my thesis. I would like to express my gratitude to Dr. Yong Kim, whose support and encouragement as a professor and teacher during my residency at UCLA have been invaluable. I would also like to acknowledge and express my gratitude to my co-investigators, Dr. Joseph Mullen, and Dr. Tyler Brennan, as well as the dedicated members of our study team. Their commitment and countless hours invested in bringing this project to fruition are deeply appreciated.

Furthermore, I would like to acknowledge the support received from 3M Investigator Sponsored Research, who provided funding for this project under grant #2020-ISR-000199. Their financial support has been instrumental in the successful completion of this study.

Finally, I would like to express my heartfelt gratitude to my wife and lifelong companion, Sara, for being my unwavering source of support and strength throughout my educational journey. Her unwavering belief in my aspirations and sacrifices have made it possible for me to pursue my dreams of pursuing a master's and undergoing orthodontic training at UCLA. Her contribution to my achievements is immeasurable, and I credit her as an equal partner in my success.

Introduction

Despite significant progress in orthodontic materials and techniques, the occurrence of enamel demineralization around orthodontic brackets remains a persistent issue that adversely affects the health and appearance of affected teeth. Fixed orthodontic appliances do not directly cause damage to the teeth themselves, however, they alter the oral environment by creating additional surfaces where bacteria can accumulate while at the same time making it more challenging to maintain proper oral hygiene. This accumulation of plaque near orthodontic brackets can lower the pH in the surrounding areas, which impairs the natural process of remineralization and can eventually result in enamel decalcification. These changes contribute to an elevated risk of developing white spot lesions and dental caries [1-3].

Decalcification of enamel occurs when there is a net loss of minerals, resulting in a condition known as white spot lesion (WSL). WSLs appear as opaque and white areas on the enamel surface, and they scatter light differently compared to sound enamel (Figure 1) [2, 4]. Previous studies have shown that 23% of orthodontic patients developed WSLs during treatment [4]. Different significant risk factors have been implicated in the development of these lesions: poor oral hygiene, patients whose oral hygiene declined during treatment, treatment time in excess of 36 months, and preexisting white spot lesions [4].

When the oral cavity shifts from a healthy to a diseased state, dysbiosis of the salivary microbiome is also observed. These imbalances in the microbiome can cause it to change from being commensal and benign to dysbiotic [1]. Microbial shifts play a crucial role in the development of white spot lesions, caries, gingivitis, and periodontal disease. These conditions arise when there is a transition in the microbial community from a benign, commensal state to a community that exhibits either acidogenic characteristics, leading to caries, or inflammatory properties, contributing to periodontal disease. These complications are frequently observed as side effects of orthodontic treatments [5]. Previous studies have investigated the effect of

orthodontic appliances on the composition of oral microbial communities [5-12]. Mullen et al. reported that although orthodontic treatment initiates a shift in the oral microbiome, the nature of that change is patient specific [5]. Campobasso et al. performed a comprehensive analysis of studies on the oral microbiome during orthodontic treatment. Based on their systematic review, fixed orthodontic appliances were found to cause an increase in the presence of pathogenic periodontal bacteria, particularly in subgingival plaque, during the early stages of treatment. However, no significant differences were observed in the long-term levels of these bacteria in saliva during orthodontic treatment with brackets [6]. According to some studies, orthodontic treatment does not appear to have a long-term impact on the microbial composition and biodiversity at the salivary level [6]. However, others have reported patients with fixed appliances showed a significant change in oral microbiome. One study found that patients with fixed orthodontic appliances show a significant increase in the numbers of streptococci and lactobacilli, indicating a higher risk of caries development [7]. Another study compared the composition of oral microbiota in individuals undergoing orthodontic treatment with those who were considered healthy. They utilized PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis) technique to analyze the microbial diversity, and real-time PCR to quantify the abundance of dominant species. The study findings demonstrated a notable distinction in microbial diversity between orthodontic patients and healthy individuals. Specifically, certain species such as *Pseudomonas*, *Veillonella*, and *Burkholderia* were exclusively detected in orthodontic patients, whereas *Streptococcus* and *Neisseria* species were present in both the orthodontic and healthy groups [8]. Jing et al. examined the changes in populations of cariogenic bacteria, specifically *Streptococcus mutans* and *Lactobacillus*, in the saliva of patients undergoing fixed orthodontic treatment. The findings of the study indicated that the overall level of bacteria remained stable over the 18-month treatment period. However, there was a slight increase in the quantity of *Lactobacillus*, although this increase was not

statistically significant. In contrast, the level of *S. mutans* remained stable during the initial 6 months but showed a significant increase thereafter [10]. Jung et al. conducted a study to assess the changes in salivary levels of *S. mutans* and *S. sobrinus* following orthodontic treatment with fixed appliances, using real-time PCR analysis. The study revealed that the levels of both *S. mutans* and *S. sobrinus* in saliva significantly increased after orthodontic treatment, despite improved oral hygiene practices and a decrease in total bacteria count in saliva. Interestingly, the study also found that the salivary levels of these bacteria during orthodontic treatment were lower compared to the levels observed after the completion of orthodontic treatment [11]. A systematic review examining the relationship between orthodontic appliances and changes in the oral microbiota reported a moderate to high association between orthodontic appliances and alterations in the oral microbiota. The review concluded that orthodontic appliances have an impact on the oral microbiota, leading to an increase in the counts of *S. mutans* and *Lactobacillus* spp., as well as an increase in the percentage of potentially pathogenic gram-negative bacteria such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* [12].

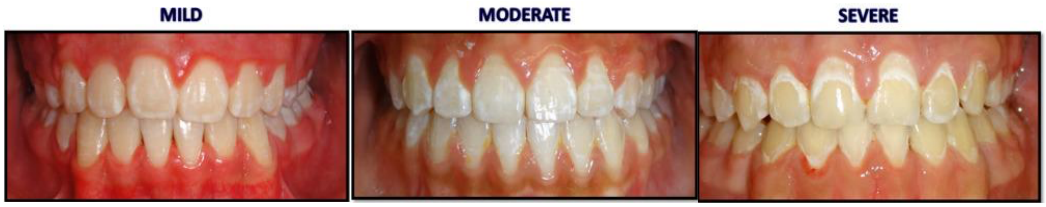


Figure 1: Appearance of white spot lesions at different stages of severity[13]

To identify clinically meaningful changes in the oral microbiome, species of interest include *Streptococcus mutans* and *Lactobacillus* which are main etiological bacteria in the development of dental caries [14]. While the initial development of carious lesion has been attributed to *S. mutans*, *Lactobacillus* species are particularly involved in the progression of advanced caries lesions in both children and adults [15]. Increased levels of *S. mutans* and

Lactobacillus species have been reported to exist in the oral cavity after bonding orthodontic appliances, with a positive correlation between caries and the degree of bacterial infection [8]. Other studies showed the salivary *S. mutans* level significantly increases during orthodontic treatment, while its level decreases after debonding during the retention period [9, 10]. Yet, Jung et al. reported that salivary *S. mutans* levels during orthodontic treatment are lower than those after orthodontic treatment. This study showed that salivary *S. mutans* and *S. sobrinus* levels significantly increase after orthodontic treatment despite a decrease in total saliva bacteria counts [11]. Some research has also evaluated the synergistic effects of other microbes in enhancing the pathogenicity of *S. mutans*. Grzegocka et al. found that treatment with orthodontic appliances promotes candida yeast colonization with a dominance of *Candida albicans* [16]. In contrast, another study found fixed appliances had no influence on the presence or level of colonization by *C. albicans* [17]. Analysis of plaque containing *S. mutans* alone compared to those containing both *S. mutans* and *C. albicans* found greater biofilm mass and CFU counts in the co-species biofilm [18]. Koo et al. also reported a synergistic effect between *S. mutans* and *C. albicans* to cause dental caries and increase biofilm formation [19].

Changes in the buffering capacity, pH, and flow rate of saliva are crucial factors in maintaining proper oral function and preventing the occurrence of caries. These properties of saliva help to regulate the oral environment, neutralize acids produced by bacteria, and facilitate the remineralization process of tooth enamel [20]. Orthodontic treatment, however, can have an impact on the physical and chemical properties of saliva. The presence of orthodontic appliances such as braces or retainers can alter the flow rate of saliva and create areas of stagnation or pooling, which may affect the distribution of saliva and its buffering capacity. Additionally, the presence of orthodontic appliances can contribute to changes in the pH of saliva due to factors such as plaque accumulation, difficulty in cleaning oral surfaces, and the influence of dietary factors [21]. With regard to salivary properties, both long- and

short-term studies have reported contradictory outcomes about alterations on buffering capacity, pH and flow rate upon initiation of orthodontic treatment [20]. Kouvelis et al. reported that, although buffering capacity and saliva flow rate tend to increase after the placement of fixed orthodontic appliances, there is not any significant change either in pH or in the oral microbial flora [20]. On the other hand, Alessandri Bonetti et al. found that the placement of fixed orthodontic appliances does not change the salivary pH, buffer capacity and flow rate after 1 year of treatment [22].

While there have been numerous clinical trials evaluating white spot lesions (WSLs) during orthodontic treatment, there is very little information on the use of fluoride treatment for WSLs after orthodontic therapy. Du et al. studied the efficacy of 5% NaF varnish in reverting white spot lesions (WSLs) after fixed orthodontic treatment. They found that application of topical fluoride varnish is effective in reversing WSLs after debonding. They suggested using NaF varnish as a routine caries prevention measure after orthodontic treatment [23]. After removal of the orthodontic brackets, some level of natural remineralization of white spot lesions occurred, and daily use of fluoride toothpaste may be helpful for this process. However, not all patients experienced this remineralization, and treatment with fluoride varnish induced greater remineralization of white spot lesions [24]. Singh et al. evaluated the efficacy of fluoride toothpaste alone and in combination with fluoride varnish and CPP-ACP plus crème, which is a non-fluoridated milk-derived complex utilized to deliver calcium and phosphate ions to the tooth surface, in the remineralization of post-orthodontic WSLs. They reported that the use of 5% NaF varnish in addition to twice daily use of 1000 ppm of fluoride toothpaste had no additional beneficial effect in the remineralization of post-orthodontic WSLs [25]. Hochli et al. did a systematic review and meta-analysis to study the effects of various interventions to treat post-orthodontic WSLs. They found that fluoride varnish seems to be effective as the monthly use of fluoride varnish improved the lesion area and enamel

fluorescence of WSLs, but they concluded that further research is needed to elucidate its clinical relevance [26]. There is no consensus regarding the effects of NaF varnish after debonding orthodontic brackets. In addition, the limitation of the current literature is that previous studies did not assess the effects of removing fixed appliances on salivary parameters and oral microbiome. Changes in salivary properties and the oral microbial community may influence the ability of the saliva to revert WSLs with the use of fluoride after orthodontic appliances are removed. The relative lack of research in this area represents a knowledge gap that warrants further investigation.

Objectives and Specific Aims

The goal of this study is to assess how white spot lesion therapy using two different types of varnishes affects WSL size and appearance, key salivary parameters, such as pH, buffering capacity, flow rate, and salivary levels of micro, following the removal of fixed orthodontic appliances. Our hypothesis was that the use of traditional sodium fluoride (5% NaF) varnish or the novel application of light-cured fluoride varnish (LCFV) will reduce white spot lesions after treatment with fixed brackets will impact salivary parameters and the oral microbiome. To test this hypothesis, we have developed the following specific aims:

1. To characterize the salivary parameters of pH, flow rate, and buffering capacity after orthodontic treatment.
2. To evaluate whether there are microbial shifts, particularly for *S. mutans* and *C. albicans*, in the first six months following the completion of orthodontic treatment.
3. To examine the effectiveness of light-cured fluoride varnish (LCFV), 5% NaF varnish, and placebo varnish in treating white spot lesions (WSL) measured by DIAGNOdent scores.

Design and Methodology

A randomized clinical trial was carried out using a split-mouth design to evaluate the effectiveness of WSL therapy with fluoride varnish on different salivary parameters. The study recruited potential subjects who met specific inclusion criteria and were in the final stages of their orthodontic treatment. These criteria include:

1. Males and females who are between the ages of 12 and 27, inclusive, at the time the Assent and Informed Consent Form is signed
2. Systemically healthy, as determined by the investigator
3. Subject is nearing the end of their orthodontic treatment using metal fixed oral appliances (brackets) on at least the maxillary arch
4. Subject has at least two visible white spot lesions in separate quadrants at the time of recruitment on teeth treated with orthodontic brackets
5. Subject is willing to use only oral care products that fall within the standard of care (manual toothbrush and NaF toothpaste) for the duration of the study
6. Subject must be willing to delay dental cleanings between the baseline and final visit (the standard of care was upheld as subjects received cleanings 6 months apart at both the baseline and final visit)
7. Subject is willing and able to comply with oral hygiene and diet instructions
8. Subject can understand and sign the Assent and/or Informed Consent Form prior to initiation of study procedures
9. Subject can communicate with the investigator/study personnel, understand, and comply with the study requirements, and is willing to return for protocol-specified visits at the appointed times

The exclusion criteria are as follows:

1. Advanced periodontal disease

2. Medical condition (e.g., artificial heart valve, history of infective endocarditis, cardiac transplant with valvular dysfunction, congenital heart disease or total joint replacement) for which antibiotics are recommended prior to dental visits and/or procedures
3. Pathologic lesions of the oral cavity (suspected or confirmed)
4. Use of systemic antibiotics, topical oral antibiotics, or use of other drugs, which in the opinion of the investigators could influence the study outcome, within 30 days prior to screening
5. Presence of any condition or concurrent illness, which in the opinion of the investigators, would compromise normal immune function ((e.g., diabetes, rheumatoid arthritis, lupus, liver disease, organ transplant, etc.), interfere with the use of study dentifrice and oral care products, or interfere with the ability to comply with study requirements, or jeopardize the safety of the subject or the validity of the study results. Eligibility for the study was determined by the investigators through chart review, phone conversation with the patients, or during the screening process in the orthodontic clinic. Once a subject is determined eligible, informed consent was obtained and they were assigned a unique study identification number. All teeth with WSL present was identified and recorded. After eligibility is confirmed, the subject was assigned randomly to one of the three groups using stratified permuted block randomization (Table 1):

Group 1

a. LCFV (Vanish™ XT Extended Contact Varnish, 3M Oral Care, St. Paul, MN, USA) on upper left (UL) and lower right (LR) quadrants (Figure 2A)
b. 5% NaF varnish (Acclean 5% Sodium Fluoride Varnish, Henry Schein Inc., Melville, NY, USA) on upper right (UR) and lower left (LL) quadrants (Figure 2B)

Group 2

c. Placebo varnish (petroleum jelly) on UL and LR quadrants

d. LCFV on UR and LL quadrants

Group 3

e. 5% NaF varnish on UL and LR quadrants

f. Placebo varnish on UR and LL quadrants

Table 1. Group Identification

Group	Quadrants	Treatment
A	UL, LR	LCFV
	UR, LL	5% NaF
B	UL, LR	Placebo
	UR, LL	LCFV
C	UL, LR	5% NaF
	UR, LL	Placebo

UL: upper left, LR: lower right, UR: upper right, LL: lower left

Eight strata were identified for the study to balance the age and gender distribution of the groups: (1) 12–15-year-old males, (2) 12–15-year-old females, (3) 16–19-year-old males, and (4) 16–19-year-old females, (5) 20-23-year-old males, (6) 20-23-year-old females, (7) 24-27-year-old males, and (8) 24-27-year-old females. The allocation sequence for this study was determined using a random number and sequence generator [27], with permuted block sizes ranging from 1 to 3, until the final allocation sequence is achieved. To assign patients to treatment groups, a printed group assignment was placed in a lined, sealed envelope that is labeled with the strata and sequence number. When a patient was recruited, the investigators recorded themselves writing the subject's name or ID number on the sealed envelope, before opening it to assign them to a treatment group.

Figure 2. Varnishes utilized in the study. A) 3M Vanish XT Extended Contact Varnish. B) Henry Schein Acclean 5% Sodium Fluoride Varnish

To achieve sufficient statistical power and obtain statistically significant results, the sample size must account for the following factors: the split-mouth design, the fact that each patient provides data for multiple teeth and measuring each tooth individually. However, calculating an appropriate sample size in a linear mixed model that assumes correlation within a subject requires reliable information on within-subject correlation, which can only be obtained from preliminary data with sufficient samples. Furthermore, adjusting the Type I error rate to account for multiple comparisons is a standard practice in statistical analysis and was appropriate in this case. While a sample size of 7 per group may be sufficient to detect large differences between groups, it is generally recommended to have a larger sample size to increase the power of the study and reduce the risk of type II error. Moreover, smaller sample sizes, especially those below 10, may raise doubts about their reliability. Considering these factors, along with practicality, cost, and difficulties in recruitment, a sample size of 10 participants per group was estimated to provide enough power.

The study includes a total of 4 visits: T0 (baseline, within a month after braces are removed), T1 (30 days after T0), T2 (90 days after T0), and T3 (180 days after T0). Patients were advised not to brush their teeth on the morning of each time point to ensure sufficient

plaque buildup. At the start of every appointment, patients completed a questionnaire on their use of fluoride products in the preceding six months and answer other relevant questions (Appendix B). During the initial study visit, each participant filled out an oral health questionnaire. To determine the salivary consistency, the investigators visually assessed the resting salivary consistency in the oral cavity. The subjects were then instructed to spit the saliva into a collection cup. The investigators then measured the salivary pH using pH strips (Saliva-Check Buffer Testing Matt, GC America Inc., Alsip, IL, USA) (Figure 3). They took the pH strip, place one end of it into the sample of resting saliva for 10 seconds, and then checked the color of the strip. Afterward, the patients were given a piece of wax to chew. After 30 seconds, the subjects were instructed to spit into the collection cup.

Figure 3. Saliva-Check BUFFER test for Saliva quality, pH, and buffering capacity

The patients continued chewing the wax for an additional 5 minutes and expectorate every 15-20 seconds into the provided cup until 5ml of saliva was collected. The collected saliva was used to measure the pH and buffering capacity of the stimulated saliva. To test the pH of stimulated saliva, the investigators took the pH test strip, placed the unused end into the sample of saliva for 10 seconds, and then checked the color of the strip.

The investigators measured the buffering capacity of stimulated saliva using a buffer test foil pack and a pipette to draw up a small amount of saliva from the cup. They dispensed

one drop of saliva from the cup onto each of the three test pads and drained excess saliva onto a tissue by turning the test strip on its side. After waiting for two minutes, they compared the color of each pad with the reference table, added up the three scores, and recorded the results. After collection, the samples were taken to the laboratory without using any subject identifiers. The samples were be plated for CFU analysis. After the saliva samples were taken, a dental prophylaxis was performed with a prophy cup and paste (containing no fluoride), and hand scalers as needed. The subjects were asked to rinse their mouths with water. Teeth that were identified as having a WSL present were scored using the DIAGNOdent Pen 2190 laser (KaVo, Biberach an der Riss, Germany) (Figure 4). Each lesion was scored three times and measurements were recorded on the Laser Fluorescence Data Sheet. The scores were averaged to provide a final score for each lesion (Appendix C).

The varnishes were applied to all teeth in each quadrant of the mouth by following the instructions provided by the manufacturer. The split-mouth design was used in this study, wherein two different varnishes were applied to each subject. After drying the teeth with air for 15 seconds, the 5% NaF and placebo varnishes were placed on the buccal surfaces of appropriate quadrants using an applicator brush, avoiding contact with soft tissue, and using brush strokes from gingival to occlusal. The LCFV was applied by rinsing the teeth with water for 15 seconds and then using a dry cotton applicator to remove excess water. Next, the LCFV was mixed for 15 seconds and applied in a thin layer to the teeth. To maintain patient blinding, each quadrant was light cured, regardless of the type of varnish applied. Finally, before leaving, the patients were provided with an Oral Hygiene Study Instructions sheet (Appendix D), a new soft-bristled toothbrush, and a tube of over-the-counter NaF toothpaste.

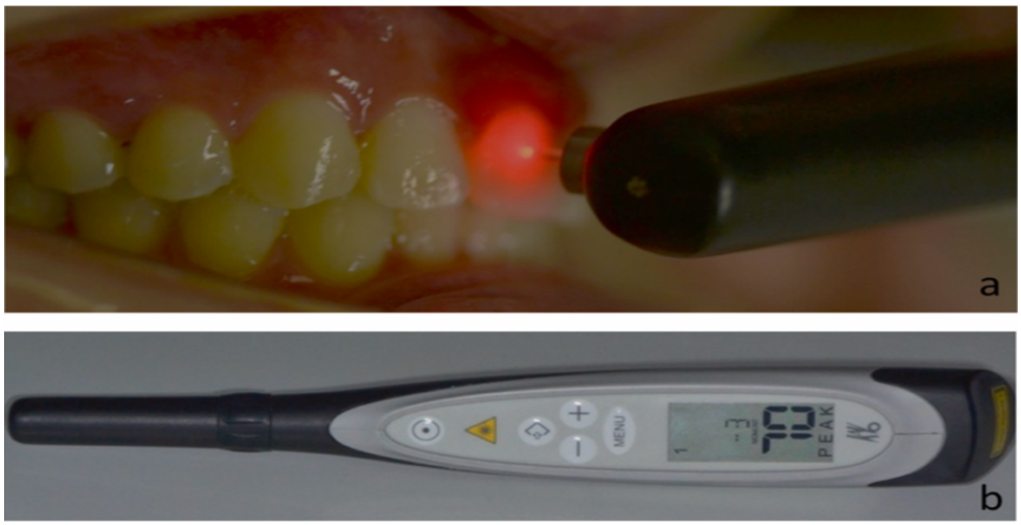


Figure 4- DIAGNOdent pen. (a) Use for WSL detection. (b) device calibrated to use[28]

At every recall visit, the same procedures were followed, which involved filling out the Oral Health Questionnaire (Appendix B), collecting plaque, cleaning the buccal surfaces using a prophy cup, obtaining DIAGNODent scores for all WSL, and reapplying the varnishes. Although it is not clinically necessary for the LCFV, the varnish was removed and reapplied at each timepoint to preempt any possible obscuring effect it may have on the DIAGNODent measurements. Another new toothbrush was given to the participants at T2, as per the ADA recommendation of changing toothbrushes every three months. At T3, the participants received another complete dental prophylaxis, including hand scaling as necessary, for both buccal and lingual surfaces.

Salivary samples collected from each subject were plated to calculate the number of colony-forming units (CFUs) of both *S. mutans* and *C. albicans*, as well as the total culturable microorganisms. The samples were be plated on Brain Heart Infusion (BHI) to assess total bacterial load, Sabouraud Dextrose (SAB) to approximate the load of *C. albicans*, and *Mitis Salivarius*- Bacitracin (MSB) to approximate the load of *S. mutans*. The saliva samples were vortexed for more than 30 seconds and aliquot 750 µL to a 1.5 mL micro-centrifuge tube and placed on ice. For each saliva sample, 100µL of vortexed saliva was mixed with 900µL of

phosphate-buffered saline (PBS) at a dilution ratio of 1:10. The mixture was vortexed for 15 seconds to ensure thorough mixing. Subsequently, a serial dilution was performed using a 1:10 dilution factor, resulting in a total of 7 dilutions. Control cultures of *S. mutans* 140 were also subjected to the dilution process. For the culturing step, Brain Heart Infusion (BHI) medium was used, and the dilutions were initiated from an optical density (OD) of 1. A volume of 100μL from each of the serially diluted samples was plated onto respective plates. Glass rods were used to distribute the liquid evenly throughout the plate by gently moving the rod in all directions for approximately 1 minute. This ensured the uniform distribution of the samples on the plates for subsequent growth and analysis.

Before performing the statistical analysis, the normality of the data was assessed using Shapiro-Wilk analysis, and appropriate further analyses were selected for each measurement [29]. The power of the study was calculated with the G∗Power statistical analysis program using the number of saliva samples [30]. Statistical significance was determined using a Kruskal Wallis test with the Bonferroni correction method for the DIAGNOdent scores, salivary physicochemical characteristics, as well as Colony Forming Unit (CFU) counts at level of $P \leq 0.05$. The random missing data that resulted from patients missing appointments were addressed by using regression imputation [31].

Results

Demographics

The study presented in this context is still in progress, and the data provided are from a subset of 17 participants that have been collected thus far, consisting of eight males and nine females. The average age of the subjects was 17 years and 0 months at the time of the initial data collection. Table 2 provides additional details, including the total number of samples collected at each time point. The analysis primarily focused on the 147 white spot lesions (WSLs) identified within the study population. Among these lesions, 39 teeth with WSLs

received placebo treatment, 47 received 5% NaF treatment, and 61 received LCFV treatment. Further distribution of WSL's is listed in Table 3.

Table 2: Subject demographic data

Number of Subjects	17
Average Age of Subjects (at T1)	17y 0m
Number of Males	8
Number of Females	9
Total Number of Sample Collections	53
T1	17
T2	14
T3	13
T4	9

Table 3: Characteristics of teeth included in the study

	# Teeth	Maxillary	Mandibular
Placebo	39	19	20
5% NaF	47	24	23
LCFV	61	29	32
Total	147	72	75

DIAGNOdent Results

The normality of the DIAGNOdent scoring data was assessed with the Shapiro-Wilk test. The results indicated a significant departure from normality ($W(449) = 0.62$, $p < 0.001$). Due to the lack of normal distribution, the Kruskal Wallis test was employed to examine the statistical significance. Although not statistically significant, there were notable trends observed in the data. For the WSLs treated with the placebo varnish, there was a 22.41% decrease in scores from T1 to T4. Similarly, the 5% NaF group showed a 19.15% decrease in scores. The LCFV group appeared to have potential outliers in the data, specifically the DIAGNOdent scores for two teeth in subject 5 at T3/T4. After excluding extreme outliers, the LCFV group showed a small, but statistically nonsignificant, increase (10.65%) in

DIAGNOdent scores during the first 6 months after orthodontic treatment completion (Figure 5, Table 4-5).

Table 4. Average DIAGNOdent scores at different timepoints

Average DIAGNOdent Scores (9 completed cases)					
	T1	T2	T3	T4	T1-T4% Change
Placebo	5.8	6.3	3.7	4.5	-22.41
5% NaF	9.4	12	15.2	7.6	-19.15
LCFV	5.61	6.3	9.1	5.67	10.65

Table 5. p-values comparing changes in DIAGNOdent scores from T1 to T4

	p-values (calculated from Kruskal Wallis Test)					
	T1-T2	T1-T3	T1-T4	T2-T3	T2-T4	T3-T4
Placebo	0.9131	0.0902	0.3017	0.0713	0.2535	0.5084
5% NaF	0.5246	0.972	0.255	0.5473	0.0823	0.2409
LCFV	0.4403	0.8235	0.7124	0.5806	0.2569	0.5541

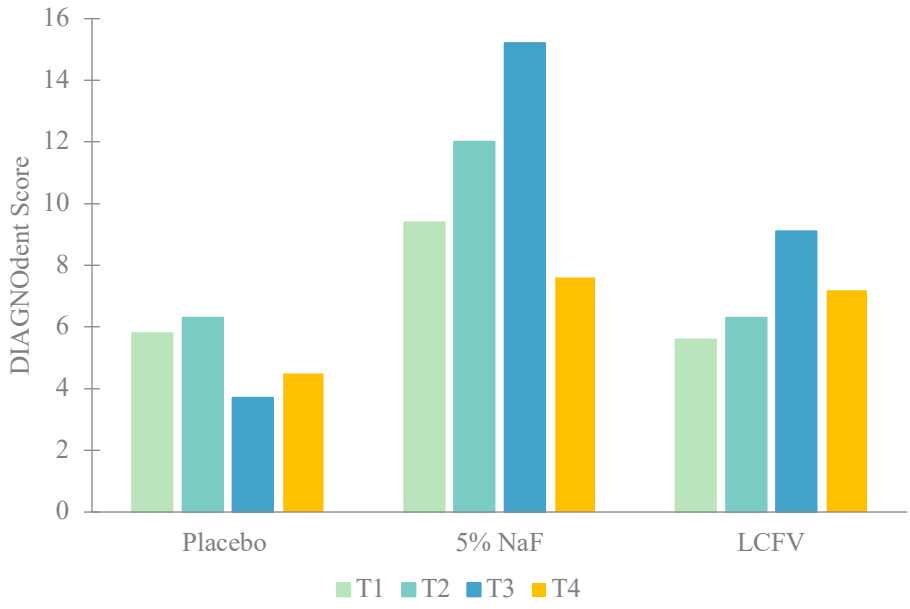


Figure 5. DIAGNOdent Scoring Across Treatments and Timepoints

The DIAGNOdent data were analyzed using the Kruskal-Wallis test to compare the DIAGNOdent scores among different treatments at different time points. The results of the analysis showed no statistically significant difference in the DIAGNOdent scores across the various time points (Table 6).

Table 6. multiple comparisons for the DIAGNOdent scores from T1 to T4

	p-values (calculated from Kruskal Wallis Test)		
	Placebo-LCFV	Placebo-5% NaF	5% NaF-LCFV
T1	0.9867	0.9856	0.9395
T2	0.9963	0.5089	0.5115
T3	0.8248	0.0549	0.1721
T4	0.5312	0.3990	0.9435

Salivary Results

Physicochemical Results

Based on the resting pH data within each group (Figure 6A), the LCFV treatment and the 5% NaF treatment had a similar impact on the resting pH values, as the pH values for these treatments are relatively close to each other. In T4, both 5%NaF and LCFV treatments resulted in a slightly higher resting pH compared to T1. The stimulated pH values for LCFV and 5% varnish treatments are 7.1 and 7.4, respectively, indicating a difference of 0.3 in favor of the 5% varnish treatment. The findings suggest that the 5% varnish treatment generally results in slightly higher stimulated pH values compared to the LCFV treatment. However, the differences in pH between the treatments are relatively small and are not clinically significant (Figure 6B). Figure 6C displays the changes in salivary pH over the study duration.

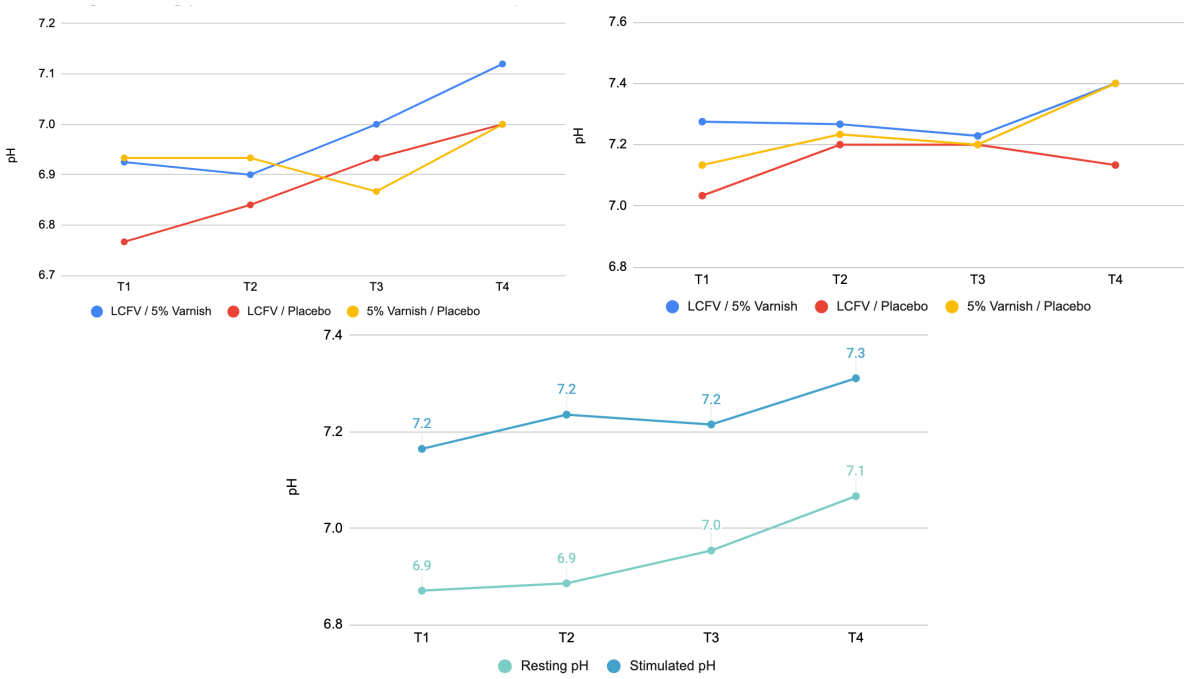


Figure 6. Time and treatment-dependent variations in the pH of saliva. A) Resting pH B) Stimulated pH; C) Average pH values of all treatment groups over time

Analysis of the buffering capacity data within each group revealed that the differences in buffering capacity between the treatments vary. At some points, such as T1 and T4, the 5% NaF treatment shows a higher buffering capacity compared to the corresponding alternative treatments. In T2, the LCFV treatment has a slightly higher buffering capacity than the placebo treatment. However, in T3, the 5% varnish treatment exhibits a higher buffering capacity compared to the placebo treatment (Figure 7A, Table 6).

Table 7. Salivary physicochemical parameters at different timepoints

Resting pH	LCFV / 5% NaF	LCFV / Placebo	5%NaF / Placebo
T1	6.9	6.8	6.9
T2	6.9	6.8	6.9
T3	7.0	6.9	6.9
T4	7.1	7.0	7.0

Stimulated pH	LCFV / 5% NaF	LCFV / Placebo	5% NaF / Placebo
T1	7.3	7.0	7.1
T2	7.3	7.2	7.2
T3	7.2	7.2	7.2
T4	7.4	7.1	7.4

Buffering Capacity	LCFV / 5% NaF	LCFV / Placebo	5% NaF / Placebo
T1	10.1	8.8	11.0
T2	11.0	10.2	10.3
T3	8.7	8.3	10.0
T4	10.4	9.3	12.0

Throughout the study, fluoride varnish treatments did not show any notable effects on the buffering capacity of saliva (Figure 7B, Table7).

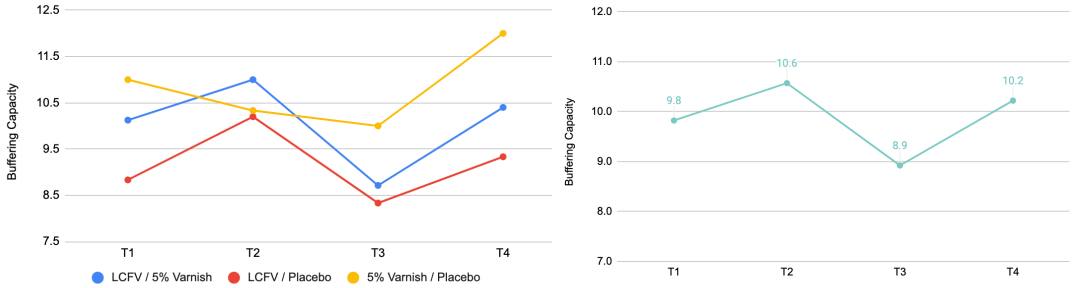


Figure 7. Time and treatment-dependent variations in the buffering capacity of saliva. A) variations across different groups and time points; B) Average values over time

Table 8. Multiple comparisons of the physicochemical properties of saliva from T1-T4

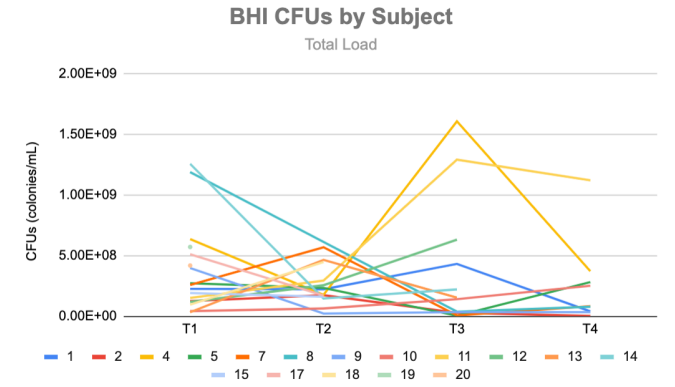
	p-values (calculated from Kruskal Wallis Test)					
	T1-T2	T1-T3	T1-T4	T2-T3	T2-T4	T3-T4
Group 1	0.8621	0.6061	0.2395	0.5167	0.2069	0.4906
Group 2	0.5573	0.1998	0.0513	0.4504	0.1614	0.5637
Group 3	0.7604	0.5935	0.8715	0.8191	0.7059	0.5898

	p-values (calculated from Kruskal Wallis Test)					
	T1-T2	T1-T3	T1-T4	T2-T3	T2-T4	T3-T4
Group 1	0.8255	0.6357	0.2113	0.8207	0.1696	0.1019
Group 2	0.1594	0.2023	0.4093	0.946	0.7129	0.6967
Group 3	0.4795	0.6714	0.2713	0.7773	0.5485	0.4237

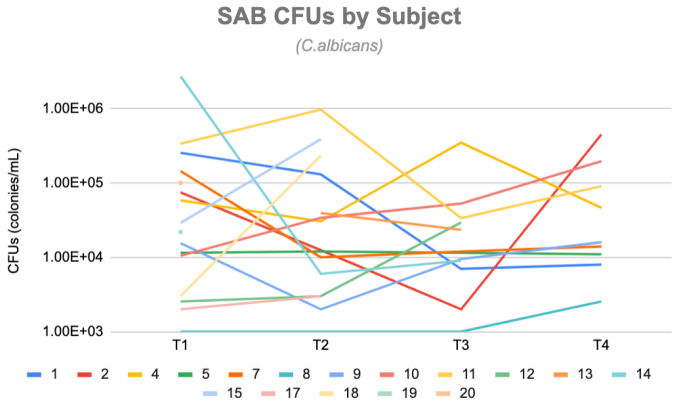
	p-values (calculated from Kruskal Wallis Test)					
	T1-T2	T1-T3	T1-T4	T2-T3	T2-T4	T3-T4
Group 1	0.1924	0.2873	0.4307	0.2412	0.674	0.087
Group 2	0.1594	0.2023	0.4093	0.946	0.7129	0.6967
Group 3	0.1836	0.6169	0.7208	0.1125	0.4493	0.4576

CFU Results

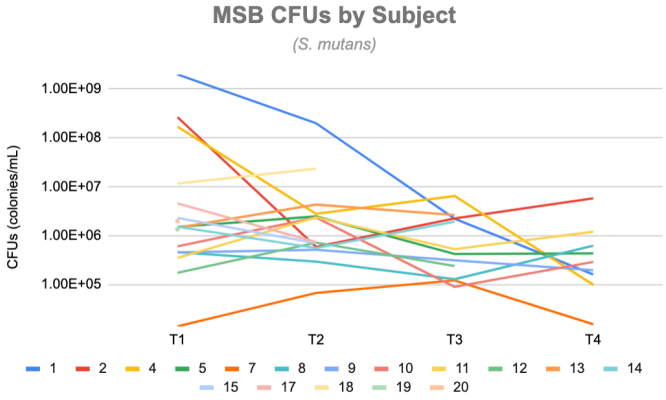
The total microbial colony counts on BHI agar showed a relatively stable trend across the four time points. However, when examining individual subjects, there are different patterns of change. For some subjects, such as subjects 1, 2, 5, 7, 8, and 18, there are fluctuations in the BHI CFUs over time. These fluctuations indicate variations in the bacterial population during the course of the study. On the other hand, some subjects, like subjects 11, 13, and 14, show a consistent increase in total microbial levels from T1 to T4. This suggests a growth or accumulation of bacteria in these subjects over time. The results of colony count on BHI agar suggest individual differences in the dynamics of bacterial growth and colonization. There may also be potential outliers in the data, such as the CFU count for Subject 5 at T4. These outliers could be attributed to factors like plating errors or contamination during the experiment (Figure 8A).



A



B



C

Figure8. salivary CFU counts by subject. A) salivary total bacteria (BHI); B) C. albicans (SAB); C) S. mutans (MSB)

The trend of changing *C. albicans* CFU counts on SAB agar across subjects and time points was variable. Most subjects showed consistent decreases in *C. albicans* levels over time, while others exhibited fluctuations, which may be due to wearing orthodontic retainers, plating

errors, or contamination during the experiment. In general, a decline in the salivary loads of *C. albicans* was observed among the majority of subjects (Figure 8B).

Looking at *S. mutans* colony counts on MSB, the CFU counts across all subjects showed a decreasing trend from T1 to T2, followed by relatively stable scores from T2 to T3, and further decreasing scores from T3 to T4, which showed a decline in *S. mutans* bacterial population over the course of study. This data suggests that in some subjects, the application of fluoride varnish had a suppressing effect on the growth and prevalence of *S. mutans* bacteria (Figure 8C).

The data presented in Figure 9 and Table 9 represent the specific counts or colony-forming units (CFUs) for each microbial species on the respective agar plates in all samples. The data reveal an overall downward trend in microbial load from T1 to T4. Specifically, there was a non -significant 9.75% increase in total microbial load as measured on BHI agar, an 81.66% decrease in *C. albicans* load as measured on SAB agar, and an 81.35% decrease in *S. mutans* load as measured on MSB agar (Tables 8-9). The average SAB scores showed a decline from T1 to T4, indicating a potential reduction of *C. albicans* in saliva. This suggests that the application of fluoride varnish may have influenced the population of *C. albicans*, leading to favorable changes in the composition of the oral microbiome. Moreover, the data demonstrated a significant decrease in the measured levels of *S. mutans* in the saliva samples from T2 to T4 (Figure 9, Table 9).

Table 9. The average colony forming units (CFUs) for each agar medium (BHI, SAB, MSB)

Average CFUs (colonies/mL)					
	T1	T2	T3	T4	T1-T4 % Change
BHI - Total Bacteria	2.7E+08	2.43E+08	3.81E+08	2.81E+08	9.75%
SAB - *C. albicans*	2.63E+05	1.35E+05	5.18E+04	4.82E+04	-81.66%
MSB - *S. mutans*	2.03E+06	3.46E+06	1.44E+06	3.78E+05	-81.35%

Table 10. Multiple comparisons for *CFUs from T1 to T4 for each medium (BHI, SAB, MSB)*

p-values (calculated from Kruskal Wallis Test)						
	T1-T2	T1-T3	T1-T4	T2-T3	T2-T4	T3-T4
BHI - Total Bacteria	0.2133	0.9535	0.5187	0.3853	0.7426	0.6516
SAB – *C. albicans*	0.5688	0.3893	0.4335	0.3829	0.4016	0.9339
MSB – *S. mutans*	0.4605	0.5656	0.03	0.06	**0.0002**	0.1295

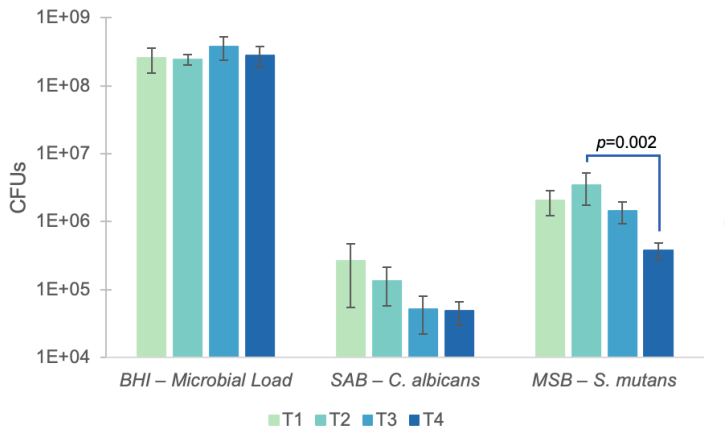


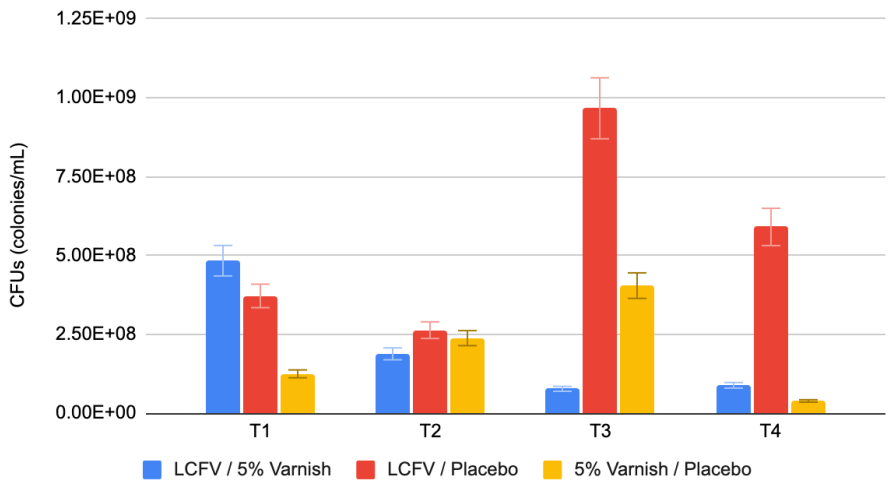
Figure 9. Salivary CFU counts at different time points.
A) total bacteria (BHI); B) C. albicans load (SAB); C) S. mutans load (MSB)

When comparing the total bacterial counts across the different groups, a noticeable trend emerged. In groups 1 and 3, there was a clear decrease in total bacterial counts from T1 to T4, indicating a potential reduction in the overall bacterial load over time. On the other hand, in group 2, there was a slight increase in total bacterial counts from T1 to T4, although this

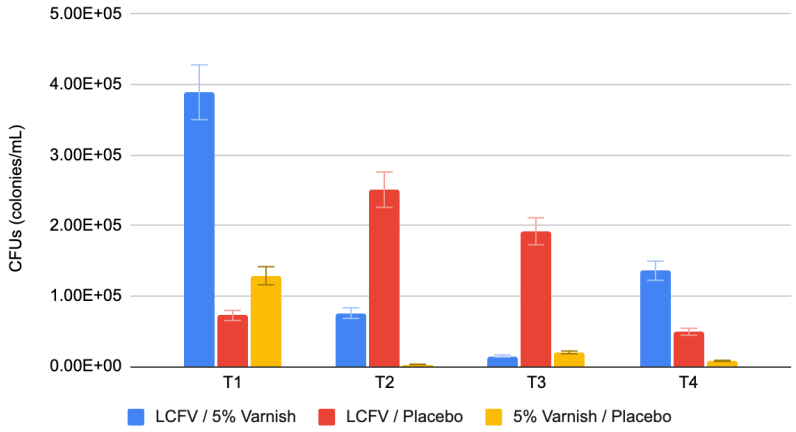
increase was not statistically significant. The potential reasons behind the trends in the total bacterial counts could be attributed to several factors. One possible reason is the relatively small sample size, which may limit the power to detect significant differences. Another factor to consider is the possibility of examiner errors during the counting of bacterial colonies. Additionally, contamination issues during the experiment could introduce unwanted bacteria into the samples, leading to inaccurate colony counts and potentially affecting the observed trends (Figure 10A, Table 10).

Candida albicans colonies on SAB agar exhibited a consistent decrease in CFU counts from T1 to T4, suggesting a potential reduction in the presence of *C. albicans* over time. However, there were some fluctuations observed across groups at different time points like in group 1 there was a slight increase at T4, in group 2 a slight increase at T2, and in group 3 an increase in colony counts at T3. This increase may be attributed to factors such as individual variations among subjects, variation in oral hygiene, diet or hydration of the subject among others (Figure 10B, Table 10).

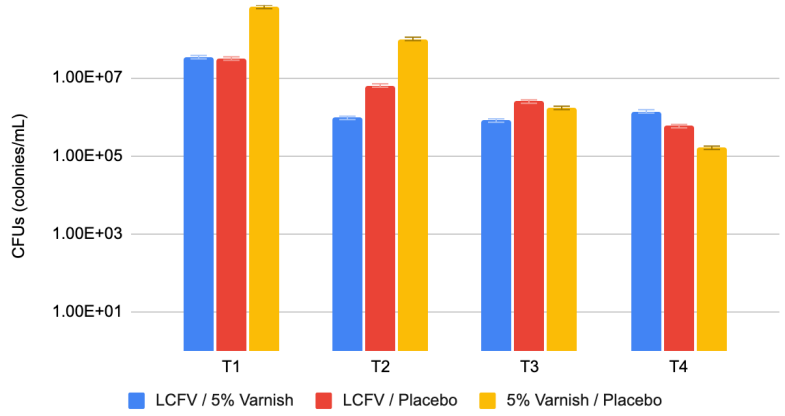
Similar to *C. albicans* colony counts on SAB agar, CFU counts for *S. mutans* colonies exhibited a consistent decreasing trend from T1 to T4, suggesting a potential reduction in the number of *S. mutans* over time. However, in group 2, there was a slight fluctuation with a small increase from T1 to T2. The observed decline in the colonization of *S. mutans* in saliva indicates that the natural adjustment resulting from the removal of orthodontic brackets may have influenced the presence of *S. mutans* in the oral cavity (Figure 10C, Table 10).



A



B



C

Figure 10. Salivary CFU counts across different treatment groups. A) total bacteria (BHI); B) C. albicans (SAB); C) S. mutans (MSB)

Upon analyzing the proportions of *S. mutans* and *C. albicans* in relation to the total microbial load, an interesting pattern emerged. There was an overall decreasing trend observed for *S. mutans* in proportion to the total microbial load, indicating a potential reduction in *S.*

mutans prevalence. Likewise, the presence of *C. albicans* demonstrated a general decrease, although not statistically significant, relative to the overall microbial population, indicating a possible inhibition of *C. albicans* colonization or a return to pretreatment levels following removal of orthodontic fixed appliances (Figure 11, Table 12).

Table 11. Salivary CFU counts across different treatment groups from T1 to T4

		Average CFUs (colonies/mL)			
		T1	T2	T3	T4
LCFV/ 5% NaF	BHI- Total Bacteria	4.84E+08	1.88E+08	7.73E+07	1.10E+08
	SAB – *C. albicans*	3.89E+05	7.58E+04	1.49E+04	5.74E+04
	MSB – *S. mutans*	3.38E+07	9.42E+05	8.05E+06	2.81E+05
LCFV/ Placebo	BHI- Total Bacteria	3.19E+08	2.63E+08	9.66E+08	5.90E+08
	SAB – *C. albicans*	7.52E+04	2.51E+05	1.92E+05	4.93E+04
	MSB – *S. mutans*	3.87E+06	6.35E+06	2.74E+06	5.79E+05
5%NaF/ Placebo	BHI- Total Bacteria	1.25E+08	2.55E+08	4.05E+08	3.89E+07
	SAB – *C. albicans*	1.29E+05	3.00E+03	2.00E+04	8.00E+03
	MSB – *S. mutans*	6.56E+06	7.20E+05	1.70E+06	1.61E+05

Table 12- Multiple comparisons of *Proportions of S. mutans and C.albicans*

p-values (calculated from Kruskal Wallis Test)						
	T1-T2	T1-T3	T1-T4	T2-T3	T2-T4	T3-T4
SAB – *C.albicans*	0.445	0.9215	0.3098	0.5469	0.7285	0.3882
MSB – *S. mutans*	0.3159	0.461	0.196	0.8099	0.0327	0.0586

Furthermore, when analyzing the ratio of *S. mutans* and *C. albicans* to the total microbial load across different groups (Figure 12, Table 13), similar patterns were observed. In all three groups, there was an overall downward trend in *S. mutans* load, suggesting that the different fluoride treatments applied eventually led to a significant reduction in the population

of *S. mutans*. The results indicated that the population and proportion of *C. albicans* remained relatively consistent across all groups and relatively stable throughout the observation period.

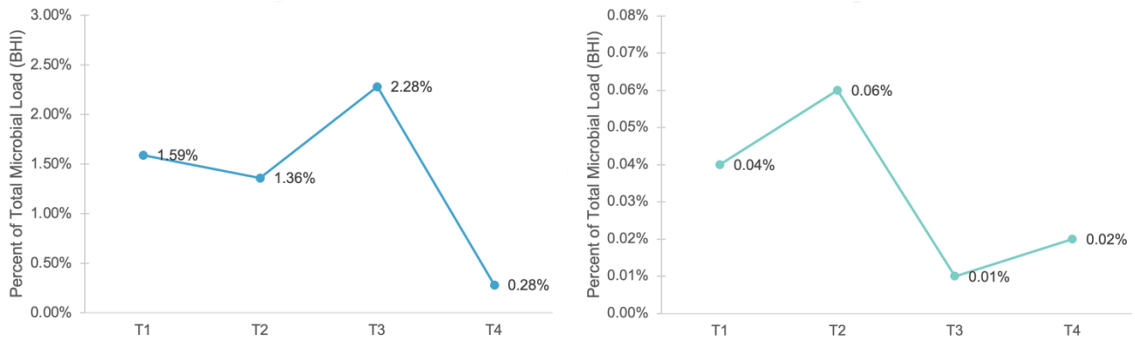


Figure 11. Proportions of A) S. mutans and B) C. albicans relative to total microbial load

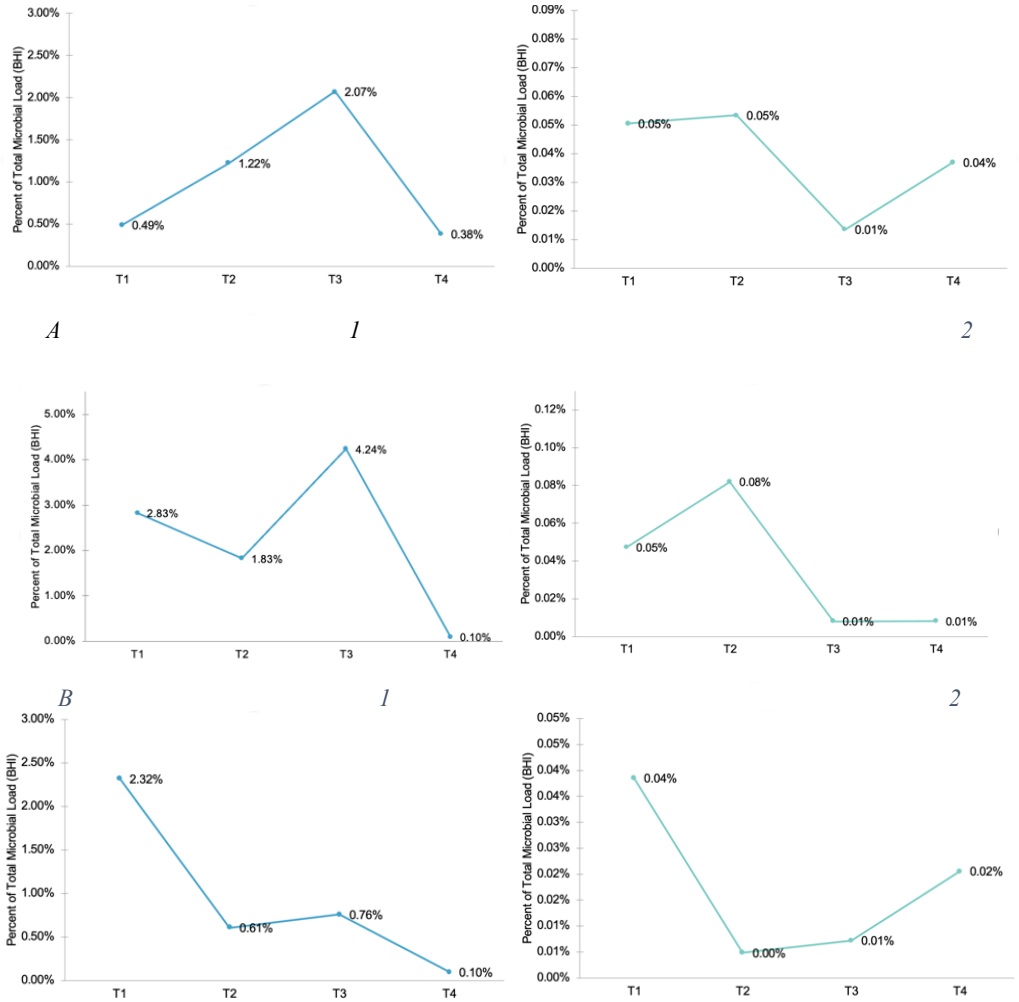


Figure 12. Proportions of S. mutans and C.albicans relative to total microbial load across treatment groups. A) LCFV/5% NaF; B) LCFV/Placebo; C) 5% NaF/Placebo. 1) S. mutans, 2) Candida albicans

Table 13- Proportions of S. mutans and C.albicans across treatment groups

p-values (calculated from Kruskal Wallis Test)							
		T1-T2	T1-T3	T1-T4	T2-T3	T2-T4	T3-T4
5% NaF-LCFV	SAB – *C.albicans*	0.8706	0.6255	0.7235	0.7393	0.6174	0.4348
	MSB – *S. mutans*	0.3983	0.1549	0.9841	0.5633	0.462	0.2104
LCFV - Placebo	SAB – *C.albicans*	0.2155	0.7482	0.5673	0.5384	0.6167	0.8702
	MSB – *S. mutans*	0.465	0.8032	0.0602	0.3778	0.012	0.1449
5%NaF-Placebo	SAB – *C.albicans*	0.5403	0.2636	0.0667	0.6547	0.1824	0.2888
	MSB – *S. mutans*	0.8383	0.7656	0.7389	0.9406	0.8676	0.9062

Discussion

Studies have indicated that white spot lesions (WSLs) tend to undergo a certain level of natural remineralization after the removal of orthodontic brackets. This natural remineralization may be further facilitated by the regular use of fluoride toothpaste. However, it is important to note that not all patients experience this remineralization, and the application of fluoride varnish as a treatment resulted in more substantial remineralization of white spot lesions [24]. Previous findings for the use fluoride varnish to improve remineralization of WSLs are rather conflicting. One study found that the use of 5% NaF varnish in addition to twice daily use of 1000 ppm of fluoride toothpaste had no additional beneficial effect in the remineralization of post-orthodontic WSLs [23]. However, a systematic review has suggested that regularly applying sodium fluoride (NaF) varnish on a monthly basis is beneficial for both preventing dental caries and improving white spot lesions (WSLs), specifically in terms of reducing lesion area and enhancing enamel fluorescence after orthodontic treatment [26]. The

existing literature highlights the importance of conducting further research to obtain a thorough understanding of the clinical significance and practical implications of these findings [23, 26].

The ideal treatment approach for white spot lesions (WSLs) aims to remineralize the lesions starting from the deeper layers towards the outer surface of the enamel. This strategy enhances the likelihood of achieving successful and aesthetically pleasing treatment outcomes. When using fluoride topically, it is important to determine the appropriate fluoride concentration and frequency of application. According to a previous study, the application of highly concentrated fluoride (2.23%) improves the remineralization of bleached enamel. This suggests that higher fluoride concentrations might be more effective for the remineralization process. However, other studies have indicated that excessive fluoride doses can physically hinder the penetration of calcium ions into the subsurface layers beneath the enamel surface. It seems that higher levels of fluoride,225 ppm, are more effective in preventing lesion formation and treating deep WSLs, while lower concentrations, 50 ppm, are suitable for remineralizing superficial lesions and controlling lesion progression. In this study, patients received fluoride varnish treatments which included novel use of LCFV, 5% NaF varnish or a combination of both varnishes in a spilt-mouth design, at base line, one month, and three months, which provided a higher fluoride concentration to support the remineralization process. Fluoride treatment was further supported by the daily use of fluoride toothpaste with a lower fluoride concentration to allow for slower penetration of calcium and fluoride ions into the white spot lesion (WSL) [24].

The DIAGNOdent is a dental instrument that utilizes laser fluorescence to measure the extent of enamel demineralization. The device emits laser light at a specific wavelength of 655 nm, which is absorbed by the organic and inorganic components of the tooth. When caries or tooth decay is present, the fluorescence levels increase, and this change is detected as a higher digital value. DIAGNOdent readings should be interpreted with caution, as they can be

influenced by factors such as stains, calculus, plaque, and bacterial metabolites that may not necessarily reflect the issues perceived by patients or dentists [25]. To minimize the impact of these factors, in this study a dental prophylaxis procedure was conducted for all participants before assessing white spot lesions (WSLs) using the DIAGNOdent pen. These measures were taken to reduce potential confounding effects and ensure more accurate DIAGNOdent readings specifically related to the WSLs being evaluated. However, the findings of the current study suggest that the DIAGNOdent pen may not be the most reliable tool for examining white spot lesions (WSLs) due to potential inaccuracies related to calibration and operator technique. This is further supported by the presence of significant outliers in the DIAGNOdent scores, indicating variability in the quantitative accuracy of the device. As such, it is important to understand the limitations and interpret the DIAGNOdent scores in conjunction with other diagnostic tools rather than solely relying on its numerical readings [23, 28].

Prior research on traditional 5% NaF varnish reported an average DIAGNODent score reduction of 7.56 (from 17.66 ± 5.36 to 10.10) in contrast to a mean score reduction of 3.09 (from 16.19 ± 5.70 to 13.10) with a placebo [23]. The findings from the DIAGNOdent measurements in this study revealed interesting trends. In the placebo varnish and 5% NaF varnish groups, there was an approximate 20% decrease in scores for the treated WSLs over the observation period. However, the group treated with LCFV showed a slight increase in DIAGNOdent scores during the first 6 months after completing orthodontic treatment. This unexpected finding indicates that the LCFV may not have produced the same continuous remineralization effect as observed with the placebo and 5% NaF varnishes. The expected result of treating teeth with 5% NaF varnish is a continuous remineralization effect of varnish on tooth enamel surface, leading to a reduction in white spot lesions (WSLs) and improvement in DIAGNOdent scores.

It is well-established that saliva plays a significant role in the remineralization process by acting as a reservoir of calcium and phosphate ions and providing minerals to the tooth damaged enamel replenish the minerals lost from the tooth surface [24]. Therefore, the decrease in DIAGNOdent scores observed in the placebo treatment group can be potentially attributed to the natural remineralization process facilitated by regular use of fluoride toothpaste during the study period. The LCFV group showed a slight, but statistically nonsignificant, increase in DIAGNOdent scores during the first 6 months after orthodontic treatment completion. It has been reported that the use of light-cured fluoride varnish (LCFV) offers a longer duration of protection against demineralization on the tooth surface compared to conventional fluoride varnish [32]. This extended presence of LCFV may contribute to its effectiveness in preventing demineralization of enamel but not helpful in treating white spot lesions (WSLs) because the high concentration of fluoride in LCFV could create a barrier on the surface layer of WSLs, preventing the penetration of calcium ions into the subsurface layer. This could inhibit further remineralization process in the deeper layers of WSLs [33, 34].

Changes in the buffering capacity, pH, and flow rate of saliva are important factors in maintaining oral health and preventing caries. It has been reported that Orthodontic treatment might affect the physical and chemical properties of saliva. Studies investigating these effects have shown contradictory results with some suggesting that the placement of brackets can increase the buffering capacity and saliva flow rate, but others reporting no changes in salivary pH, buffering capacity, and flow rate during orthodontic treatment. To our knowledge no study has assessed the salivary properties after removing orthodontic appliances. In the present study, we found no significant changes from T1 to T4 in salivary resting pH, stimulated pH, as well as saliva buffering capacity despite some fluctuations, which indicates that wearing orthodontic retainers after removing brackets does not affect salivary properties.

In order to assess changes in the oral microbiome that could be relevant for WSL development, it is important to focus on key microbial species such as *S. mutans* and *C. albicans*, which are known to play an important role in the development of dental caries. In the present study, we examined the microbial data both as a whole and across different groups and time points to specifically analyze the effects of the varnish on salivary microbial composition. Interestingly, we observed that the total bacterial load remained relatively stable during the 6-month observation period after the removal of orthodontic appliances. When comparing the total bacterial counts across the different groups, in groups 1 and 3, there is an apparent decrease in total bacterial counts from T1 to T4, indicating a potential reduction in the overall bacterial load over time. On the other hand, in group 2, there is a slight increase in total bacterial counts from T1 to T4, although this increase is not statistically significant. The findings in group 1 and 3 are consistent with a previous study that reported a decrease in salivary levels of total bacteria following debonding brackets [11]. The differences observed in the results of group 2 could be attributed to several factors. One possible factor is the limited sample size in the current study, which may have introduced variability and affected the accuracy of the results. Another factor could be examiner errors during the counting of bacterial colonies, as this process requires precision and can be subject to human error. Additionally, contamination issues during the experiment may have led to inaccurate colony counts and influenced the observed trends.

CFU counts for *S. mutans* colonies show a downward trend from T1 to T4, indicating a potential reduction in the population of *S. mutans* over time. This trend aligns with the decrease in *C. albicans* colony counts observed on SAB agar. Our results are in agreement with most studies that reported salivary *S. mutans* tends to decrease during the retention period of orthodontic treatment [9, 10, 14]. This suggests a positive impact on the overall oral health and microbial composition, potentially resulting from improved ability of the subjects to manage

and maintain oral hygiene in the absence of orthodontic appliances. However, our findings oppose the results of one study that found a significant higher level of salivary *S. mutans* after orthodontic treatment, despite a decrease in the overall bacterial count in saliva. [11]. The disparity in results could potentially be attributed to different techniques used to quantify the total bacterial load or patient population. The decreasing trend in *C. albicans* observed in the study may be attributed to the potential antifungal properties of fluoride [35]. While fluoride varnish is primarily used for its antibacterial effects, it could also have some impact on fungal organisms like *C. albicans*. However, the specific mechanisms and extent of this antifungal activity are not well understood and require further investigation.

In the current study the ratio of *S. mutans and C. albicans* to the total microbial load in the different groups reveals that there is a general decrease in the *S. mutans* load over time, indicating that the various fluoride treatments administered ultimately result in a significant reduction in the population of *S. mutans*. On the other hand, the proportion of *C. albicans* population remains relatively stable. These findings emphasize the potential impact of fluoride on reducing the prevalence of cariogenic bacteria *like S. mutans* in saliva after removing orthodontic brackets.

The findings of the current study contradict the suggestion of complex interactions between *S. mutans* and *C. albicans* in the oral microbiome, where these species can influence each other's growth and virulence [19]. Despite the observed decrease in *S. mutans* levels in the study, there was no corresponding decrease in *C. albicans* levels. These results indicate that the relationship between these two species may be more nuanced and further research is needed to fully understand the underlying mechanisms and the clinical significance of the observations.

Limitations of Study

A notable limitation of this study is that due to its ongoing nature the sample size is still relatively small and unevenly distributed among groups. However, ongoing efforts to recruit additional subjects will help address this limitation and lead to a larger dataset. By increasing the sample size, the variability in the data can be minimized, allowing for more rigorous statistical analyses. Furthermore, a larger sample size will enhance the ability to identify and analyze significant findings within the data, providing more comprehensive and reliable results.

In the current study, the identification of *S. mutans* was done by assessing colony morphology on Mitis-Salivarius Bacitracin agar (MSB). However, this method may have limitations in terms of accuracy as is allows some growth of other streptococci, and distinction of *S. mutans* from other streptococcal species is not always trivial [11, 36]. Similarly, SAB agar is commonly used to select and quantify *C. albicans*, but this selective medium is not entirely specific to this organism. Other fungal microorganisms, including other *Candida* species like *C. tropicalis* may also be present [37]. Despite these limitations, both MSB and SAB agar are widely recognized in the field of microbiology as suitable choices for estimating the presence of *C. albicans* and *S. mutans* within a microbial community. In future studies, it is suggested that real-time polymerase chain reaction (PCR) could be a more efficient and sensitive approach with minimum risk of contamination for the quantification and detection of *S. mutans* or other bacterial species in the oral microbiome. PCR technique allows for precise measurement of targeted bacteria, and its various applications can be conducted simultaneously, minimizing the risk of contamination.

Another limitation of this study is the inconsistency in the type of retainers worn by the subjects throughout the observation period. Some patients wore clear retainers, such as Essix retainers, while others wore Hawley retainers, which are acrylic retainers with wires.

Additionally, some patients wore clear retainers during the day and Hawley retainers at night. It is important to note that different types of retainers may have varying effects on salivary physiochemical and microbial properties. Studies have reported that the use of clear retainers leads to an increase in the acidity (pH) of saliva. Additionally, there was an observed increase in the oral microbiota, specifically with higher numbers of *S. mutans* and *Lactobacillus* colonies [38]. In another study, it was found that there was a statistically significant decrease in the salivary *S. mutans* level in the samples taken at 5 and 13 weeks after debonding in the clear retainer group. However, in the Hawley retainer group, the *S. mutans* levels increased at week 5 after debonding and decreased at week 13 [39]. In future studies, it would be advantageous to categorize patients based on the type of retention appliance they use or to perform separate analyses according to the specific type of retainer worn by patients. This would allow for a more comprehensive understanding of the potential effects of different retainer types on salivary properties and microbial composition.

An additional limitation of the current study is the use of fluoride toothpaste by the patients on a daily basis, which is considered the standard of care. While this practice aligns with recommended oral hygiene practices, it could potentially act as a confounding factor in the study. The presence of fluoride in toothpaste can contribute to the remineralization process and potentially influence the outcomes related to the effectiveness of the interventions being studied.

The last limitation of this study is the inclusion of xylitol in the 5% NaF varnish used. Xylitol is known to have antimicrobial properties against *S. mutans* in the oral cavity and can potentially impact the composition of salivary microbiota [31]. It would have been preferable to use a 5% NaF varnish without xylitol; however, the sodium fluoride varnish without xylitol currently available in the market has not received FDA approval for use in the United States.

Conclusion

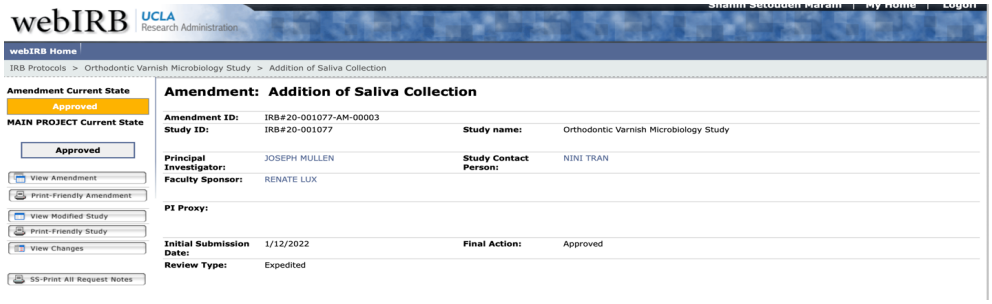
This study presents encouraging findings regarding the improvement of white spot lesions (WSLs) and the reduction of cariogenic bacteria, specifically *S. mutans,* during the initial 6 months following the completion of orthodontic treatment with fixed appliances. This decrease is determined through the quantification of colony-forming units (CFUs) in saliva samples cultured on different media. Although LCFV has been used as a protective barrier against demineralization and acid erosion, its effectiveness in remineralizing white spot lesions (WSLs) after the removal of orthodontic brackets is currently not clear. Further research with an increased sample size is needed to determine whether the alternative use of LCFV on WSLs can effectively promote remineralization of the lesions compared to 5% NaF and placebo varnish.

Disclosures

This study was funded by a grant from 3M Investigator Sponsored Research, #2020-ISR- 000199. The authors are grateful to 3M for sponsoring this research project, and a financial breakdown of the funds/products supplied can be found in Appendix E.

Appendix A

The approval notice for the study by the UCLA IRB and the key personnel.



List the key personnel and study staff below.

Note: All personnel listed below are required to complete CITI training courses (except for Fund Managers and Regulatory Coordinators). Please verify CITI training completion for all personnel prior to submitting a New Study application or Amendment application to add personnel. Verify using the Training Log tab in the application workspace (accessible by clicking the Exit button at the bottom of this page). HIPAA training is also required if personnel will be accessing protected health information.
Please make sure to have all personnel update their webIRB profile and contact information. Instructions on how to update the webIRB profile are available here.

	Name	Department	Role	Other Role (if applicable)	Will Obtain Consent?	Manage device accountability?	Access to personally identifiable info?	Access to code key?
View	Shahin Setoudeh Maram	DENTISTRY	Co-Investigator		yes	Yes	Yes	Yes

Appendix B

University of California Los Angeles

Oral Health Questionnaire

Name: _____ Visit #: 1 2 3 4

Have you been treated with any systemic antibiotics (e.g., pills) or topical antibiotics (e.g., mouth rinse/ ointment) in the past 30 days (or since your last visit)?

YES (please describe _____) NO

Have you used any fluoride-containing products BESIDES over-the-counter sodium fluoride toothpaste in the past 30 days (or since your last visit)?

YES (please describe _____) NO

Have you used any other adjunctive oral hygiene therapies (e.g., mouthrinse, hydrogen peroxide) in the past 30 days (or since your last visit)?

YES (please describe _____) NO

In the past 30 days (or since your last visit), have you been using a manual or electric toothbrush?

MANUAL ELECTRIC

In the past 30 days (or since your last visit), how many times a day have you brushed your teeth on average?

_____

In the past 30 days (or since your last visit), how many times a day have you flossed your teeth on average?

_____

In the past 30 days (or since your last visit), which of the following interdental cleaning products have you used? (Circle all that apply)

REGULAR FLOSS FLOSS PICKS WATERPIK INTERDENTAL BRUSHES

Since your last visit, have you noticed any change in the white spots on your teeth?(Describe where in mouth)

IMPROVED_____ STAYED THE SAME_____ GOTTEN WORSE_____

When was your last professional dental cleaning? _____

What Kind of Retainer have been wearing since the last study visit? (Circle one for each arch)
Upper retainer: HAWLEY ESSIX Lower retainer: HAWLEY ESSIX

Appendix C

Data Collection Sheet

Orthodontic White Spot Lesion Varnish Study
Data Collection Sheet

Note: Do NOT include any patient identifying information

Subject ID#: _____ Operator Name: _____

Date: _____ Study Visit _____
(1, 2, 3, 4)

Did they brush their teeth? Yes No

Did they fast (food/drink)? Both Food only Drink only No

Tooth number	DiagnoDent Score			Tooth number	DiagnoDent Score		
2				18			
3				19			
4				20			
5				21			
6				22			
7				23			
8				24			
9				25			
10				26			
11				27			
12				28			
13				29			
14				30			
15				31			

Time plaque collected: _____ Time plaque processed: _____

Time saliva collected: _____ Time saliva processed: _____

Label collection tubes:
[subject ID][X=maxillary, D=mandibular][visit][R=right, L=left]
Saliva: [subject ID][S][visit]

SALIVA MEASURES (circle one for each)

1) **Resting Flow Rate**: dry lower lip, time how long until droplets form

LOW (>60sec) Normal (30-60sec) High (<30sec)

2) **Salivary Consistency**: assess resting saliva in mouth

High Viscosity (Sticky/Frothy) Increased Viscosity (Frothy/Bubbly) Normal Viscosity (Watery/clear)

3) **pH (Resting Saliva)**: Spit pooled saliva into small cup, insert one end of pH strip for 10sec, save other end

RECORD pH: _____

Highly Acidic (5.0-5.8) Moderately Acidic (6.0-6.6) Healthy Saliva (6.8-7.8)

4) **Quantity of Stimulated Saliva**: chew wax 30sec, spit into STERILE TUBE, continue spitting until either >5mL collected or 5min pass, then spit small amount extra in second small cup

mL collected (if <5mL): _____

Very Low (<3.5mL) Low (3.5-5.0mL) Normal (>5.0mL)

5) **pH (Stimulated Saliva)**: insert other end pH strip 10sec into cup

RECORD pH: _____

Highly Acidic Moderately Acidic Healthy Saliva

6) **Buffering Capacity**: use pipette to place 3 drops stimulated saliva onto strip, turn strip on side to drain excess onto gauze, wait 2 minutes then score

RECORDED SCORE: _____

Very Low (0-5) Low (6-9) Normal (10-12)

Green = 4 points
Green/Blue = 3 points
Blue = 2 points
Blue/Red = 1 point
Red = 0 points

Appendix D

University of California Los Angeles

ORAL HYGIENE STUDY INSTRUCTIONS

Investigating the ability of light-cured fluoride varnish to remineralize post-orthodontic white spot lesions and alter the oral microbiome

You have enrolled in a study being conducted by Joseph Mullen, DDS, Renate Lux, PhD, Nini Tran, DDS, PhD, and associates from the School of Dentistry at the University of California, Los Angeles. As a part of your participation in this study, we ask that you comply with the following oral hygiene instructions, which are consistent with the standard of care for oral hygiene and for patients receiving treatment with fluoride varnish:

Instructions for today:

- Do not brush or floss your teeth in the next 4 hours
- Eat only soft food for the next 4 hours
- Do not consume hot drink or alcohol (including mouth rinses) for at least 4 hours
- Brush your teeth and floss before going to bed this evening

Instructions for the duration of the study:

- Brush your teeth twice each day (morning and night) using a soft-bristled manual toothbrush and a pea-sized amount of over-the-counter sodium fluoride toothpaste.
- Floss your teeth twice each day (morning and night).
- Refrain from using supplemental fluoride or antibiotic therapies (e.g., fluoride mouth rinse, antibiotic mouth rinse) for the duration of your involvement in the study, unless they are prescribed by your dentist or physician. If you are prescribed any antibiotic therapy (systemic or topical) by your dentist or physician, follow their instructions, and inform Dr. Joseph Mullen by calling him at (916) 770-9035.
- At today's visit you received a professional dental cleaning. Please refrain from receiving additional professional cleanings for the next six months, since you will receive another professional cleaning at your final study visit. This is consistent with the standard of care, which is to receive a cleaning every six months.

If you have any questions, comments, or concerns about the research, you can talk to the one of the researchers. Please contact Tyler Brennan, DDS, at (208) 985-0444, Shahin Setoudeh Maram, DMD, MSD at (424)542-4977, or the faculty sponsor, Renate Lux, PhD, at (310) 206-5660.

UCLA Office of the Human Research Protection Program (OHRPP):

If you have questions about your rights as a research subject, or you have concerns or suggestions and you want to talk to someone other than the researchers, you may contact the UCLA OHRPP by phone: (310) 206-2040; by email: participants@research.ucla.edu or by mail: Box 951406, Los Angeles, CA 90095-1406.

References:

1. Kilian, M., et al., *The oral microbiome - an update for oral healthcare professionals.* Br Dent J, 2016. **221**(10): p. 657-666.
2. Proffit, W.R., *Contemporary orthodontics.* . 2013, St. Louis, Missouri: St. Louis, Mo. : Elsevier/Mosby, c2013.
3. Gorton, J. and J.D. Featherstone, *In vivo inhibition of demineralization around orthodontic brackets.* Am J Orthod Dentofacial Orthop, 2003. **123**(1): p. 10-4.
4. Julien, K.C., P.H. Buschang, and P.M. Campbell, *Prevalence of white spot lesion formation during orthodontic treatment.* Angle Orthod, 2013. **83**(4): p. 641-7.
5. Mullen, J.V., E.; Chang Chien, K.; Duong, E.; Rizvi, M.; Hajian, P.; Li, H.; Shi, B.; Ting, K.; Shi, W.; Lux, R.; Wu, T., *Clinical and Microbial Changes in Orthodontic Patients Using Clear Aligners Vs. Fixed Appliances.* Journal of the California Dental Association, 2019. **47**(12): p. 783-791.
6. Campobasso, A., et al., *Taxonomic Analysis of Oral Microbiome during Orthodontic Treatment.* Int J Dent, 2021. **2021**: p. 8275181.
7. Mummolo, S., et al., *Salivary levels of Streptococcus mutans and Lactobacilli and other salivary indices in patients wearing clear aligners versus fixed orthodontic appliances: An observational study.* PLoS One, 2020. **15**(4): p. e0228798.
8. Sun, F., et al., *Comparison of oral microbiota in orthodontic patients and healthy individuals.* Microb Pathog, 2018. **123**: p. 473-477.
9. Rosenbloom, R.G. and N. Tinanoff, *Salivary Streptococcus mutans levels in patients before, during, and after orthodontic treatment.* Am J Orthod Dentofacial Orthop, 1991. **100**(1): p. 35-7.
10. Jing, D., et al., *Effect of fixed orthodontic treatment on oral microbiota and salivary proteins.* Exp Ther Med, 2019. **17**(5): p. 4237-4243.

11. Jung, W.S., et al., *Quantitative analysis of changes in salivary mutans streptococci after orthodontic treatment.* Am J Orthod Dentofacial Orthop, 2014. **145**(5): p. 603-9.

12. Lucchese, A., et al., *Changes in oral microbiota due to orthodontic appliances: a systematic review.* J Oral Microbiol, 2018. **10**(1): p. 1476645.

13. Miller, M.J., et al., *Demineralized white spot lesions: An unmet challenge for orthodontists.* Seminars in Orthodontics, 2016. **22**(3): p. 193-204.

14. Lemos, J.A., et al., *The Biology of Streptococcus mutans.* Microbiol Spectr, 2019. **7**(1).

15. Wen, Z.T., et al., *Lactobacilli and human dental caries: more than mechanical retention.* Microbiology (Reading), 2022. **168**(6).

16. Grzegocka, K., et al., *Candida prevalence and oral hygiene due to orthodontic therapy with conventional brackets.* BMC Oral Health, 2020. **20**(1): p. 277.

17. Sanz-Orrio-Soler, I., S. Arias de Luxan, and C.C. Sheth, *Oral colonization by Candida species in orthodontic patients before, during and after treatment with fixed appliances: A prospective controlled trial.* J Clin Exp Dent, 2020. **12**(11): p. e1071-e1077.

18. Falsetta, M.L., et al., *Symbiotic relationship between Streptococcus mutans and Candida albicans synergizes virulence of plaque biofilms in vivo.* Infect Immun, 2014. **82**(5): p. 1968-81.

19. Koo, H., D.R. Andes, and D.J. Krysan, *Candida-streptococcal interactions in biofilm-associated oral diseases.* PLoS Pathog, 2018. **14**(12): p. e1007342.

20. Kouvelis, G., et al., *A Prospective Cohort Study Assessing the Impact of Fixed Orthodontic Appliances on Saliva Properties and Oral Microbial Flora.* Oral Health Prev Dent, 2021. **19**(1): p. 67.

21. Dhiman, S., et al., *The relevance of physico-chemical and diagnostic properties of saliva during orthodontic treatment.* Med Rev, 2014. **1**: p. 5.

22. Alessandri Bonetti, G., et al., *Effect of fixed orthodontic appliances on salivary properties.* Prog Orthod, 2013. **14**: p. 13.

23. Du, M., et al., *Randomized controlled trial on fluoride varnish application for treatment of white spot lesion after fixed orthodontic treatment.* Clin Oral Investig, 2012. **16**(2): p. 463-8.

24. He, T., et al., *Comparative assessment of fluoride varnish and fluoride film for remineralization of postorthodontic white spot lesions in adolescents and adults over a 6-month period: A single-center, randomized controlled clinical trial.* Am J Orthod Dentofacial Orthop, 2016. **149**(6): p. 810-9.

25. Singh, S., et al., *Effects of various remineralizing agents on the outcome of post-orthodontic white spot lesions (WSLs): a clinical trial.* Prog Orthod, 2016. **17**(1): p. 25.

26. Hochli, D., et al., *Interventions for orthodontically induced white spot lesions: a systematic review and meta-analysis.* Eur J Orthod, 2017. **39**(2): p. 122-133.

27. Haahr, M. *True Random Integer Generator. RANDOM.ORG: True Random Number Service 2021*. 2021;

28. Oliveira, R.S., et al. *Laser surface engineering in dentistry*. 2015.

29. Shaphiro, S. and M. Wilk, *An analysis of variance test for normality.* Biometrika, 1965. **52**(3): p. 591-611.

30. Faul, F., et al., *G* Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences.* Behavior research methods, 2007. **39**(2): p. 175-191.

31. Kang, H., *The prevention and handling of the missing data.* Korean journal of anesthesiology, 2013. **64**(5): p. 402.

32. Shah, M., et al., *Comparative assessment of conventional and light-curable fluoride varnish in the prevention of enamel demineralization during fixed appliance therapy: a split-mouth randomized controlled trial.* Eur J Orthod, 2018. **40**(2): p. 132-139.

33. Jafari, K., S. Hekmatfar, and M. Fereydunzadeh, *In vitro Comparison of Antimicrobial Activity of Conventional Fluoride Varnishes Containing Xylitol and Casein Phosphopeptide-Amorphous Calcium Phosphate.* J Int Soc Prev Community Dent, 2018. **8**(4): p. 309-313.

34. Edunoori, R., et al., *Comparison of the efficacy of Icon resin infiltration and Clinpro XT varnish on remineralization of white spot lesions: An in-vitro study.* J Orthod Sci, 2022. **11**: p. 12.

35. Flisfisch, S., et al., *Effects of fluorides on Candida albicans.* Oral Dis, 2008. **14**(4): p. 296-301.

36. Zeng, Y., et al., *Identification of Non- Streptococcus mutans Bacteria from Predente Infant Saliva Grown on Mitis-Salivarius-Bacitracin Agar.* J Clin Pediatr Dent, 2020. **44**(1): p. 28-34.

37. Weerasekera, M.M., et al., *Culture media profoundly affect Candida albicans and Candida tropicalis growth, adhesion and biofilm development.* Mem Inst Oswaldo Cruz, 2016. **111**(11): p. 697-702.

38. Kasibut, P., et al., *Oral Microbiome in Orthodontic Acrylic Retainer.* Polymers (Basel), 2022. **14**(17).

39. Eroglu, A.K., Z.M. Baka, and U. Arslan, *Comparative evaluation of salivary microbial levels and periodontal status of patients wearing fixed and removable orthodontic retainers.* Am J Orthod Dentofacial Orthop, 2019. **156**(2): p. 186-192.