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EFFECT OF THREE DIFFERENT FLUORIDE VARNISHES ON STREPTOCOCCUS MUTANS AND LACTOBACILLUS BIOFILM: AN IN VITRO STUDY

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KEYWORDS

Fluoride-releasing material, Fluoride varnish, Lactobacillus, Streptococcus mutans

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ABSTRACT

Introduction: Fluoride varnish is quickly and easily applied without the need for bulky mouth trays or suctioning of saliva. This is especially helpful for infants and toddlers. **Aim:** The aim of this study was to evaluate and compare the effect of three different fluoride varnishes (Durashield, Flour Protector and Bifluoride 10) on *streptococcus mutans and lactobacillus* count (in vitro study). **Materials and methods**: Total of 144 acrylic discs were divided into two main equal groups of 72 discs in each (group A: for examination of *Streptococcus mutans* and group B: for examination of *Lactobacillus*); each group was further subdivided into four equal subgroups based on the type of varnish applied to the disc surface (A1, B1: Durashield; A2, B2: Fl protector; A3, B3: Biflouride; A4, B4: untreated discs served as control). Saliva collected from single volunteer was poured into tubes until the discs were completely immersed and incubated for 24h, 48h and 5 days. Six discs from each subgroup were taken out from tubes every time and sonicated in saline. Suspensions were been plated on selective media for *Streptococcus mutans* and *Lactobacillus*. The numbers of colony-forming units (CFU/mL) on suitably diluted plates were been determined. **Results**: For all tested subgroups, the lowest amount bacterial count of *Streptococcus mutans* and *Lactobacillus*was observed during the first 24 h, followed by a significant increase over the following 4 days. Biflouride subgroups had the lowest values of viable *Streptocoocusmutans* and *Lactobacillus*counts during all test periods. In comparison Durashield with Flour protector subgroup difference of viable counts of *streptocoocusmutans* and *lactobacillus* was observed but statistically non-significant. **Conclusions**: Biflouridevarnish exhibited the greatest antibacterial effect (*Streptococcus mutans* and *Lactobacillus*) compared to Durashield and flour protector. Fluor protector varnish showed the same inhibitory effect of Durashildvarnish despite Flour protector had lower fluoride concentration than Durashield.

INTRODUCTION

Oral biofilms are an essential component in the etiology of dental caries and periodontal disease. Dental plaque biofilm is a deposit of proteins, cell-free enzymes, and bacteria embedded in expolysaccharides that adhere firmly to the tooth surface[(1)].

About 700 different bacteria species have been identified from the human oral microbiome. Oral *streptococci*, especially of group *mutans* and lactic acid bacteria (*Lactobacillus* spp.) play an important role in

the pathogenesis of dental caries. It is believed that bacteria of the species *Streptococcus mutans*is the main factor that initiates caries and very important factor of enamel decay. *Mutans streptococci* and *lactobacilli* are characterized by the ability to grow in an acidic environment and the property of rapid metabolism of sugars supplied in the diet to organic acids, including lactic acid[2,3].

Lactobacilli are isolated from deep caries lesions but rarely just before the development of dental caries and in the early tooth decay. It is believed that they are pioneering microorganisms in the caries progress, especially in dentin[4]. Studies have shown that *Lactobacilli* are a dominant part of the flora inhabiting the deep cavities, and their number correlates with the amount of carbohydrates[5,6] .

The prevention of dental caries is targeted at the control of dental plaque. Chemical agents could represent a valuable complement to mechanical plaque control. The active agents should prevent biofilm formation without affecting the biological equilibrium within the oral cavity[7,8].

Fluoride varnish is quickly and easily applied without the need for bulky mouth trays or suctioning of saliva. This is especially helpful for infants and toddlers, some developmentally disabled individuals, or people with severe gag reflexes who otherwise might not tolerate the use of trays or the bulkiness of gels or foams[9-11]. When fluoride Varnish brushed onto the teeth, provides a highly concentrated dose of fluoride and maintains prolonged contact with enamel to inhibit caries. Fluoride varnish has been used for over 30 years since its introduction in the 60s[12,13].

The study was designed to evaluate and compare the effect of three different fluoride varnishes (Durashield, Flour Protector and Bifluoride 10) on *streptococcus mutans* and *lactobacillus* count, in vitro study.

MATERIALS AND METHODS

The protocol of the research project followed the guidelines of scientific committee of Faculty of Dentistry, Suez Canal University.

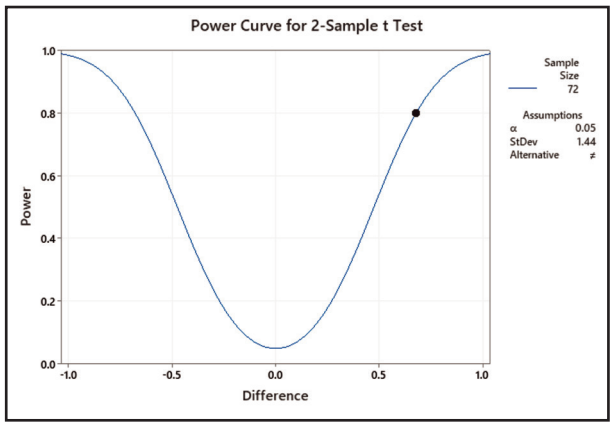
Table (1) *Dental varnishes used in this study*

	Active ingredients	Other ingredients	Manufacturers
Durashield	5% Sodium Fluoride	Ethanol Rosin	Sultan Healthcare, NJ, USA
Flour Protector	1%Difluorosilane	Polyurethane and Ethyl acetate	Ivoclar Vivadent, Schaan, Liechtenstein
Bifluoride 10	6% Sodium Fluoride 6% Calcium Fluoride	——	Voco, Cuxhaven, Germany

Sample size selection[14]

Sample Size	Power	Difference
72	0.8	0.676970

The sample size is for each group.



Study design

Total 144 standard acrylic discs were prepared and divided into two main equal groups of 72 discs in each (group A: for examination of *Streptococcus mutans* count and group B: for examination of *Lactobacillus* count); each group was further subdivided into four equal subgroups (18 discs in each) based on the type of varnish applied to the disc surface nd untreated discs [18] served as control. **(Fig. 1)**

Equal volumes of unstimulated saliva were Collected from single volunteer with the following inclusion criteria: [15]

- age = 6 year
- Had newly erupted four first permanent molars (free of caries)
- Had high caries index (dmfs=18).
- Did not receive any medication during the two weeks preceding the study

Saliva was poured into 144 tubes until the discs were completely immersed. Tubes shaken for 1min (six times) at room temperature and incubated for 24h, 48h and 5 days at 37°C. Discs were taken out from tubes (6 discs every time) and they been immersed in 5 mL of saline. Then discs were sonicated for 1 min (using an ultrasonic water bath to detach the bacteria from the surface). Samples from the suspensions were plated on selective media for streptococcus and Lactobacillus. Mitis Salivarius Bacitracin Agar was used as the selective medium for culturing *Streptococcus mutans* and Rogosa Agar *was* used for culturing *Lactobacillus*. The plates were been incubated for 48 h at 37°C, and the numbers of colony-forming units (CFU/mL) were been determined.

Fig. (1) The experimental factorial design of in vitro study

Preparation of standard acrylic discs [16]:

Standard molds were prepared from dental plaster with holes filled with acrylic mix. After 24h, the discs were removed from plaster molds, polished and packaged for autoclaving by subjecting them to pressurized saturated steam at 121°C (250°F) for around 20 minutes (autoclave brand: bench-top E7 EURONDA)

The discs were divided into 2 groups; each group was further subdivided into four equal subgroups (18 discs in each) based on the type of varnish applied to each disc surface: Durashield, Flour Protector and Bifluride10. Untreated discs [18] served as controls.

Bacteriological studies

Mitis Salivarius Bacitracin Agar was used as the selective medium for culturing *Streptococcus mutans* and Rogosa Agar *was* used for culturing *Lactobacillus*. **(Fig. 2&3)**

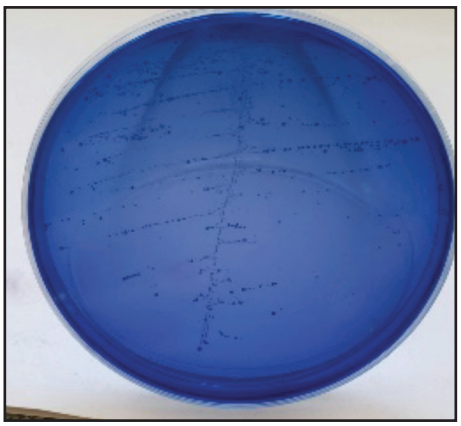


Fig. (2) Streptococcus mutans on Streptococcus Mitis Bacitracin agar plate

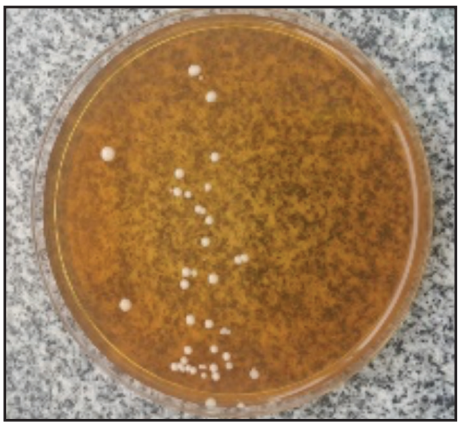


Fig. (3) Lactobacilli on Rogosa agar

Processing of the specimen:

All bacteriological analysis were performed in microbiology department, faculty of medicine, Suez Canal university. All subgroups were incubated for 24h, 48h and 5days, 6 discs from each subgroup (3 times) were removed from saliva, taken out from tubes, washed and immersed in 5 ml sterile saline. Specimens were sonicated for 1 min in ultrasonic water bath (Elma, Germany) to detach biofilm-forming bacteria from the surface of the discs. Using sterile disposable calibrated loops 1/100 and 1/1000 ml, samples from the suspensions were inoculated on freshly prepared MSB and Rogosa agar plates. Dilution 1:100 or 1:1000 with phosphate buffered saline (PBS) before inoculation was done only if needed according the turbidity of the suspension. After incubation, a colony counter with magnifying glass was used to count the number of colonies and they were expressed as number of colony forming units per ml (CFU/ml) of saliva. By multiplying the actual colony count by 10^2 or 10^3 (according to the used calibrated loop) quantification of the number of colonies was done. [15]

Statistical method

All data were collected, tabulated and statistically analyzed. Data was presented as mean ± standard deviation for numerical variables. The numbers of colonies were presented as millions. ANOVA test was used to compare changes inside each subgroup within time intervals. T-paired test was used to

compare changes between two subgroups according to time intervals. The Friedman test was used to compare changes between two subgroups over time. The significance level was set as P value <=0.05 is significant. Statistical analysis was performed using SPSS version 16.

RESULTS

ANOVA test revealed that there was a significant increase in the viable counts of *Streptococcus mutans* and *Lactobacillus* during all test periods in all subgroups. For all tested subgroups, the lowest amount bacterial count was observed during the first 24 h, followed by a significant increase over the following 4 days. **(Table. 2&3)**

Table (4): showed comparison between *Streptococcus mutans* count in subgroup A1 (control) and subgroups A2, A3 and A4 individually in all time parts. T-paired test revealed that a marked decrease in the viable counts of *Streptococcus mutans* during the test periods was observed between A1 (control) subgroup and other tested subgroups, which was statistically significant. Among the tested subgroups, A4 subgroup had the lowest values of viable *Streptocoocus mutans* counts during all test periods.

Table (5): showed comparison between *Lactobacillus* count in subgroup B1(control) and other tested subgroups B2, B3 and B4 individually in all time parts using (T-paired) test. A marked difference of the viable counts of *Lactobacillus* during all test periods was observed between A1(control) subgroup and other tested subgroups, which was statistically significant. Among the tested subgroups, B4 subgroup had the lowest values of viable *Lactobacillus* counts during all test periods.

Table (2) *Descriptive statistics of* ***Streptococcus mutans*** *biofilm count in CFU/ml at different examination times:*

Duration	Control (A1) Mean ± SD	Durashield (A2) Mean ± SD	Flour Protector (A3) Mean ± SD	Biflouride (A4) Mean ± SD
24 hours (n=6)	3.83 ± 0.72	2.42 ± 0.36	2.41 ± 0.39	1.98 ± 0.28
48 hours (n=6)	4.63 ± 0.14	3.79 ± 0.09	3.24 ± 0.09	2.64 ± 0.16
5 days (n=6)	8.32 ± 0.13	7.38 ± 0.13	6.85 ± 0.1	6.19 ± 0.12
P value	**0.002***	**0.001***	**0.003***	**0.001***

Table (3) *Descriptive statistics of* ***Lactobacillus*** *biofilm count in CFU/ml at different examination times:*

Duration	Control (B1) Mean ± SD	Durashield (B2) Mean ± SD	Flour Protector (B3) Mean ± SD	Biflouride (B4) Mean ± SD
24 hours (n=6)	2.69 ± 0.09	2.15 ± 0.05	2.02 ± 0.06	1.92 ± 0.06
48 hours (n=6)	3.52 ± 0.26	3.25 ± 0.05	3.14 ± 0.05	2.85 ± 0.05
5 days (n=6)	5.72 ± 0.05	5.13 ± 0.05	5.08 ± 0.05	4.88 ± 0.05
P value	**0.003***	**0.001***	**0.002***	**0.002***

Regarding the comparison between all subgroups of *Streptococcus mutans* and *Lactobacillus* at different test times

Table (4) *Multiple Comparison of Streptococcus mutans counts between two Subgroups individually during all test periods parts using T-paired test:*

	24 Hours		48 Hours		5 Days	
	MEAN ± SD	**P VALUE**	**MEAN ± SD**	**P VALUE**	**MEAN ± SD**	**P VALUE**
A1 : A2	3.83 ± 0.72 2.42 ± 0.36	0.021*	4.63 ± 0.14 3.79 ± 0.09	0.031*	8.32 ± 0.13 7.38 ± 0.13	0.003*
A1 : A3	3.83 ± 0.72 2.41 ± 0.39	0.001*	4.63 ± 0.14 3.24 ± 0.09	0.02*	8.32 ± 0.13 6.85 ± 0.1	0.001*
A1 : A4	3.83 ± 0.72 1.98 ± 0.28	0.000*	4.63 ± 0.14 2.64 ± 0.16	0.000*	8.32 ± 0.13 6.19 ± 0.12	0.000*
A2 : A3	2.42 ± 0.36 2.41 ± 0.39	0.931	3.79 ± 0.09 3.24 ± 0.09	0.831	7.38 ± 0.13 6.85 ± 0.1	.621
A2 : A4	2.42 ± 0.36 1.98 ± 0.28	0.043*	3.79 ± 0.09 2.64 ± 0.16	0.01*	7.38 ± 0.13 6.19 ± 0.12	0.006*
A3 : A4	2.41 ± 0.39 1.98 ± 0.28	0.041*	3.24 ± 0.09 2.64 ± 0.16	0.01*	6.85 ± 0.1 6.19 ± 0.12	0.004*

Table (5) *Multiple Comparison of Lactobacillus counts between two subgroups individually during all test periods using T-paired test:*

	24 Hours		48 Hours		5 Days	
	MEAN ± SD	**P VALUE**	**MEAN ± SD**	**P VALUE**	**MEAN ± SD**	**PVALUE**
B1 : B2	2.69 ± 0.09 2.15 ± 0.05	0.001	3.52 ± 0.26 3.25 ± 0.05	0.031	5.72 ± 0.05 5.13 ± 0.05	0.004
B1 : B3	2.69 ± 0.09 2.02 ± 0.06	0.041*	3.52 ± 0.26 3.14 ± 0.05	0.009*	5.72 ± 0.05 5.08 ± 0.05	0.005*
B1 : B4	2.69 ± 0.09 1.92 ± 0.06	0.000*	3.52 ± 0.26 2.85 ± 0.05	0.000*	5.72 ± 0.05 4.88 ± 0.05	0.001*
B2 : B3	2.15 ± 0.05 2.02 ± 0.06	0.631	3.25 ± 0.05 3.14 ± 0.05	0.931	5.13 ± 0.05 5.08 ± 0.05	0.613
B2 : B4	2.15 ± 0.05 1.92 ± 0.06	0.023*	3.25 ± 0.05 2.85 ± 0.05	0.02*	5.13 ± 0.05 4.88 ± 0.05	0.005*
B3 : B4	2.02 ± 0.06 1.92 ± 0.06	0.020*	3.14 ± 0.05 2.85 ± 0.05	0.02*	5.08 ± 0.05 4.88 ± 0.05	0.003*

DISCUSSION

Antibacterial varnishes are widely used for various reasons, such as the greater ease of usage in young patients and their ability to contact inaccessible areas, including interdental points, compared with other methods[17]. Many studies have compared the long-term prophylactic effect between fluoride and chlorhexidine varnishes[18].

Streptococcus mutans and *Lactobacillus* were chosen because they are the major pathogenic bacteria associated with dental biofilms. *Streptococcus mutans* has been implicated most of the initiator of dental caries. *Lactobacilli* are implicated as important contributory bacteria in tooth decay. They are considered secondary invaders rather than initiators of the caries process[19,20].

This study model mimics several of the environmental conditions in the oral cavity such as saliva, bacteria and in situ polysaccharide production which affect bacterial adhesion to surfaces[16]. Saliva samples were used to determine counts of *streptococcus muatns* and *lactobacillus*. It is considered the most reliable method in children due to higher odds ratio between level of the count in plaque and saliva, which confirmed by **Sengupta P et al [21].**

In the present study saliva was obtained from a volunteer (6 years) with high caries index (dmfs=18) according to American Dental Association which recommended fluoride varnish application for individuals at risk of dental caries. [15] The use of oral stimulants when collecting saliva is not recommended due to the possibility of causing assay interference or alteration of levels of some analytics, even chewing on unflavored paraffin/wax could affect flow-dependent analytics.[22] Therefore, unstimulated saliva was collected to minimize unnecessary sources of variation in saliva test results. Mitis Salivaris agar and Rogosa agar were chosen because of their highly accurate results in showing counts of bacterial colonies in the saliva sample, this finding agreed with **Daniela et al [23]**.

In the present study, ANOVA test revealed a significant increase in the viable counts of *Streptococcus mutans* and *Lactobacillus* during the test periods in control subgroup (A1and B1), other tested subgroups A2, B2, A3, B3, A4 and B4. These results may be due to the bacteria that survived and continued to grow produced an extracellular matrix. It is thought that, as the biofilm thickness was increasing during the 5 days, the penetration of antimicrobials through the biofilm could be blocked and that pH differences in the plaque layers could decrease the antibacterial efficacy of the test varnishes[24,25].

For all tested subgroups, the lowest bacterial count was observed during the first 24 h, followed by a significant increase over the following 4 days. These findings agreed with **Erdem et al[16],** they compared the effect of two fluoride varnishes and one fluoride/chlorhexidine varnish on *Streptococcus mutans* and *Streptococcus sobrinus* biofilm formation, and concluded that the fluoride concentration decreased while the viable bacterial counts increased. Thus, it is possible that the rapid release of fluoride from the varnishes resulted in too low remaining concentrations of fluoride to exert an antibacterial effect or to inhibit biofilm formation. Similar results were observed with both biofilms. Sustained-release systems -including varnishes- generally show an initial burst, with rapid release of the active agent, followed by a slower phase of release[26].

In the present study, when the antibacterial effects of the three varnishes were compared, a significant decrease in the viable counts of *Streptococcus mutans* and *Lactobacillus* during the test periods was observed between control (A1 and B1) subgroups and other tested subgroups, indicating

obvious antibacterial activity of fluoride varnishes, these findings agreed with many studies [27-29].

In the present study, among the tested subgroups, Biflouride (A4 and B4) had the lowest values of viable *Streptococcusmutans* and *Lactobacillus* count during all test periods, followed by Flour Protector (A3 and B3) while Durashield (A2 and B2) showed the least inhibitory effect. This might be attributed to the higher fluoride content of Bifluorid (6%) as compared to Durashield (5%) and FlourProtector(1%) and the comparatively low fluoride content. This results agreed with **Munshi et al, [30]; Shalin et al,[31]** ; **Chau et al,[32]** , they reported that (Biflouride (6%NaF+6%NaF), Cavityshield (5%NaF) and Flor Opal (5%NaF)) varnishes, reduced affected *Streptococcus mutans* adhesion (67-98% reduction), CFU count, water-insoluble and extracellular polysaccrides amount. While, Flour Protector (1%NaF) did not affect *Streptococcus mutans* adhesion. These results may be related to the fluoride-release pattern of the fluoride varnishes tested.

The previous results disagreed with **Erdem et al, [16]** who found that Bifluoride had the lowest inhibitory effect against *Streptococcus mutans* biofilms during the experimental period, although it had the highest fluoride concentration. This may due to the consistency of previous editions of Biflouride varnish had a higher viscosity than Flour protector and Flour protector plus cervitec (chlorhexidine) varnishes, which may have resulted in a thicker layer on the acrylic surface. The adherence of the bacteria to this surface may have been easier than that in the other groups. Newly edition of Biflouride was improved to become less viscous and give minimal thickness on enamel surface with optimal adhesion and longer fluoride release pattern[33,34].

Fluor Protector contains polyurethane-based compound difluorosilane, has a low pH and formes a thin transparent film on the disc surface. Although Fluor Protector contained a lower fluoride concentration than Durashield, the difference of bacterial count was statistically non-significant; this may be explained by its silane content.

CONCLUSION

Flouride varnishes (Durashiled, Flour protector and Biflouride) had antibacterial property. Biflouride varnish exhibited the greatest antibacterial effect compared to Durashield and flour protector. Fluor protector varnish showed the same inhibitory effect of Durashildvarnish despite Flour protector had lower fluoride concentration than Durashield.

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