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Colonization of *Streptococcus mutans* and *Lactobacillus* around orthodontic ligation systems with or without antiseptic application

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Abstract:

The presence of acid producing oral microbes colonizing around the orthodontic attachments bonded on the tooth surface lead to enamel demineralization which often leads to alteration in the appearance of the enamel surface. Therefore, it is of interest to evaluate the *Streptococcus mutans* and *Lactobacillus* colonization around 5 orthodontic ligation systems in 12 patients undergoing fixed orthodontic treatment. Five ligation systems were stainless steel ligature, Super-Slick elastomeric ring, Safe-T-Tie, Mini Stick and self-ligating brackets. Plaque samples were collected after 21 days without chlorhexidine and again after 21 days of chlorhexidine use. Chlorhexidine significantly reduced cariogenic bacterial CFUs in all groups ($p < 0.05$). Thus, data shows that self-ligating brackets, when combined with chlorhexidine mouthwash, may reduce biofilm accumulation and enamel demineralization risk during orthodontic therapy.

Keywords: Chlorhexidine (CHX), colony-forming units, *Lactobacillus*, orthodontic ligation systems, self-ligating brackets, *Streptococcus mutans*

Background:

Enamel decalcification is a common problem in orthodontics. The presence of acid producing oral microbes colonizing around the orthodontic attachments bonded on the tooth surface lead to enamel demineralization which often leads to alteration in the appearance of the enamel surface [1-4]. Fifty percent of individuals undergo iatrogenic enamel demineralisation subsequent to orthodontic treatment. These painful lesions-or, in severe cases, cavitation-may lead patients to discontinue treatment prematurely. Patients receiving orthodontic treatment face changes in oral ecology characterised by a decreased pH and increased retention of food particles, potentially leading to elevated proportions and absolute counts of salivary *Lactobacilli* and *Streptococcus mutans* [5]. Fixed appliances augment the accumulation of bacteria on dental surfaces by generating additional retention areas and hindering their access to tooth surfaces for optimal cleaning. Biofilm retention is markedly affected by the ligation procedure of orthodontic arch wires. Elastomeric rings have been shown to harbour a greater number of microorganisms in the plaque surrounding brackets than steel ligatures. Fluoride-releasing elastomeric rings have been created to enhance enamel remineralisation in difficult-to-clean regions adjacent to the bracket base and to reduce biofilm formation, hence mitigating demineralisation. In recent years, companies have employed Metafasix technology (Super Slick™) to alter the surface characteristics of elastomeric modules. It has been proposed that these alterations diminish bacterial adhesion [6-8]. While biofilm formation persists, self-ligating brackets, which secure archwires using a metal clip instead of elastomeric or stainless-steel ligatures, are considered to be less susceptible to bacterial colonisation. Orthodontic patients are advised to utilise fluoridated or chlorhexidine-based mouthwashes and to bond brackets with fluoride-containing composites or glass ionomer cements to inhibit cariogenic biofilm production and enamel demineralisation. Chlorhexidine (CHX) di-gluconate has been

selected as a standard chemical therapy agent owing to its proven efficacy [9]. Therefore, it is of interest to compare and evaluate the microbial colony count of *Streptococcus mutans* and *Lactobacillus* in the plaque formed around brackets with the use of different ligation systems with or without use of chlorhexidine.

Material and Methods:**Study design and population:**

The comparative *in vivo* study was conducted among patients undergoing fixed orthodontic treatment aged between 16 to 25 years who were selected from the postgraduate clinic the Department of Orthodontics and Dentofacial Orthopaedics.

Sample size:

The sample size (n) is derived by using the comparing two means formula:

$$n = \frac{2 \left(\frac{Z_{\alpha}}{2} + Z_{\beta} \right)^2 \cdot \sigma^2}{d^2}$$

Where $Z_{\alpha/2}$ -critical value at confidence level of 95%, α is 0.05 and the critical value is 1.96,

Z_{β} - for a power of 80%, B is 0.2 and the critical value is 0.84),

σ^2 is the population variance,

d - Mean difference between 2 groups (master article reference - Silver elastics)

Substituting the values in the above formula,

A total sample size of 12 patients was derived.

$$n = \frac{2(1.96 + 0.84)^2 \cdot (3.8)^2}{(97.97 - 80.3)^2}$$

Inclusion criteria:

[1] 1st or 2nd premolar extraction cases.

[2] Fixed orthodontic treatment with metal brackets.

- [3] The age group of 16 to 25 years.
 [4] Permanent dentition free of plaque & patients motivated to maintain good oral hygiene.

Exclusion criteria:

- [1] Presence of decalcification or risk of caries
 [2] Halitosis
 [3] Poor periodontal status

Methodology:

First Phase - For ease of analysis, a split-mouth design will be used. Stainless steel 0.022" slot MBT brackets (leone) were bonded in both the arches except the premolars to be extracted which were bonded with self-ligating brackets (selfy) and 0.012 / 0.014 Nitinol wire was placed. In each subject the modified elastomeric rings and the self-ligating brackets were placed as follows:

- [1] Group A - Stainless steel ligature was placed in the 1st quadrant.
 [2] Group B - Super-slick elastomeric ring was placed in the 2nd quadrant.
 [3] Group C - Safe-T-Tie was placed in the 3rd quadrant.
 [4] Group D - Mini Stick was placed in the 4th quadrant.
 [5] Group E - Self-ligating bracket to be bonded on the 1st or 2nd premolar whichever is to be extracted. Second Phase - After 21 days, the patients were called back and the ligatures were made carefully and aseptically. Removed and self-ligating brackets were de-bonded without affecting the plaque surrounding the brackets. The de-bonded bracket and elastomeric ligatures were placed in a sterile vial and inoculated on *Lactobacilli* and *Streptococcus mutans* culture media. The patient was instructed to use mouthwash containing chlorhexidine after the new ligature ties and self-ligating brackets as mentioned in the aforementioned groups were installed. After the following 21 days, the samples were gathered once more and sent for microbiological examination.

Microbiological analysis:

To assess the colonisation of *Streptococcus mutans* and *Lactobacillus*, respectively, specimens were collected in aseptic conditions and inoculated on mitis salivary agar and tomato juice agar. A platinum loop was used to quickly spread a decimal dilution of the specimen made with 0.01 millilitres of

sterile water across the entire medium on the plates. The plates were then incubated for 48 hours.

Statistical analysis:

Microsoft Excel (v.2013) was used to enter and sort the data. The Statistical Package for Social Sciences (SPSS) program (v.21.0) was used for statistical analysis. Colony forming units (CFUs) in various groups were subjected to descriptive and frequency statistics. The One-way Analysis of Variance (ANOVA) and the post hoc Tukey's test were used to compare the subgroups A, B, C, D and E to see whether there were any significant differences. Comparison of the CFUs amongst two groups; with and without chlorhexidine was done using unpaired t-test or Independent samples t-test to assess significant differences between various subgroups. All statistical tests were run at 95% confidence intervals; keeping a p-value of less than 0.05 as statistically significant.

Results:

The frequency distribution of *Streptococcus mutans* and *Lactobacillus* colony-forming units (CFUs) without chlorhexidine showed that Groups B, C and E had a higher proportion of samples with CFU counts $<10^2$ while Group D had relatively higher CFU counts. *Lactobacillus* was distributed equally among the five ligation systems (**Table 1**). In all groups, the CFU values of *Streptococcus mutans* and *Lactobacillus* changed to the lower CFU range ($<10^2$) after use of chlorhexidine. Group E showed the highest reduction in microbial counts, while Group D still had relatively higher counts (**Table 2**). Intergroup comparison of CFUs between different subgroups A, B, C, D and E showed statistically significant differences (p-value < 0.05) between for with & without chlorhexidine groups between different subgroups (**Table 3**). Multiple pairwise Intergroup comparison of *Streptococcus mutans* and *Lactobacillus* CFUs between different subgroups A, B, C, D, and E in the without Chlorhexidine group showed statistically significant differences (p value < 0.05) between Group D and all other groups (**Table 4**). Multiple pairwise Intergroup comparison of *Streptococcus mutans* between different subgroups A, B, C, D, and E in with Chlorhexidine group showed statistically significant differences (p value < 0.05) between Group D and all other groups and in case of *Lactobacillus* showed statistically significant differences (p value < 0.05) only between group D and group A (**Table 5**).

Table 1: Frequency distribution of *Streptococcus mutans* and *Lactobacillus* Colony Forming Units (CFUs) without Chlorhexidine

CFU Range	Group A	Group B	Group C	Group D	Group E
	Strep / Lacto	Strep / Lacto	Strep / Lacto	Strep / Lacto	Strep / Lacto
No growth	0 / 0	0 / 0	0 / 0	0 / 0	1 (8.3%) / 0
$<10^2$	5 (41.7%) / 6 (50.0%)	7 (58.3%) / 8 (66.7%)	7 (58.3%) / 7 (58.3%)	5 (41.7%) / 0	9 (75.0%) / 10 (83.3%)
10^2 - 10^5	7 (58.3%) / 6 (50.0%)	5 (41.7%) / 4 (33.3%)	5 (41.7%) / 5 (41.7%)	7 (58.3%) / 6 (50.0%)	2 (16.7%) / 2 (16.7%)
$>10^5$	0 / 0	0 / 0	0 / 0	0 / 6 (50.0%)	0 / 0
Total	12	12	12	12	12

Table 2: Frequency distribution of *Streptococcus mutans* and *Lactobacillus* Colony Forming Units (CFUs) with Chlorhexidine

CFU Range	Group A	Group B	Group C	Group D	Group E
	Strep / Lacto	Strep / Lacto	Strep / Lacto	Strep / Lacto	Strep / Lacto
$<10^2$	8 (66.7%) / 8 (66.7%)	9 (75.0%) / 9 (75.0%)	9 (75.0%) / 9 (75.0%)	0 / 0	11 (91.7%) / 11 (91.7%)
10^2 - 10^5	4 (33.3%) / 4 (33.3%)	3 (25.0%) / 3 (25.0%)	3 (25.0%) / 3 (25.0%)	9 (75.0%) / 9 (75.0%)	1 (8.3%) / 1 (8.3%)

>10 ⁵	0 / 0	0 / 0	0 / 0	3 (25.0%) / 3 (25.0%)	0 / 0
Total	12	12	12	12	12

Table 3: Intergroup comparison of CFU's between different subgroups A, B, C and D & E

Comparison groups		Microorganism	Sum of Squares	Df	Mean Square	F	pvalue
A vs B vs C vs D vs E	Without Chlorhexidine	<i>Streptococcus mutans</i>	15.600	4	3.900	14.709	.000*
		<i>Lactobacillus</i>	13.333	4	3.333	13.836	.001*
	With Chlorhexidine	<i>Streptococcus mutans</i>	10.400	4	2.600	13.839	.000*
		<i>Lactobacillus</i>	.500	4	.125	.640	.037*

*p value <0.05 statistically significant

Table 4: Multiple pairwise intergroup comparison of *Streptococcus mutans* and *Lactobacillus* CFUs between different subgroups (A-E) without chlorhexidine

(I) Group	(J) Group	<i>Streptococcus mutans</i> Mean Difference (I-J)	p-value	<i>Lactobacillus</i> Mean Difference (I-J)	p-value
A	B	0.16667	0.932	0.16667	0.920
A	C	0.16667	0.932	0.08333	0.994
A	D	-1.00000*	0.000*	-1.00000*	0.000*
A	E	0.50000	0.137	0.33333	0.465
B	C	0.00000	1.000	-0.08333	0.994
B	D	-1.16667*	0.000*	-1.16667*	0.000*
B	E	0.33333	0.513	0.16667	0.920
C	D	-1.16667*	0.000*	-1.08333*	0.000*
C	E	0.33333	0.513	0.25000	0.724
D	E	1.50000*	0.000*	1.33333*	0.000*

*p value <0.05 statistically significant

Table 5: Multiple pairwise intergroup comparison of *Streptococcus mutans* and *Lactobacillus* CFUs between different subgroups (A-E) with chlorhexidine

(I) Group	(J) Group	<i>Streptococcus mutans</i> Mean Difference (I-J)	p-value	<i>Lactobacillus</i> Mean Difference (I-J)	p-value
A	B	0.08333	0.990	0.00000	1.000
A	C	0.08333	0.990	0.08333	0.090
A	D	-0.91667*	0.000*	0.08333*	0.010*
A	E	0.25000	0.622	0.25000	0.640
B	C	0.00000	1.000	0.08333	0.990
B	D	-1.00000*	0.000*	0.08333	0.990
B	E	0.16667	0.879	0.25000	0.640
C	D	-1.00000*	0.000*	0.00000	1.000
C	E	0.16667	0.879	0.16667	0.887
D	E	1.16667*	0.000*	0.16667	0.887

*p value <0.05 statistically significant

Discussion:

Fixed orthodontic appliances provide new areas of retention for colonization of bacteria and increase in *Streptococcus mutans* and *lactobacilli* [10]. Many studies have assessed the effect of fixed orthodontic appliances on microbial flora and periodontal health, but few have studied the effect of different methods of ligation [11]. Electrostatic and hydrophobic interactions, physicochemical properties of bacteria and bracket surfaces, and salivary composition and flow affect bacterial adherence [12]. In addition, patients undergoing orthodontic treatment are prone to enamel white spot lesions because metallic brackets reduce pH, increase plaque accumulation and promote *S. mutans* colonization [13]. Stainless steel has a higher critical surface tension than ceramic brackets and therefore shows higher bacterial adhesion [14]. In view of these observations, the present study assessed the microbial colonisation with respect to various ligation techniques and changes in the *Streptococcus mutans* and *Lactobacillus* colony-forming units (CFUs) with and without chlorhexidine mouthwash. In the literature, bacterial interactions in biofilms have been suggested to play a role in disease progression once a critical bacterial threshold is reached [15]. The present study demonstrated Group E (self-ligating brackets) to have the least bacterial colonisation with maximum CFUs in the range of 10²-10⁵ for both *Streptococcus mutans* and

Lactobacillus irrespective of chlorhexidine use. Similar results were reported by Dias *et al.* [9], with less bacterial colonisation in self-ligating brackets and similar antimicrobial activity of chlorhexidine in both conventional and self-ligating systems. Biofilms of *S. mutans* remained susceptible to chlorhexidine in both systems. However, Baka *et al.* [16] did not observe significant differences in the plaque retention between self-ligating brackets and conventional brackets ligated by stainless steel ligature. Orthodontic patients usually have difficulty in maintaining oral hygiene, which leads to increased biofilm accumulation and changes in the oral microflora [17]. The present study is in agreement with the findings of several investigators who reported increased adhesion of *S. mutans* to elastomeric ligatures [18]. Self-ligating brackets had less bacterial accumulation than conventional brackets which is consistent with reports that self-ligating brackets provided better hygiene due to its design and absence of elastomeric ligatures [19]. The results indicated that chlorhexidine was effective in reducing viable bacteria on both conventional and self-ligating brackets, confirming its usefulness as an adjunct for biofilm control during orthodontic treatment. However, long term use of chlorhexidine may lead to staining of teeth and tongue and alteration of taste [20]. Surface modified elastomeric ligatures have been developed to minimise bacterial adhesion. The Metafasix

technology produces a smooth hydrogel-coated surface that limits bacterial adherence and the OrthoShield technology incorporates silver ions that disrupt bacterial metabolism and provide antimicrobial activity for approximately 30 days [21]. The present study compared Group B (Super Slick), Group C (Safe-T-Ties) and Group D (Mini Stick) with stainless steel ligatures (Group A) and self-ligating brackets (Group E). Unmodified elastomeric ligatures showed greater bacterial colonisation than surface modified elastomers. Both surface-modification technologies led to reduced bacterial adhesion, as shown by comparable reductions in *Streptococcus mutans* and *Lactobacillus* counts in Group C and Group B, without statistically significant differences. The results are in accordance with Bai and Vaz [10] but in contradiction with the findings of Magno *et al.* [6] and Kim *et al.* [8] possibly because of differences in study design, evaluation time, microbiological methods and host related factors. Corbette *et al.* also noted that there was a lack of direct comparisons between different antimicrobial technologies [22]. Pandis *et al.* [15] reported conflicting findings with increased accumulation of *S. mutans* on self-ligating brackets perhaps owing to variations in clinical settings. *Streptococcus mutans* was chosen because of its role in dental caries and *Lactobacillus* was chosen because of its association with caries progression. Lundstrom and Krasse [23] observed significant reduction of *S. mutans* but not *Lactobacillus* with chlorhexidine while Sari and Birinci [24] observed reduction in *S. mutans* alone. In contrast, the present study showed reduced levels of both microorganisms following chlorhexidine application. Forsberg *et al.* [25] reported a significantly greater bacterial accumulation with elastomeric ligatures than steel ligatures and recommended avoiding elastomeric rings in patients with poor oral hygiene because of increased risk of caries and gingivitis. However, Sukontapatipark *et al.* [26] and Türk kahraman *et al.* [27] did not find significant effect of the ligation material on microbial colonisation. Study conducted by Aggarwal *et al.*, found that there was no significant difference in adherence of *Lactobacillus* and *Streptococcus mutans* on titanium and plastic brackets whereas, significant difference present in retention of *Lactobacillus* among all the other groups of bracket [28]. The strengths of the present study are the evaluation of *Streptococcus mutans* and *Lactobacillus* CFUs and the evaluation of the antimicrobial effect of chlorhexidine in different ligation systems. Limitations include the estimation of bacterial adhesion without molecular characterization of the flora surrounding the implants. Future studies using larger sample sizes, DNA isolation and polymerase chain reaction are recommended.

Conclusion:

Chlorhexidine significantly reduced *S. mutans* viability in both self-ligating and conventional brackets compared with controls. Self-ligating brackets showed lower bacterial colony counts, suggesting less cariogenic biofilm adhesion and better oral hygiene outcomes.

Advancement to knowledge:

This study presents new clinical data on the microbial colonization of five orthodontic ligation systems and the adjunctive effect of chlorhexidine mouthwash *in vivo*. These findings improve our understanding of how ligation design and antimicrobial adjuncts affect oral microbial ecology during orthodontic treatment and offer clinically relevant advice for reducing biofilm accumulation, enamel demineralization and white spot lesion development in orthodontic patients.

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