Original Article

Microbiologic Changes in Subgingival Plaque After Removal of Fixed Orthodontic Appliances

Dong-Soon Choi[a]; Bong-Kuen Cha[b]; Paul-Georg Jost-Brinkmann[c]; Si-Young Lee[d]; Beom-Seok Chang[e]; Insan Jang[f]; Jae-Seok Song[g]

ABSTRACT

Objective: To evaluate changes that occur in the subgingival microbiota after removal of fixed orthodontic appliances using polymerase chain reaction (PCR).

Materials and Methods: Thirty orthodontic patients (11 males and 19 females; aged 20 ± 7.3 yr) were included in this study. Subgingival plaque samplings were gathered from the disto-buccal gingival crevice of the left upper central incisors and the left lower central incisors, and from the mesio-buccal gingival crevice of the left upper first molars and the left lower first molars, at two different times: 2 weeks before appliance removal (T1), and 3 months after appliance removal (T2). DNA was extracted from the samples and the 16S rRNA-based PCR detection method was used to determine the prevalence of *Actinobacillus actinomycetemcomitans*, *Tannerella forsythia*, *Campylobacter rectus*, *Eikenella corrodens*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Treponema denticola*, which are considered as putative periodontopathogens.

Results: The frequency of positive sites at T1 and T2 was 65% and 43.3% for *C. rectus*, and 53.3% and 30.8% for *E. corrodens*, respectively. For the other bacteria, the frequency tended to be reduced between times.

Conclusion: Periodontopathogens during orthodontic treatment were significantly reduced within 3 months of appliance removal. However, how long it takes to return to the preorthodontic composition of the subgingival microbiota and whether it happens at all remain to be seen. (*Angle Orthod.* 2009;79:1149–1155.)

KEY WORDS: Periodontopathogens; Gingivitis; PCR; Orthodontic appliance

INTRODUCTION

It has been shown that adverse changes in microflora occur shortly after placement of orthodontic appliances, and these are mirrored by increased plaque, bleeding, and probing depth.[1–5] These problems have been related to difficulties in maintaining oral hygiene, caused by the presence of orthodontic appliances, which can cause accumulation of bacterial plaque.[2,4,6] Some studies have reported that the placement of orthodontic appliances affects the subgingival microbial composition, thereby increasing the prevalence of periodontopathogens.[1,4,7–11]

However, it was reported that inflammatory and hyperplastic changes in the gingiva that occurred during orthodontic treatment are reversible upon appliance removal.[1–3,5,12] Long-term retrospective clinical studies also have concluded that temporary minor damage to periodontal structures can be observed during orthodontic treatment.[13,14]

However, only a few studies have focused their attention on microbiologic changes that occur in subgingival plaque after removal of fixed orthodontic appliances.[12,15,16] Consequently, very little information is available on the change in diversity of microorganisms, according to specific sites of the dental arch. The purpose of this microbiologic study was to evaluate changes that occur in the subgingival microbiota after

[a] Assistant Professor, Department of Orthodontics, College of Dentistry, Kangnung National University, Gangneung, South Korea.
[b] Professor, Department of Orthodontics, College of Dentistry, Kangnung National University, Gangneung, South Korea.
[c] Professor, Department of Orthodontics and Dentofacial Orthopedics, Center for Dental and Craniofacial Sciences, Charité-Universitätsmedizin Berlin, Berlin, Germany.
[d] Professor, Department of Microbiology and Immunology, Kangnung National University, Gangneung, South Korea.
[e] Professor, Department of Periodontics, College of Dentistry, Kangnung National University, Gangneung, South Korea.
[f] Research Fellow, Department of Orthodontics, College of Dentistry, Kangnung National University, Gangneung, South Korea.
[g] Associate Professor, Department of Preventive Medicine and Public Health, College of Medicine, Kwandong University, Gangneung, South Korea.
Corresponding author: Professor Bong-Kuen Cha, Department of Orthodontics, College of Dentistry, Kangnung National University, 120 Gangneung Daehangno, Gangneung, Kangwon 210-702, South Korea
(e-mail: korth@kangnung.ac.kr)

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the orthodontic appliances are removed with use of the polymerase chain reaction (PCR) method.

MATERIALS AND METHODS

Subjects and Clinical Procedures

The experimental group included 30 orthodontic patients (11 males and 19 females; aged 20.0 ± 7.3 yr [mean ± SD]). Subjects were enrolled according to the following criteria: (1) fixed orthodontic appliances to be removed within 1 month; (2) healthy systemic condition; and (3) no use of antimicrobial and anti-inflammatory drugs within 3 months before the baseline examination. All subjects had bands cemented with polyacid-modified composite resin (Ultra Band-Lok; Reliance Orthodontic Products Inc, Itasca, Ill) on molars and brackets bonded with composite resin (Transbond XT; 3M Unitek, Monrovia, Calif) on the other teeth for orthodontic treatment.

Subgingival plaque samplings were gathered from the disto-buccal gingival crevice of the left upper central incisors (U1) and the left lower central incisors (L1), and from the mesio-buccal gingival crevice of the left upper first molars (U6) and the left lower first molars (L6), at two different times: at baseline, 2 weeks before appliance removal (T1); and 3 months after appliance removal (T2).

The control group included 30 gingivally healthy subjects (13 males and 17 females; aged 16.7 ± 6.5 yr) without orthodontic appliances. The inclusion criteria for a healthy gingival condition included a periodontal probing depth of less than 4 mm, and a plaque index[17] and a gingival index[17] of less than 1. The gingival condition was assessed by one periodontist. Subgingival plaque samplings were taken from the same sites as in the experimental group.

Sampling sites were isolated with sterile cotton rolls and were dried by a gentle air stream. Then sterile paper points (DiaDent, Seoul, Korea) were inserted about 1 mm into the gingival crevice and were left in situ for 30 seconds. These paper points were transferred immediately into an Eppendorf tube that contained 250 mL distilled water and then were kept in a freezer at less than −20°C.

Bacteriologic Methods

DNA was extracted from the samples through the method described by Matto and coworkers.[18] In this study, the 16S rRNA-based PCR detection method was used to determine the prevalence of *Actinobacillus actinomycetemcomitans*, *Tannerella forsythia*, *Campylobacter rectus*, *Eikenella corrodens*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Treponema denticola*, which are considered putative periodontopathogens.[12,15,16,19,20]

Table 1. Species-Specific Primers Used for PCR[19]

Primer Pairs (5′-3′)	Size of Amplification, bp
Actinobacillus actinomycetemcomitans	
AAA CCC ATC TCT GAG TTC TTC TTC ATG CCA ACT TGA CGT TAA AT	557
Tannerella forsythia	
GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	641
Campylobacter rectus	
TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	598
Eikenella corrodens	
CTA ATA CCG CAT ACG TCC TAA G CTA CTA AGC AAT CAA GTT GCC C	688
Porphyromonas gingivalis	
AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	404
Prevotella intermedia	
TTT GTT GGG GAG TAA AGC GGG TCA ACA TCT CTG TAT CCT GCG T	575
Prevotella nigrescens	
ATG AAA CAA AGG TTT TCC GGT AAG CCC ACG TCT CTG TGG GCT GCG A	804
Treponema denticola	
TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA	316

PCR was performed in a reaction mixture that contained 1 μL of bacterial genomic DNA, 0.5 μM of primer 1, 0.5 μM of primer 2, 2 μl of 10× PCR buffer with $MgCl_2$, 0.5 mM of dNTP, 1 unit of Taq polymerase (Bioneer, Daejeon, Korea), and 13.8 μL of distilled water, for a final volume of 20 μL. The species-specific PCR primers used in this study and the sizes of base sequence and amplification products are shown in Table 1.

PCR amplification was performed in a DNA thermal cycler (GeneAMP PCR System 9700; PerkinElmer, Waltham, Mass). PCR temperature profiles included initial denaturation at 95°C for 2 minutes, followed by 36 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and an extension step at 72°C for 1 minute for *T. forsythia*, *C. rectus*, *E. corrodens*, *P. gingivalis* and *T. denticola*, as well as an extension step at 72°C for 2 minutes for *A. actinomycetemcomitans*, *P. intermedia*, and *P. nigrescens*. After completion of the final cycle, the PCR products finally were extended at 72°C for 2 minutes and 72°C for 10 minutes, respectively.

PCR amplification products were electrophoresed on 1% agarose gel and were stained with ethidium bromide for 30 minutes; they then were visualized and

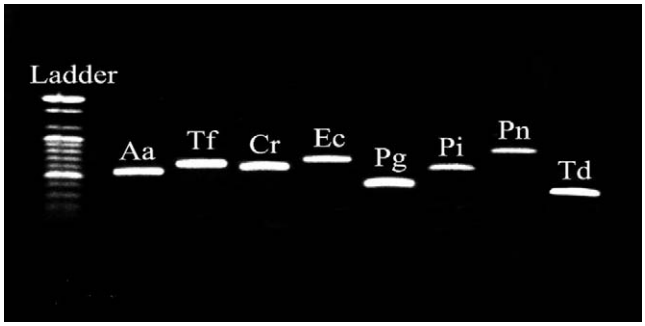

Figure 1. Electrophoresis results of polymerase chain reaction (PCR) amplification. A single DNA and the predicted size were obtained by PCR with the use of a specific primer pair against the target organism. Lane 1: 100 bp ladder; lane 2-9: Aa, *Actinobacillus actinomycetemcomitans*; Tf, *Tannerella forsythia*; Cr, *Campylobacter rectus*; Ec, *Eikenella corrodens*; Pg, *Porphyromonas gingivalis*; Pi, *Prevotella intermedia*; Pn, *Prevotella nigrescens*; and Td, *Treponema denticola*.

photographed by ultraviolet (UV) transillumination (Figure 1).

Statistical Analysis

The McNemar test was used for pairwise comparisons of the frequency of periodontopathogens between periods. Logistic regression analysis of ordinal data was used to assess the effect of appliance removal on bacterial colonization between sites. The odds ratio and the 95% confidence interval for the improved change (O-X), representing microorganisms existing at T1 but having disappeared at T2, were calculated, as was the frequency of unchanged pattern (X-X and O-O). Because sites are categorical variables, they were treated to dummy variables, and U1 and L6 were set as reference sites. Differences were considered significant at P values less than .05. Data were processed with SAS, version 9.12 for Windows (SAS Institute Inc, Cary, NC).

RESULTS

Results are presented as the frequency (percentage) of sites positive for each species. The frequency of sites positive at T1 and T2 of the experimental group and the control group is summarized in Table 2. The frequency of sites positive at T1 tended to be higher than that of the control group, and the frequency for *T. forsythia*, *C. rectus*, and *E. corrodens* showed significant differences. The frequency of *E. corrodens* was reduced significantly at T2 compared with T1. No statistically significant difference in frequency was seen for all investigated species between T2 and the control group (Figure 2).

The frequency of sites positive for all eight periodontopathogens at T1 was 18.3% at the left upper incisor (U1), 32.5% at the left upper first molar (U6), 30.4% at the left lower incisor (L1), and 28.3% at the left lower first molar (L6); a statistically significant difference was noted between sites (Table 3).

The frequency of unchanged pattern, X-X and O-O comparing T1 and T2, was 61.6% and 7.6%, respectively (Figure 3). A changed pattern, O-X and X-O, was observed in 19.8% and 11.0%, respectively (Figure 3). Improved changes (O-X) were compared between sites. This change was more distinct at the upper and lower first molars and at the lower central incisors than at the upper central incisors ($P < .05$; Table 4).

DISCUSSION

Most longitudinal studies found that orthodontic treatment has no detrimental effect on dental health.[2,3,5,13,14] In contrast, some studies have reported a statistically significant increase in the mean loss of clinical attachment in postorthodontic patients compared with untreated controls.[4,21] These contradictory findings may be explained by the different clinical assessment techniques used to evaluate the periodontal attachment level. In addition, very little is known about the effects of brackets and bands on specific subgingival bacteria.

The PCR method is much more sensitive and has greater specificity compared with other microbiological identification techniques such as cell culturing and the DNA probe method, especially in the detection of anaerobic bacteria.[19] It is surprising, however, that only a few studies have used PCR methods to evaluate microbial alterations after orthodontic appliances were removed.[15,16]

Specific microorganisms are associated with specific periodontal diseases (eg, *A. actinomycetemcomitans* is linked with localized juvenile periodontitis).[22] However, adult periodontitis is not associated with a single organism, and it is likely that a consortium of bacteria is responsible.[23,24] The eight microorganisms tested in this study are known to have synergistic relationships that can foster their survival and may enhance their harmful effects on the host.[19] The periodontopathogens investigated in this study were *T. forsythia*, *P. gingivalis*, and *T. denticola*, called ''red complex'' species, which are related to the severity of a periodontal disease,[20] along with other virulent periodontopathogens such as *A. actinomycetemcomitans*, *P. intermedia*, and *P. nigrescens*.[20,22] In addition, *C. rectus* and *E. corrodens* were investigated because they are associated with gingivitis.[19,25]

The frequency of sites positive for each species at T1 was higher than the frequency in the gingivally healthy control group (Figure 2). Results confirm many those of previous reports, that is, that fixed orthodontic

Table 2. Frequency of Periodontopathogens in Subgingival Plaque at Two Weeks Before Appliance Removal (T1) and Three Months After Appliance Removal (T2), and in Control Subjects

	T1		T2		Control		*P* Value		
	n	%	n	%	n	%	T1 vs T2[a]	T1 vs Control[b]	T2 vs Control[b]
A. actinomycetemcomitans									
Total (n = 120)	7	5.8	4	3.3	2	1.7			
U1 (n = 30)	2	6.7	0	0.0	1	3.3	—	.554	.313
U6 (n = 30)	2	6.7	0	0.0	0	0.0	—	.150	—
L1 (n = 30)	0	0.0	3	10.0	1	3.3	—	.313	.301
L6 (n = 30)	3	10.0	1	3.3	0	0.0	.500	.076	.313
T. forsythia									
Total (n = 120)	32	26.7	20	16.7	9	7.5			
U1 (n = 30)	3	10.0	2	6.7	1	3.3	1.000	.301	.554
U6 (n = 30)	11	36.7	5	16.7	3	10.0	.109	.015*	.448
L1 (n = 30)	10	33.3	8	26.7	3	10.0	.804	.028*	.095
L6 (n = 30)	8	26.7	5	16.7	2	6.7	.549	.038*	.228
C. rectus									
Total (n = 120)	78	65.0	52	43.3	53	44.2			
U1 (n = 30)	16	53.3	9	30.0	11	36.7	.092	.194	.584
U6 (n = 30)	22	73.3	16	53.3	12	40.0	.236	.009*	.301
L1 (n = 30)	22	73.3	17	56.7	17	56.7	.302	.176	1.000
L6 (n = 30)	18	60.0	10	33.3	13	43.3	.096	.196	.426
E. corrodens									
Total (n = 120)	64	53.3	37	30.8	43	35.8			
U1 (n = 30)	12	40.0	8	26.7	8	26.7	.424	.273	1.000
U6 (n = 30)	17	56.7	11	36.7	12	40.0	.238	.196	.791
L1 (n = 30)	18	60.0	11	36.7	16	53.3	.118	.602	.194
L6 (n = 30)	17	56.7	7	23.3	7	23.3	.013*	.008*	1.000
P. gingivalis									
Total (n = 120)	16	13.3	8	6.7	8	6.7			
U1 (n = 30)	1	3.3	2	6.7	0	0.0	1.000	.313	.150
U6 (n = 30)	6	20.0	2	6.7	2	6.7	.219	.129	1.000
L1 (n = 30)	5	16.7	2	6.7	3	10.0	.375	.448	.640
L6 (n = 30)	4	13.3	2	6.7	3	10.0	.500	.688	.640
P. intermedia									
Total (n = 120)	10	8.3	7	5.8	6	5.0			
U1 (n = 30)	3	10.0	2	6.7	1	3.3	1.000	.301	.554
U6 (n = 30)	2	6.7	2	6.7	1	3.3	1.000	.554	.554
L1 (n = 30)	3	10.0	3	10.0	3	10.0	1.000	1.000	1.000
L6 (n = 30)	2	6.7	0	0.0	1	3.3	—	.554	.313
P. nigrescens									
Total (n = 120)	34	28.3	44	36.7	31	25.8			
U1 (n = 30)	5	16.7	10	33.3	7	23.3	.267	.519	.390
U6 (n = 30)	11	36.7	12	40.0	8	26.7	1.000	.405	.273
L1 (n = 30)	8	26.7	14	46.7	11	36.7	.210	.405	.432
L6 (n = 30)	10	33.3	8	26.7	5	16.7	.804	.136	.347
T. denticola									
Total (n = 120)	22	18.3	7	5.8	14	11.7			
U1 (n = 30)	2	6.7	0	0.0	2	6.7	—	1.000	.150
U6 (n = 30)	7	23.3	1	3.3	3	10.0	.070	.166	.301
L1 (n = 30)	7	23.3	5	16.7	5	16.7	.754	.519	1.000
L6 (n = 30)	6	20.0	1	3.3	4	13.3	.063	.488	.161

[a] McNemar test.
[b] Chi-square test.
* $P < .05$.

appliances affect the subgingival microbial composition, increasing the prevalence of periodontopathogens.[1,4,7–12]

The frequency for each of the species *A. actinomycetemcomitans*, *T. forsythia*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, and *T. denticola* was only slightly reduced between T1 and T2, and no significant differences were seen (Figure 2, Table 2). However, other similar studies for these six species reported high frequencies before appliance removal and a significant

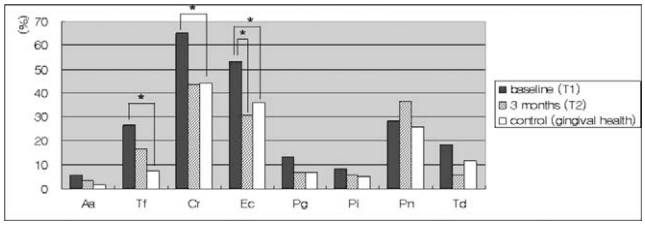

Figure 2. Frequency of sites positive for subgingival microorganisms at baseline (T1) and at 3 months after appliance removal (T2) and in the control group. Aa, *Actinobacillus actinomycetemcomitans*; Tf, *Tannerella forsythia*; Cr, *Campylobacter rectus*; Ec, *Eikenella corrodens*; Pg, *Porphyromonas gingivalis*; Pi, *Prevotella intermedia*; Pn, *Prevotella nigrescens*; Td, *Treponema denticola*. *$P < .05$; the McNemar test was used to compare the frequency of T1 and T2; the Chi-square test was used for comparison with the control group.

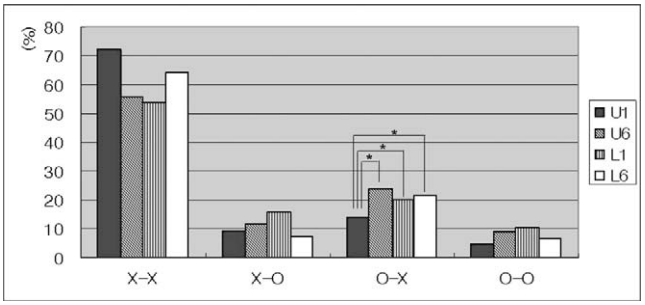

Figure 3. Comparison of subgingival microorganisms at each site. X-X, Microorganisms did not exist at T1 and T2; X-O, microorganisms did not exist at T1 but appeared at T2; O-X, microorganisms existed at T1 but disappeared at T2; O-O, microorganisms existed at T1 and T2; U1, the left upper central incisor; U6, the left lower first molar; L1, the left lower central incisor; and L6, the left lower first molar. *$P < .05$; logistic analysis was used.

reduction after appliance removal—from about 67% to 10% for *A. actinomycetemcomitans*,[15] from 94.1% to 63.2% for *T. forsythia*, and from 72.1% to 36.8% for *T. denticola*.[16] These contradictory findings probably stem from differences in sampling sites, sampling techniques, observation time, and prophylactic measures. Subjects selected in other studies were patients with signs of gingival inflammation,[15,16] and this difference can result in higher frequency than was seen in the results reported here. Our subjects were randomly selected and consequently must be considered a representative sample of orthodontic patients. Other studies have used curettes for subgingival plaque sampling,[15,16] which is believed to remove the largest number of microorganisms (up to 90% of the subgingival plaque) and would be indicated if an estimate of total pocket contents is required. However, this might bring into question whether samples should be taken for posttreatment assessment. Moreover, PCR requires only small but reproducible samples of microbiota. We wanted to prevent the sampling itself from having an effect on bacterial growth in the pocket, which is more likely to occur with curettes[26] than with paper points.

Moreover, the frequency of these five species except *T. forsythia* was not higher than that in the control group, suggesting that these periodontopathogens are not major components of subgingival plaque in orthodontic patients. This finding is in agreement with most longitudinal clinical studies, hinting that the gingival alterations produced by orthodontic appliances are transient, and that no permanent damage to periodontal structures is observed.[2,3,5] However, the result that the frequency of *T. forsythia* at T1 is higher than that of gingivally healthy control subjects may suggest the possibility of periodontal problems, especially in the lower incisor, lower molar, and upper molar areas, during orthodontic treatment.

This study evaluated two additional microorganisms: *C. rectus* and *E. corrodens*. Ashimoto et al[19] and Socransky et al[23,24] reported that *C. rectus* showed positive associations with red and orange complexes such as *T. forsythia*, *T. denticola*, *P. intermedia*, and *P. nigrescens*, which were related strongly to pocket depth and bleeding on probing. Other studies have reported that *C. rectus* and *E. corrodens* tend to occur at higher prevalence in adult gingivitis, as well as in advanced periodontitis,[19,27] suggesting that these may be considered endogenous pathogens that occasionally contribute to the development of periodontitis.

The frequency of *C. rectus* and *E. corrodens* at 65% and 53.3% for T1 was reduced to 43.3% and 30.8% for T2 (Figure 2, Table 2; $P < .05$ for *E. corrodens*) results at normal levels. This is the first study that presents evidence of transient changes in these micro-

Table 3. Difference in Frequency by PCR for Eight Periodontopathogens at T1 Between Sampled Sites

	PCR+	PCR−	*P* Value
U1 (n = 240)	44 (18.3%)	196 (81.7%)	
U6 (n = 240)	78 (32.5%)	162 (67.5%)	
L1 (n = 240)	73 (30.4%)	167 (69.6%)	
L6 (n = 240)	68 (28.3%)	172 (71.7%)	
Total (n = 960)	263 (27.4%)	697 (72.6%)	.003[a]*

[a] Chi-square test.
* $P < .01$.

Table 4. Odds Ratio of the Improved Change (O-X)[a] Between Sites

Site	Odds Ratio	95% Confidence Limits[b]	
U1-U6	−0.362	−0.601	−0.122*
U1-L1	−0.279	−0.525	−0.033*
U1-L6	−0.270	−0.511	−0.028*
L6-U6	−0.092	−0.309	0.125
L6-L1	−0.009	−0.234	0.215

[a] O-X, Microorganisms existed at T1 but disappeared at T2.
[b] Statistically significant difference between each site were tested using logistic analysis of ordinal data.
* $P < .05$.

organisms, confirming that inflammatory and hyperplastic changes reported in the gingiva during orthodontic treatment in other studies were reversible upon appliance removal.[1,2,3,5]

However, frequency in gingivally healthy subjects remained at a relatively high level, indicating that this organism may be part of the normal oral flora of subgingival pockets and is frequently implicated in gingivitis and periodontitis.[25,28]

The frequency of sites positive at T1 was lowest at the upper incisor (18.3%) followed by 32.5% at the upper molar, 30.4% at the lower incisor, and 28.3% at the lower molar ($P < .01$; Table 3). These results match those of previous reports indicating that gingival hyperplasia associated with brackets and bands is greater in the posterior than in the anterior teeth.[2,5] Reasons for this include the following: (1) difficult accessibility for cleaning the posterior teeth[29]; (2) greater likelihood of food impaction posterior between the archwire and soft tissue; and (3) different effects of orthodontic bands and brackets on gingival health.[1,2,6,7] It is interesting that frequency in the lower incisor is almost the same as that in the posterior teeth. Whether better oral hygiene may have resulted in different microbial findings remains unknown.

A major aim of this study was to evaluate whether the sites positive for periodontopathogens would return to normal (negative) after appliance removal. The improved change (O-X) with periodontopathogens found at T1 but not at T2 was 19.8% (Figure 3), and this change was more distinct in the upper molar, lower molar, and lower incisor area than in the upper incisor area (Table 4).

It has been reported that the frequency of sites with periodontopathogens was significantly reduced within 1 month after appliance removal and oral hygiene instruction[16] or additional prophylaxis.[15] However, the results reported by Yang et al[16] (63.2% for *T. forsythia*, 36.8% for *P. gingivalis*, and 36.8% for *T. denticola*) and Sallum et al[15] (10% for *A. actinomycetemcomitans*, 50% for *T. forsythia*, 20% for *P. gingivalis*, 10% for *P. intermedia*, and 33% for *P. nigrescens*) were still higher than the frequency of gingivally healthy subjects in this study (Table 2).

The medium-term evaluation (3 months after appliance removal) in the present study proved that the overall frequency of positive sites tends to return to levels found in gingivally healthy subjects (Figure 2). However, the frequency for *T. forsythia* and *P. nigrescens* shows still higher than normal levels, although it did not reach statistical significance, suggesting that complete recovery of microbial composition associated with gingival health may require additional time. Moreover, the undesired changes; new development (X-O) and unchanged positive sites (O-O) were measured in 11.0% and 7.6%, respectively (Figure 3). Therefore, it is recommended that all orthodontic patients must receive oral hygiene instruction and professional prophylaxis even after appliance removal as well as during orthodontic treatment.

CONCLUSIONS

- Periodontopathogens present during orthodontic treatment were significantly reduced within 3 months of appliance removal.
- Plaque control after appliance removal as well as during orthodontic treatment is still important for maintaining gingival health; 11% deteriorated after appliance removal and 7.6% remained periodontopathogen positive.

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