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Quantitative analysis of changes in salivary mutans streptococci levels after orthodontic treatment

교정치료 후 타액 내 치아 우식증 원인균 변화에 대한 정량 분석

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ABSTRACT

Quantitative analysis of changes in salivary mutans streptococci levels after orthodontic treatment

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Objectives: This study was designed to analyze the initial changes in salivary *Streptococcus mutans*, *Streptococcus sobrinus* levels after orthodontic treatment with fixed appliances. **Methods:** The subjects consisted of 58 adult patients. Unstimulated whole saliva and simplified oral hygiene index (OHI-S) were obtained at the following 4 time points: at the time of debonding (T1), 1 week after debonding (T2), 5 weeks after debonding (T3), and 13 weeks after debonding (T4). Repeated measures ANOVA was used to determine time-related differences in salivary bacterial levels and OHI-S among 4 time points after quantifying salivary levels of *S. mutans*, *S. sobrinus*, and total bacteria using real-time polymerase chain reaction. **Results:** OHI-S and total bacteria significantly

decreased, but salivary mutans streptococci (MS) levels significantly increased after orthodontic treatment. The amount of total bacteria in saliva significantly decreased at T3 (T1, T2 > T3, T4) and OHI-S decreased at T2 (T1 > T2, T3, T4). However, salivary *S. mutans* and *S. sobrinus* significantly increased at T3 and T4, respectively (T1, T2 < T3 < T4). Furthermore, the proportion of MS to total bacteria significantly increased at T4 (T1, T2, T3 < T4). **Conclusions:** This study suggests that careful hygienic procedures are needed to reduce the risk for dental caries after orthodontic treatment, despite overall improved oral hygiene status.

Keywords: mutans streptococci, *Streptococcus mutans*, *Streptococcus sobrinus*, saliva, orthodontic treatment, real-time PCR

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I. INTRODUCTION

One of the most common side effects during orthodontic treatment is enamel demineralization around fixed appliances.[1] Mutans streptococci (MS) are generally considered the major cause of enamel demineralization due to their ability to adhere to the tooth surface and produce lactic acid through carbohydrate fermentation.[2-4] Among them, *Streptococcus mutans* and *Streptococcus sobrinus* are commonly found in human oral cavity.[5,6] Therefore, it is important to understand changes in *S. mutans* and *S. sobrinus* numbers in the oral cavity in order to prevent enamel demineralization in orthodontic patients.

Various methods have been used to identify MS, including colony morphology on mitis-salivarius bacitracin agar, biochemical tests, and immunological methods. However, these techniques are inaccurate, time-consuming, or laborious. Recently, real-time polymerase chain reaction (PCR) has emerged as a more rapid and sensitive method of quantifying and detecting specific bacterial species. Real-time PCR can detect absolute numbers of targeted bacteria and the various applications including amplification, measurement, and quantification can be conducted simultaneously, which can minimize chances of contamination.[7]

Patients with fixed orthodontic appliances are subject to changes in the oral environment including increased MS and decreased pH, because MS preferentially

colonize teeth and orthodontic appliances and the complex design of orthodontic appliances impedes proper access for cleaning.[8-11] Although the evaluations of changes in MS during orthodontic treatment have been extensively investigated, few studies have investigated changes in MS after orthodontic treatment. Considering the importance of MS levels in determining the risk of enamel demineralization and dental caries, we undertook this study investigating changes in MS levels after orthodontic treatment to help determine caries risk during the retention period. The purpose of this *in vivo* prospective study was to analyze the changes in salivary levels of *S. mutans*, *S. sobrinus*, and total bacteria after orthodontic treatment with fixed appliances using real-time PCR.

II. REVIEW OF LITERATURE

1. Cariogenic bacteria in oral cavity

1.1. Cariogenic bacteria

Culture-dependent and culture-independent methods have estimated that about 600 species of bacteria inhabit the human oral cavity.[12] The oral bacteria play critical roles in oral health and are directly linked to diseases such as dental caries. Streptococcal species, Lactobacilli, Actinomyces and occasionally Candida yeasts have been implicated in dental caries.[13] MS are generally considered the major causative organism of enamel demineralization.[2-4,14,15] The MS group consists of seven species: *S. cricetus, S. rattus, S. mutans, S. sobrinus, S. downei, S. macacae*, and *S. ferus*.[16] Among them, *S. mutans* and *S. sobrinus* are most frequently found in human oral cavity.[4-6] Also, Many studies reported that frequency of *S. mutans* is much higher than that of *S. sobrinus* in oral cavity.[4,17,18]

1.2. *Streptococcus mutans*

S. mutans is a Gram-positive organism that is the primary causative agent in the formation of dental caries in humans.[4] Gram-positive bacteria are those that are stained violet by Gram staining. This is based on the physical properties of their cell walls, as opposed to gram-negative bacteria, which cannot retain the crystal violet stain. *S. mutans* causes to initiate the decalcification of the enamel and produce an acid environment through carbohydrate degradation. *S. mutans* also has an ability to survive around low pH environment and adhere to hard surface in the oral cavity.[4,19] Ikeda and Sandham[20] found

that *S. mutans* was more prevalent on the pits and fissures, constituting 39% of the total streptococci in the oral cavity. Fewer *S. mutans* bacteria were found on the buccal surface (2–9%).

The genome of *S. mutans* UA159, a serotype c strain, has been completely sequenced and is composed of 2,030,936 base pairs. It contains 1,963 open reading frames, 63% of which have been assigned putative functions. Almost 300 appear to be unique to *S. mutans*. Previously, only three genes for glucan-binding proteins have been isolated, but genome sequencing has uncovered a potential fourth gene, *gbpD*. Genes associated with transport system are account for almost 15% of the genome. Virulence genes associated with extracellular adherent glucan production, adhesins, acid tolerance, proteases, and putative hemolysins have been identified. Strain UA159 is naturally competent and contains all of the genes essential for competence and quorum sensing. There are no bacteriophage genomes present in *S. mutans*.[21]

1.3. *Streptococcus sobrinus*

S. sobrinus is a Gram-positive, spherical shaped, catalase-negative, non-motile, and anaerobic member of the genus Streptococcus.[22] *S. sobrinus* in conjunction with the closely related species *S. mutans* are pathogenic within humans and enhances the formation of dental caries. *S. sobrinus* is more closely connected than *S. mutans* with the prevalence of caries. *S. sobrinus* is able to produce more acid environment and make a low pH rapidly.[23-25] Therefore, *S. sobrinus* is more cariogenic than *S. mutans*. *S. sobrinus* usually interact with *S. mutans* in a symbiosis relationship when biofilm develop from the

mass of colonies for both bacteria.[26]

2. Salivary levels of cariogenic bacteria

The bacteria which are present in the oral cavity are in constant contact with saliva. As soon as an organism enters the mouth, it becomes coated with specific salivary proteins, which can increase the adhesion of the bacteria to oral tissues. As well as providing receptors for adhesion, saliva can aggregate bacteria.[27,28] Many studies have analyzed salivary MS levels as one of the indicators for dental caries.[24,29-34]

Hirose et al.[24] reported that the relationship between MS in the saliva and smooth surface caries in 338 children (age range 3-5 years). The prevalence of *S. sobrinus* in saliva was more closely associated with future caries activity, especially with smooth surface caries increment, than the prevalence of *S. mutants*.

Jurela et al. analyzed to determine the difference in the levels of *S. mutans* and *S. sobrinus* in saliva in orthodontic patients with different bracket types (stainless steel and esthetic brackets) using PCR and cultivation method.[29]

Salonen et al.[30] described the distribution of salivary MS in a randomly selected adult population and the prevalence of caries. There was a correlation between MS concentration in saliva and caries. The subjects with lower concentrations showed a significantly lower mean number of decayed surfaces, compared with the individuals with higher concentrations of MS in their saliva.

Thibodeau and O'Sullivan[31] assessed the relationship between salivary MS levels and the prevalence and incidence of dental caries in 148 children. The results indicated that the prevalence of dental caries increased with salivary MS levels.

Twetman et al.[32] analyzed that caries incidence in relation to salivary MS and fluoride varnish applications in preschool children. This study confirmed that the close association between salivary MS and caries incidence in preschool children and suggests a caries-reducing effect of topical applications of the fluoride silane varnish.

Kohler et al.[33] studied that the relationship between *S. mutans* in plaque and saliva and the development of caries. Plaque samples from 10 different tooth surfaces of 10 children with varied caries experience were collected five times during 2.5 years. A stimulated saliva sample was also collected and the number of *S. mutans* per ml saliva was determined. The saliva level of *S. mutans* was shown to reflect the prevalence and proportion of this microorganism on the selected surfaces.

Sanchez-Acedo et al.[34] carried out a cross-sectional study to determine the prevalence of *S. mutans* and *S. sobrinus* and the association of the two in a random sample (n=614) of the children. Saliva samples were analyzed by real-time quantitative PCR method to study the relation of these bacteria to caries prevalence and the DMFT index. Determination of *S. mutans* and *S. sobrinus* by real-time quantitative PCR can provide valuable information for caries risk assessment in epidemiological studies.

3. Real-time polymerase chain reaction

3.1. Polymerase chain reaction

The PCR is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.[35] PCR has been developed for the rapid detection and identification of oral bacteria. Although previous methods including colony morphology, biochemical tests and immunological methods have been to identify and isolate bacteria, these techniques are inaccurate, time-consuming, and laborious to get results. PCR is a simple, rapid, and highly specific method that uses specific DNA fractions for the detection and identification of microorganisms.

PCR method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

3.2. Application of polymerase chain reaction

PCR has been applied to a large number of fields. PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. PCR can be used to analyze extremely small amounts of sample because PCR amplifies the regions of DNA that it targets. This is often critical for forensic analysis. Other

applications of PCR include DNA sequencing and early diagnosis of diseases.

3.3. Real-time polymerase chain reaction

Although PCR is more sensitive and specific method, it is expected to qualitative analysis. As it lacks the ability for precise quantification, it is unsuitable for accurate quantitative level of bacteria in oral specimens. Real-time PCR have emerged as a more rapid and sensitive method of quantifying as well as detecting bacteria.[36,37] Real-time PCR can easily detect absolute numbers of bacteria in oral specimens such as saliva, using standard curve derived from already known sample size. The processes of real-time PCR such as amplification, measurement, and quantification are able to carry out simultaneously in the same kit, which can be minimized for a chance of contamination.[7] Furthermore, the bacteria can be stored for a long time in a freezer, not being alive, which is the great advantage for researchers to handle many samples easily in vivo contrary to conventional PCR method.[37]

Real-time PCR quantifies the initial amount of the template under investigation specifically, sensitively, and reproducibly and has become a preferable alternative to conventional PCR systems, which detect the amount of final amplified product. Real-time PCR monitors the fluorescence emitted during the exponential phase of the reaction as an indicator of amplicon production during each PCR cycle as opposed to the endpoint detection by conventional PCR methods. The real-time progress of the reaction can even be viewed with some systems as the product accumulates.

3.4. Detection chemistries of real-time polymerase chain reaction

Real-time PCR is performed using various detection chemistries: TaqMan assay, SYBR Green I assay, Scorpions reaction, molecular beacon chemistry, hybridization probe, and LUX (light up extension) flourogenic primers.[38]

TaqMan probes derive their fluorescence signal from the hydrolysis of the probe by *Taq* polymerase 5'-to-3' exonuclease activity. Such probes usually utilize *Taq* polymerase, *Tth* polymerase, or indeed any enzyme with 5' nuclease activity properties.

SYBR Green I is the most commonly used DNA binding dye that incorporates into double-stranded DNA (dsDNA). It is thought that SYBR Green binds to the minor groove of dsDNA, causing fluorescence to increase. Hence, during PCR as DNA products increase exponentially, so will the fluorescence signal of SYBR Green.[39]

Scorpions are essentially fluorogenic PCR primers. They represent the most recent development in real-time PCR chemistry. Two different formats are possible, the stem-loop format and the duplex format, although the stem-loop format is the most common approach.

Molecular beacons are oligonucleotide probes capable of undergoing structural conformations to form a stem-loop like structure with a reporter dye covalently attached at one end and a quencher dye covalently attached at the other end.

The application of hybridization probes for real-time PCR monitoring involves the use of two probes to maximize specificity. Such probes utilize a single label, one with a fluorescein donor at its 3' end and a second with an acceptor fluorophore at its 5' end. The probes are designed in a manner which enables them to hybridize to a sequence of interest in a “head-to-toe” arrangement.

The use of LUX primers for detection and quantification of genes on real-time platforms represents the most recent advance in real-time chemistry design. LUX primer design is based on studies that demonstrate the effects of the primary and secondary structure of oligonucleotides on the emission properties of a conjugated fluorophore.

Maeda et al.[40] assessed the sensitivity, specificity and quantitativity of the real-time PCR using the GeneAmp Sequence Detection System with two fluorescence chemistries. TaqMan probe with reporter and quencher dye, and SYBR Green dye were used for sources of the fluorescence. The results have suggested that there was no significant difference between the TaqMan and SYBR Green and both TaqMan and SYBR Green assays showed sufficient sensitivity, specificity and quantitativity. Since the TaqMan assay required additional manipulation and cost for the probe, the SYBR Green assay, which was simpler than TaqMan assay in its manipulations, may be suitable for routine clinical examinations.

4. Changes in cariogenic bacteria during orthodontic treatment

There have been diverse studies that estimate a risk for dental caries using MS levels in

saliva during orthodontic treatment, while little has been known after orthodontic treatment.

Topaloglu-Ak et al.[11] reported that salivary *S. mutans* counts increased significantly 6 months after the insertion of fixed/removable orthodontic appliances in the oral cavity. Long-term utilization of orthodontic appliances may have a negative effect on microbial flora and increase the risk of carious lesions. Therefore, they suggested that patients should be recalled within short time intervals to be motivated for oral hygiene during their orthodontic therapy.

Rosenbloom and Tinanoff[10] evaluated that salivary *S. mutans* levels in patients before, during, and after orthodontic treatment. Although *S. mutans* levels were significantly elevated during active treatment, the levels decreased significantly to levels comparable to age-matched untreated controls in the retention phase.

Lara-Carrillo et al.[8] studied that changes in the oral environment with clinical, salivary and bacterial risk markers after placement of fixed orthodontic appliances on permanent dentition. Of all results, *S. mutans* levels increased slightly after 1 month of treatment, without statistical significance.

Pandis et al.[9] investigated the effect of conventional and self-ligating brackets on the levels of *S. mutans* and total bacteria in whole saliva of orthodontic patients. Salivary *S. mutans* levels do not seem to be significantly different between conventional and self-

ligating brackets. The pre-treatment levels of *S. mutans* are significant predictors of the levels of *S. mutans* after placement of orthodontic appliances.

Jurela et al.[29] examined the difference in the levels of *S. mutans* and *S. sobrinus* in stimulated saliva in orthodontic patients with stainless steel and plastic brackets using PCR and cultivation method. The results showed no differences in *S. mutans* and *S. sobrinus* counts among patients with different brackets at either before placement of orthodontic appliances or 12 weeks after placement.

5. Changes in cariogenic bacteria after orthodontic treatment

There have been few studies that estimate a risk for dental caries after orthodontic treatment with fixed appliances. One of reports analyzed that salivary *S. mutans* levels in 75 subjects before, during, and after orthodontic treatment.[10] Seventy-five subjects were assigned to 5 groups, each of which consisted of 15 subjects: untreated controls (age 12-15), active treatment (age 12-15), retention (age 12-15), post-retention (ager 16-21), and untreated controls (age 16-21). This previous study reported that salivary *S. mutans* level significantly increased during active orthodontic treatment, but the salivary *S. mutans* level decreased during the retention period despite the presence of removable retainers. Therefore, the report suggested that antimicrobial therapy might not be necessary to reduce *S. mutans* level after orthodontic treatment. However, this cross-sectional study was limited that compared subjects during the retention period with untreated controls of the same age.

III. MATERIAL AND METHODS

This study initially comprised 63 subjects who were poised to finish orthodontic treatment with fixed appliances in Department of Orthodontics of Seoul National University Dental Hospital. Unfortunately, 5 subjects did not attend their periodic appointments. The study population finally consisted of 58 patients (20 men and 38 women, mean age 23.4 years). Inclusion criteria at the starting point of this experiment were (1) adult patients greater than 19 and 17 years of age in males and females, respectively, (2) permanent dentition of more than 24 teeth, (3) a longer than 12-month treatment period (average 19.6 months), and (4) the following 3 bracket types with .022-inch slot: Clarity SL (3M Unitek, Monrovia, CA, USA; n = 22), Clippy-C (Tomy, Tokyo, Japan; n = 24), and Damon Q (Ormco, Orange, CA, USA; n = 12). Exclusion criteria were (1) any systematic disease, (2) any active carious lesion, (3) poor oral hygiene (simplified oral hygiene index (OHI-S) > 3.0),[41] and (4) topical fluoride application (except for fluoridated dentifrice) or antibacterial therapy within 6 months.

Immediately before debonding of the orthodontic appliances, .0175-inch twist wire fixed lingual retainers were attached to the anterior segments of both the maxillary and mandibular arches in most subjects (43 out of 58 subjects). All patients received maxillary and mandibular removable retainers after debonding and were asked to wear removable retainers 24 hours a day. The subjects received oral hygiene instructions including brushing and flossing, and maintenance methods for the removable retainers with mechanical brushing with a toothbrush every time after brushing their teeth and

rinsing with running tab water. All subjects signed informed consent forms and the Institutional Review Board of the University Hospital approved the study protocol.

Unstimulated whole saliva (UWS) was collected by the spitting method as previously described.[42] All subjects were asked to refrain from eating, drinking, tooth brushing, or mouth-rinsing at least 2 hours before saliva collection. UWS was collected at the following 4 time points according to common retention protocols as previously reported.[43] at the time of debonding (T1), 1 week after debonding when patients began to wear removable retainers (T2), 5 weeks after debonding (T3), and 13 weeks after debonding (T4). OHI-S, which measures oral hygiene status using debris and calculus deposition from 2 anterior and 4 posterior teeth,[41] was examined by a single investigator at each time point.

One milliliter of UWS was centrifuged at 13,000 rpm for 10 minutes. After removing the supernatant, the pellet was washed 3 times with 1.0 mL cold phosphate-buffered saline (PBS, pH = 7.4). The pellet was resuspended with 1.0 mL cold PBS and homogenized by sonication using three 30 seconds pulses with 30 seconds intermittent cooling stages in a chilled ice box.

Bacterial chromosomal DNA was extracted using a CellEase Bacteria II Genomic DNA Extraction Kit (Biocosm, Osaka, Japan) according to the manufacturer's instructions after mutanolysin (Sigma-Aldrich, St Louis, MI, USA) treatment for 30 minutes at 37^{o}C. A NanoVue spectrophotometer (General Electric Healthcare Life Sciences, Pittsburgh, PA,

USA) was used to assess the quality of extracted DNA after preparation.

Known specific primers that amplify the dextranase gene of *S. mutans* and *S. sobrinus* were designed from the *gtfB* and *gtfU* genes, respectively (Table 1).[44,45] A conserved sequence in the 16S rRNA was selected to quantify the number of total bacteria.[46] All primers were commercially synthesized (Takara-Korea, Seoul, Korea). In order to test primer specificities, 11 strains of MS and other Gram-positive and Gram-negative species were examined using the specific primer pairs (Table 2).

DNA was extracted from *S. mutans* UA159 and *S. sobrinus* SL1 to generate standard curves. DNA concentration was estimated by absorbance at 260 nm and a series of 10-fold dilutions ranging from 10^3 to 10^9 copies were prepared for standard curves as previously described.[47] The amount of bacterial DNA in the samples was extrapolated from the standard curve.

Real-time PCR was performed using the Bio-Rad iQ5 system (Bio-Rad, Hercules, CA, USA). The reaction mixtures contained 2 μL purified DNA from saliva samples, 100 pmol primer, and 10 μL 2x iQ SYBR Green Supermix (Bio-Rad). Distilled water was added to a final volume of 20 μL. The samples were subjected to an initial amplification for 30 seconds at 94°C, 40 cycles of denaturation for 20 seconds at 95°C, primer annealing for 45 seconds at 60°C, and extension for 10 seconds at 60°C. All data including amounts of total bacteria, *S. mutans*, and *S. sobrinus* were analyzed with iQ5 Optical System Software (Bio-Rad). All the experiments for quantifying bacteria were

performed in triplicate and independently repeated twice.

Repeated measures ANOVA was used to determine the time-related differences in OHI-S, salivary levels of total bacteria, *S. mutans*, *S. sobrinus*, and the proportion of *S. mutans* and *S. sobrinus* to total bacteria with respect to gender differences, bracket types, and the presence of fixed lingual retainers. Values were considered statistically significant when P was less than 0.05 after Scheffé's multiple comparisons.

IV. RESULTS

When analyzing primer specificity, only *S. mutans* and *S. sobrinus* strains produced single DNA fragments of 261 bp with the *gtfB* primers and 156 bp with the *gtfU* primers, respectively. Amplified DNA was not detected in other MS, Gram-positive, or Gram-negative species. All bacterial genomic DNA showed a specific DNA band around 160 bp with a universal primer set (data not shown).

Figure 1 shows the changes in salivary levels of total bacteria, *S. mutans*, and *S. sobrinus* for all subjects during the experimental period. We combined the data as one group to determine time-related changes in salivary bacterial levels, because there were no significant differences in changes from T1 to T4 according to gender, bracket type, and the presence of fixed lingual retainers (Table 3).

Salivary levels of total bacteria, *S. mutans*, *S. sobrinus*, the proportion of *S. mutans* and *S. sobrinus* to total bacteria, and OHI-S from T1 to T4 are shown in Table 4 and Figure 2. The results demonstrated that changes in OHI-S occurred faster than those in salivary bacterial levels after debonding. OHI-S significantly decreased immediately after debonding (T1 > T2, T3, T4), while the amount of total bacteria significantly decreased at 5 weeks after debonding (T1, T2 > T3, T4). Although *S. mutans* was detected in higher numbers than *S. sobrinus*, the amount of *S. mutans* and *S. sobrinus* significantly increased at 5 weeks and 13 weeks after debonding, respectively (T1, T2 < T3 < T4). The proportion of *S. mutans* to total bacteria and *S. sobrinus* to total bacteria tended to

increase with extended time, but significantly increased at 13 weeks after debonding (T1, T2, T3 < T4).

V. DISCUSSION

The present study analyzed salivary MS levels, which have been reported in many studies as one of the indicators for dental caries.[24,29-32] Measuring bacteria in saliva has many advantages, such as easy collection, noninvasiveness, analyzing generalized parameters in the oral cavity, and no restrictions to localized site compared to measuring bacteria in plaque.

Orthodontic treatments with fixed appliances change the oral environment by increasing retentive sites for bacterial accumulation and generating difficulties for maintaining oral hygiene.[8-11] These changes increase the risk for enamel demineralization and dental caries during orthodontic treatments. Therefore, removal of orthodontic appliances may reduce these undesirable risk factors by improving the oral environment. In this study, we found decreased salivary levels of total bacteria and improved oral hygiene status after debonding. In particular, OHI-S, which was used to simply evaluate clinical oral hygiene, sharply decreased at T2 and retained low scores during the experimental period (T1 > T2, T3, T4) (Table 4 and Fig 2). This was mainly due to the fact that subjects could easily manage and maintain oral hygiene due to the absence of orthodontic appliances. These results are partly explained by adjunctive treatments performed during debonding, such as removal of remnant resins, scaling, and oral prophylaxis procedures.

However, salivary levels of *S. mutans* and *S. sobrinus* significantly increased after T3

and T4, respectively (T1, T2 < T3 < T4) (Table 4 and Fig 2). The wearing of removable retainers and/or the presence of fixed lingual retainers may influence the increased salivary MS levels. In this study, however, there were no significant differences in salivary bacterial levels according to the presence of fixed lingual retainers (Table 3). This may be due to the fact that removable retainers in the oral cavity cover fixed lingual retainers and composite resins for bonding fixed retainers to teeth have a smaller surface area than the removable retainers.

Increased salivary MS levels after T3 can be explained by the wearing of removable retainers after T2. Previous studies have shown that retainer surfaces composed of acrylic resins provide favorable places for the adherence and growth of MS.[48,49] In addition, acrylic resin may promote MS accumulation via increased expression of biofilm-associated genes.[50]

Previous studies have reported that the proportion of MS to total bacteria was more meaningful to evaluate the risk for dental caries than the absolute numbers of MS alone,[44,51] and caries group exhibited 1.64% of *S. mutans* among total bacteria in adults saliva.[52] As expected from changes in salivary MS levels, the average proportion of *S. mutans* and *S. sobrinus* in the total bacteria load were 0.22% and 0.05% at T1 and significantly increased to 1.18% and 0.34% at T4, respectively (Table 4 and Fig 2). Although the average proportion of *S. mutans* to total bacteria was under 1.64% during the experimental period, the number of subjects with more than 1.64% increased from 0 at T1 to 13 at T4. In addition, the average OHI-S of the 13 subjects with over 1.64% in *S.*

mutans to total bacteria proportion was very low (0.11) at T4 (data not shown). Considering that a higher proportion of MS to total bacteria in saliva may provide a greater possibility for the adhesion of MS to tooth surfaces and there is a close association between salivary MS levels and caries incidence,[31,32] these findings indicate that a cariogenic risk exists during the retention period after orthodontic treatment despite overall improved oral hygiene status.

In this study, differences in salivary levels between absolute number of MS and proportion of MS to total bacteria were found. Salivary MS levels significantly increased at T3 and T4, while the proportion of MS to total bacteria significantly increased at T4. This may be due to the fact that the proportion of MS to total bacteria was more variable than absolute number.

The results of the present study contrast with those of a previous study which reported that salivary *S. mutans* level significantly increased during active orthodontic treatment, but the salivary *S. mutans* level decreased during the retention period despite the presence of removable retainers.[10] The previous study suggested that antimicrobial therapy might not be necessary to reduce *S. mutans* level after orthodontic treatment. Our contrasting result may be due to the differences in research design. The previous investigation was a cross-sectional study that compared subjects during the retention period with untreated controls of the same age, while the present investigation was a prospective longitudinal study with the same subjects, which may provide more valuable information on MS changes after orthodontic treatment.

The present study showed that salivary MS levels during orthodontic treatment were lower than those after orthodontic treatment. However, enamel demineralization is one of the most common side effects during orthodontic treatment with fixed appliances. This is due to the fact that orthodontic appliances provide additional places for MS binding and patients have difficulty maintaining adequate oral hygiene. The resultant increase in plaque accumulation may place patients at higher risk for enamel demineralization, mainly adjacent to the appliances. On the contrary, higher salivary MS level after debonding of orthodontic appliance may provide an increased risk for dental caries on caries susceptible area, such as occlual and/or proximal surfaces.

This study indicates that improved oral hygiene after debonding does not guarantee a reduction in cariogenic risk, which may be due to the adhesion of MS to removable retainers. Although we instructed the patients in proper cleaning methods for removable retainers via mechanical brushing and rinsing, additional cleaning methods including the use of chlorhexidine spray[49] or soaking in alkaline peroxide or chlorhexidine solutions,[53,54] which may help to reduce salivary MS levels during the retention period.

A limitation of this study is that the experimental period was relatively short. Although we analyzed salivary bacterial levels 6 months after debonding (T5) in 10 participants and no significant differences in salivary bacterial levels between T4 and T5 were identified (data not shown), a research design with a longer retention period might provide more accurate and valuable results.

VI. CONCLUSIONS

This study was performed to evaluate changes in salivary *S. mutans* and *S. sobrinus* levels after orthodontic treatment with fixed appliances using real-time PCR *in vivo*. This study showed that salivary *S. mutans* and *S. sobrinus* levels significantly increased after orthodontic treatment despite improved oral hygiene and decreased total bacteria in saliva. These findings suggest that careful hygienic procedures are needed in orthodontic patients during retention period.

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Tables

Table 1. Specific primers used in the study.

Primer		Sequence	Position	Amplicon size
Universal	Forward	5'-TGGAGCATGTGGTTTAATTCGA-3'	930-951	160bp
	Reverse	5'-TGCGGGACTTAACCCAACA-3'	1089-1071	
gtfB	Forward	5'-CTACACTTTCGGGTGGCTTG-3'	794-813	261bp
	Reverse	5'-GAAGCTTTTCACCATTAGAAGCTG-3'	1054-1031	
gtfU	Forward	5'-AAAACATTGGGTTACGATTGCG-3'	39-60	156 bp
	Reverse	5'-CGTCATTGGTAGTAGCCTGA-3'	193-951	

Table 2. Bacteria used for primer specificity testing.

Mutans streptococci	Other Gram-positive bacteria	Gram-negative bacteria
Streptococcus cricetus E49	*Streptococcus gordonii* DL1	*Escherichia coli* DH5a
Streptococcus ratti BHT 9	*Streptococcus gordonii* M5	*Actinobacillus acetemcomitans* 33385
Streptococcus ratti FA-1F	*Streptococcus mitis* 9811	*Actinomyces naslundii* 12104
Streptococcus mutans Ingbritt	*Streptococcus sanguis* 10558	*Actinomyces viscosus* 19226
Streptococcus mutans LM7	*Streptococcus sanguis* MPC1	*Fusobacterium nucleatum* 10953
Streptococcus mutans UA159		*Fusobacterium nucleatum* 27067
Streptococcus mutans OMZ175		*Lactobacillus acidophilis* 5906
Streptococcus mutans GH5IS		*Prevotella intrmedia* 25611
Streptococcus sobrinus B13		*Porphyromomas gingivalis* 381
Streptococcus sobrinus SL1		*Porphyromomas gingivalis* W50
Streptococcus sobrinus 6715		*Bacteroides intermedius* 532-70A

Table 3. Statistical significances (*P* values) in salivary bacterial levels among the different time points (T1 to T4) according to gender differences (males and females), bracket types (Clarity SL, Clippy-C, and Damon Q), and the presence of fixed lingual retainers (presence or absence).

	Gender	Bracket type	Fixed retainers
Total bacteria	0.346	0.732	0.369
Streptococcus mutans	0.223	0.126	0.062
Streptococcus sobrinus	0.583	0.295	0.553
S. mutans / Total bacteria	0.909	0.106	0.743
S. sobrinus / Total bacteria	0.659	0.557	0.303

Repeated measures ANOVA was used to determine time-related differences with Scheffé's multiple comparisons.

Table 4. The salivary levels of total bacteria, *Streptococcus mutans*, and *Streptococcus sobrinus*, the proportion of *S. mutans* and *S. sobrinus* to total bacteria, and the simplified oral hygiene index (OHI-S) at each time point. Salivary bacterial levels and OHI-S were evaluated at the following 4 time points: at the time of debonding (T1), 1 week after debonding (T2), 5 weeks after debonding (T3), and 13 weeks after debonding (T4).

	T1	T2	T3	T4	Significances
Total bacteria (Log_{10})	7.96±0.5	7.92±0.6	7.71±0.6	7.57±0.6	T1, T2 > T3, T4*
S. mutans (Log_{10}/mL)	4.73±0.6	4.79±0.5	5.02±0.5	5.22±0.4	T1, T2 < T3 < T4**
S. sobrinus (Log_{10}/mL)	4.22±0.3	4.30±0.4	4.49±0.4	4.69±0.4	T1, T2 < T3 < T4***
S. mutans / Total bacteria (%)	0.22±0.3	0.30±0.6	0.59±0.7	1.18±1.9	T1, T2, T3 < T4**
S. sobrinus / Total bacteria (%)	0.05±0.1	0.08±0.1	0.17±0.2	0.34±0.5	T1, T2, T3 < T4*
OHI-S	1.42±0.7	0.30±0.4	0.34±0.4	0.26±0.5	T1 > T2, T3,T4***

Repeated measures ANOVA was used to determine significant differences among 4 time points with Scheffé's multiple comparisons.

Significance values: $P < 0.05$ (*), $P < 0.01$(**), $P < 0.0001$(***)

The unit of total bacteria, *S. mutans* and *S. sobrinus* is the cell number in logarithm per 1.0 mL.

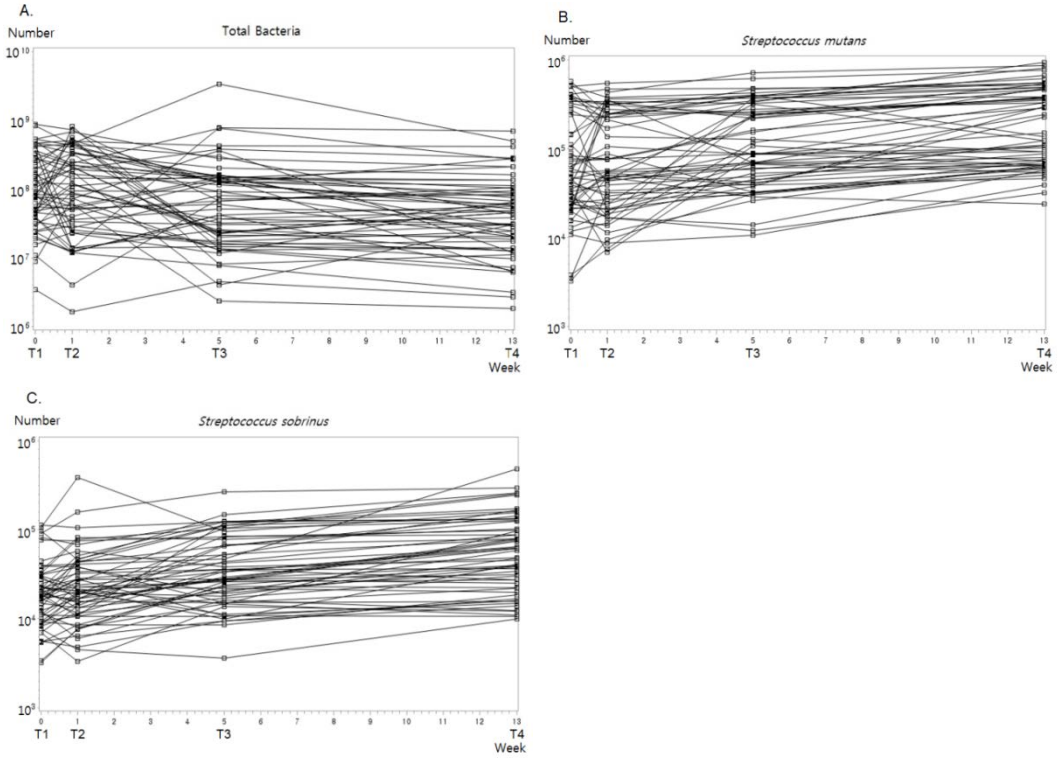

Figure 1. Changes in salivary levels of (A) total bacteria, (B) *Streptococcus mutans*, and (C) *Streptococcus sobrinus* during the experimental period in all subjects. The bacteria were derived from 1.0 mL of unstimulated whole saliva. Salivary bacterial levels were evaluated at the following 4 time points: at the time of debonding (T1), 1 week after debonding (T2), 5 weeks after debonding (T3), and 13 weeks after debonding (T4).

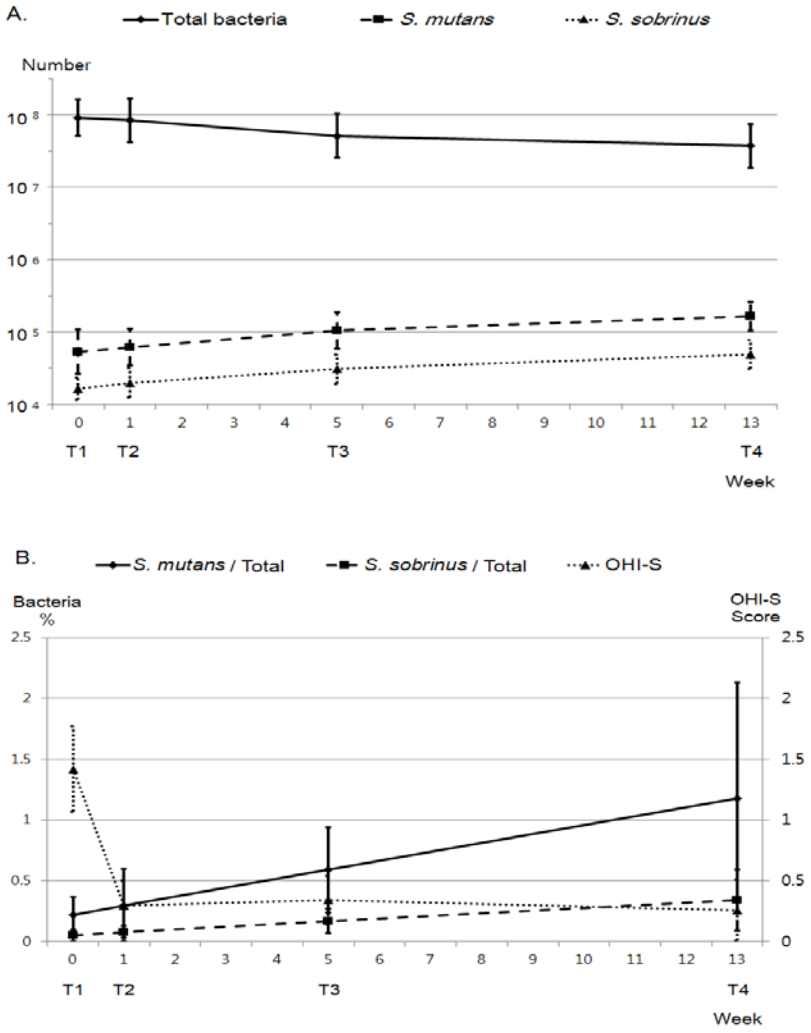

Figure 2. Changes in salivary bacteria levels and simplified oral hygiene index (OHI-S) during the experimental period: (A) total bacteria, *Streptococcus mutans*, and *Streptococcus sobrinus*, and (B) the proportion of *S. mutans*, and *S. sobrinus* to total bacteria, and OHI-S. The bacteria were derived from 1.0 mL of unstimulated whole saliva. Salivary bacterial levels and OHI-S were evaluated at the following 4 time points: at the time of debonding (T1), 1 week after debonding (T2), 5 weeks after debonding (T3), and 13 weeks after debonding (T4).

국문 초록

교정치료 후 타액 내 치아 우식증 원인균 변화에 대한 정량 분석

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목적

본 연구의 목적은 고정식 교정장치를 이용하여 교정치료를 마친 환자의 타액 내 *Streptococcus mutans, Streptococcus sobrinus*의 변화 양상을 평가하는 것이다.

방법

본 연구는 고정식 교정장치를 이용하여 교정치료를 마친 58명의 성인을 대상으로 교정장치 제거 직전(T1), 교정장치 제거 1주 후(T2), 교정장치 제거 5주 후(T3), 교정장치 제거 13주 후(T4) 시점에서 환자의 비자극성 전타액을 채취하고 simplified oral hygiene index (OHI-S) 를 평가하였다. Real-time polymerase chain reaction을 이용하여 환자의 타액 내 전체 세균, *S. mutans*, *S. sobrinus*의 양을 정량한 후, repeated measures ANOVA를 이용하여 각 시점에 따른 타액 내 세균 및 OHI-S의 변화 양상을 통계적으로 분석하였다.

결과

교정치료를 마친 후 타액 내 전체 세균의 양과 OHI-S는 통계적으로 유의하게 감소하였으나, *S. mutans* 및 *S. sobrinus*의 양은 유의하게 증가하였다. 전체 세균의 양은 T3에서 유의한 감소를 보였으며(T1, T2 > T3, T4), OHI-S 는 T2에서 유의하게 감소하였다(T1 > T2, T3, T4). 반면, *S. mutans*와 *S. sobrinus*는 각각 T3, T4에서 유의하게 증가하였다(T1, T2 < T3 < T4). 타액 내 전체 세균에 대한 *S. mutans* 및 *S. sobrinus*의 비율은 T4에서 유의하게 증가하였다(T1, T2, T3 < T4).

결론

본 연구는 고정식 교정장치를 이용하여 교정치료를 마친 환자는 구강 위생 상태가 개선됨에도 불구하고 치아 우식의 위험성을 줄이기 위해 구강 위생 관리에 주의해야 함을 보여준다.

주요어: 치아 우식증 원인균, *Streptococcus mutans, Streptococcus sobrinus*, 타액, 고정식 교정치료, real-time PCR

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