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Original article

Compositional differences in multi-species biofilms formed on various orthodontic adhesives

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Summary

Objectives: To investigate the aspects of multi-species biofilm formation on various orthodontic adhesives with different surface characteristics.

Methods: Multi-species biofilms using 13 bacterial species were grown on the surfaces of composite, compomer, and resin-modified glass-ionomer cement (RMGI). The changes in *Streptococcus mutans* (Sm), *Streptococcus sobrinus* (Ss), *Porphyromonas gingivalis* (Pg), *Aggregatibacter actinomycetemcomitans* (Aa), and total bacteria were determined at day 1 (T1) and day 4 (T2) using real-time polymerase chain reaction. Surface roughness (SR), surface free energy (SFE), and surface texture were analyzed to explain the differences in bacterial compositions among the adhesives. Repeated measures analysis of variance was used to determine time-related changes in bacterial compositions with respect to adhesive type. The Kruskal–Wallis test was used to determine differences in SR and SFE among the adhesives.

Results: There were no significant differences in the adhesion of total bacteria among the adhesives; however, the adhesion of Sm, Ss, and Pg was higher to RMGI than the other adhesives. The amount of Sm, Ss, and total bacteria increased from T1 to T2, while Pg and Aa decreased from T1 to T2. RMGI showed a rougher surface relative to composite or compomer due to the presence of micro-pores and/or flaws. Compomer had the greatest SFE followed by RMGI and composite. Interestingly, SR differences were about 10 times greater than SFE differences among the adhesives.

Conclusions: Considering the greater differences in SR than SFE among the adhesives, the rougher surface of RMGI may cause greater adhesion of Sm, Ss, and Pg.

Introduction

The common side effects of fixed orthodontic treatment are enamel demineralization and gingival inflammation (1, 2). The placement of fixed orthodontic appliances promotes biofilm formation, because it impedes access to the tooth surface for cleaning and provides retention sites for oral bacteria (3, 4). Oral biofilms are the main cause of infectious oral diseases (5). Mutans streptococci (MS), *S. mutans* and *Streptococcus sobrinus* in the biofilm are generally considered the major cause of enamel demineralization due to their ability of adherence to the tooth surface and of metabolic acid production (6). Periodontal research has reported that *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, which are commonly present in oral biofilms, play an important role in activating the immune-inflammatory response in gingival tissues (7).

Surface characteristics, including the surface roughness (SR) and surface free energy (SFE) of biomaterials are reported to influence biofilm formation and the retention of biofilms on the surface (8–11). Rough surfaces are favourable for biofilm formation because they protect bacteria from external forces and increase adhesion areas (8, 9, 11). A surface with a high SFE was reported to thermodynamically promote bacterial adhesion (8, 10). Orthodontic adhesives surrounding brackets and/or bands are a critical site for oral biofilm formation due to their rough surface and relatively high SFE (12).

Many investigators have evaluated bacterial adhesion to various orthodontic adhesives, but most studies involved the interaction of orthodontic adhesives with single bacterial species, mainly *S. mutans* (9, 13–15). However, single-species models are not appropriate to simulate the complex situation of oral biofilm on orthodontic adhesives because oral bacteria function as members of integrated microbial communities (16). In this study, a 13-species biofilm was formed on different orthodontic adhesives and compositional changes in the multi-species biofilm were analyzed using quantitative real-time polymerase chain reaction (PCR). In addition, surface characteristics were analyzed to explain differences in bacterial composition based on the different adhesives. The null hypothesis was that there would be no significant differences in biofilm formation with respect to adhesive type and incubation time.

Materials and methods

Material preparation

Three orthodontic adhesives were used in this study: composite (Transbond XT, 3M, Monrovia, CA, USA); compomer (Transbond Plus, 3M); and RMGI cement (Multi-Cure, 3M). A previous study using various types of adhesives showed that the same type of adhesives has similar surface characteristics (12). For each adhesive, a total of 61 adhesive disks were prepared with Teflon templates (a diameter of 12.7 mm and thickness of 3.0 mm); 20 for biofilm experiments, 10 for measuring SR, 30 for analyzing SFE, and one for scanning electron microscopy (SEM).

Bacterial preparation

Thirteen-species bacterial consortium containing *S. mutans* ATCC 700610, *S. sobrinus* ATCC 27607, *Streptococcus sanguinis* CCUG 17826, *Streptococcus salivarius* CCUG 50207, *Streptococcus oralis* ATCC 9811, *Actinomyces naeslundii* KCOM 1472, *Lactobacillus rhamnosus* ATCC 7469, *Veillonella dispar* KCOM 1864, *Neisseria subflava* ATCC 49275. *Fusobacterium nucleatum* ATCC 10953, *Prevotella nigrescens* ATCC 33563, *P. gingivalis* KCOM 2797, and *A. actinomycetemcomitans* ATCC 43718 was prepared due to their major prevalence in oral biofilms, their range of metabolic activity, their relevance in health and disease, and their ease of isolation and identification as previously described (17). This consortium reflects biofilm formation process on the tooth surface, which starts with the initial colonizers including streptococci and *Actinomyces* spp., followed by early-colonizing *Veillonellae* spp., middle-colonizing *Porphyromonads* spp. and *Fusobacteria* spp., and late-colonizing Gram-negative anaerobes (*P. nigrescens*, *P. gingivalis*, and *A. actinomycetemcomitans*) (18).

Each bacterial strain was individually grown to mid-exponential phase. *S. mutans*, *S. sobrinus*, *S. sanguinis*, *S. salivarius*, *S. oralis*, *A. naeslundii*, *L. rhamnosus*, *V. dispar*, and *N. subflava* were grown in a brain heart infusion (BHI, Becton Dickinson, Sparks, MD, USA) medium at 37°C with 5 per cent CO_2. *F. nucleatum*, *P. nigrescens*, and *P. gingivalis* were anaerobically grown in a tryptic soy agar (Becton Dickinson) medium supplemented with 10 μg/ml vitamin K, 5 μg/ml hemin, and 5 per cent sheep blood at 37°C for 7 days. These species were subcultured in BHI medium supplemented with 10 μg/ml vitamin K and 5 μg/ml hemin, and then grown to mid-exponential phase anaerobically at 37°C. *A. actinomycetemcomitans* was grown in a BHI medium at 37°C in an anaerobic atmosphere.

Multi-species biofilm formation

Modified McBain medium (MBM) containing 2.5 g/l porcine gastric mucin, 2 g/l proteose peptone, 2.5 g/l KCl, 1 g/l yeast extract, 1 g/l trypticase peptone, 0.1 g/l cysteine hydrochloride, 0.001 g/l hemin, 10 mM glucose, and 10 mM urea was used to supply nutrition sources and to simulate saliva as previously described (19).

A CDC biofilm reactor (BioSurface Technologies, Bozeman, MT, USA) was used for cultivating multi-species biofilms (20). This biofilm reactor is a dynamic cultivating system that simulates the oral environment by allowing the continuous flow of fresh medium and shear force. The lid of the biofilm reactor can support eight independent rods that individually hold three adhesive disks. Three different adhesive disks were randomly inserted into each rod before experimentation. After the rods with adhesive disks, the equipment, and MBM were sterilized, the reactor was set on a hot stir plate set at 37°C with a rotation speed of 60 rpm. The bacterial cell mixture listed above was added to the biofilm reactor (3.5 ml, 1% of the reactor volume) and a continuous flow of MBM was then flushed through the reactor at a rate of 100 ml/hour during the experiment.

Microbial analysis

To analyze time-related differences in biofilm composition among the three adhesives, two rods containing six adhesive disks (two sets of three adhesive disks) were removed from the reactor at two time points: days 1 (T1) and 4 (T2), which reflects early and mature biofilms, respectively. Four days are considered to be a sufficient period to form a mature biofilm, because a previous biofilm study using a CDC biofilm reactor showed that very dense mature biofilms were obtained after 72 hours of innoculation (21).

Each disk was carefully removed and transferred into a round tube before washing two times with 1.0 ml phosphate-buffered saline (PBS, pH = 7.4) to remove unbound bacteria. The biofilm was then detached from each disk by sonication with three 30-second pulses and 30-second intermittent cooling stages in a chilled ice box. After removing the disk, the bacterial cell suspension was centrifuged at 13,000 rpm for 10 minutes and washed twice with 1.0 ml PBS.

Bacterial chromosomal DNA was extracted using a CellEase Bacteria II Genomic DNA Extraction kit (Biocosm, Osaka, Japan) according to the manufacturer's instructions. A NanoVue spectrophotometer (General Electric Healthcare Life Sciences, Pittsburgh, PA, USA) was used to assess the quality of the extracted DNA after preparation.

Known specific PCR primers that amplify the dextranase genes of *S. mutans* and *S. sobrinus* were designed from the *gtfB* and *gtfU* genes, respectively (22). The PCR primers for *P. gingivalis* were designed based on the 16S rRNA gene, and the primers for *A. actinomycetemcomitans* were based on the *rpoB* gene of RNA polymerase β subunit gene. A conserved sequence in the 16S rRNA gene was selected to quantify the numbers of total bacteria (22) (Table 1). All primers were commercially synthesized (Bioneer, Daejeon, Korea).

DNA was extracted from *S. mutans* ATCC 700610, *S. sobrinus* ATCC 27607, *P. gingivalis* KCOM 2797, and *A. actinomycetemcomitans* ATCC 43718 using a G-spin Genomic DNA Extraction kit (iNtRON Biotechnology, Seongnam, Korea) according to the

Table 1. Primers and cycling conditions used.

Primer	Sequence (5′- to -3′)	Size of amplicon (base pairs)	Initial denaturation	Denaturation	Annealing	Extension	Cycles
Universal	Forward: TGGAGCATGTGGTTTAATTCGA	160	94°C	95°C	60°C	60°C	40
	Reverse: TGCGGGACTTAACCCAACA		30 seconds	20 seconds	45 seconds	10 seconds	
Streptococcus mutans	Forward: CTACACTTTCGGGTGGCTTG	261	94°C	95°C	60°C	60°C	40
	Reverse: GAAGCTTTTCACCATTAGAAGCTG		30 seconds	20 seconds	45 seconds	10 seconds	
Streptococcus sobrinus	Forward: AAAACATTGGGTTACGATTGCG	156	94°C	95°C	60°C	60°C	40
	Reverse: CGTCATTGGTAGTAGCCTGA		30 seconds	20 seconds	45 seconds	10 seconds	
Porphyromonas gingivalis	Forward: TGCAACTTGCCTTACAGAGGG	344	95°C	95°C	61°C	72°C	40
	Reverse: ACTCGTATCGCCCGTTATTC		60 seconds	5 seconds	15 seconds	33 seconds	
Aggregatibacter actinomycetemcomitans	Forward: GGCGAGCCTGTATTTGATGTGCG	113	95°C	95°C	72°C		40
	Reverse: GTGCCCGGTGCTGCGTCTTTG		10 minutes	10 seconds	30 seconds		

manufacturer's instructions. The DNA standard curve was obtained from known amounts of purified PCR product. PCR products were isolated from agarose gels using a QIAquick Gel Extraction kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instruction. DNA concentration was estimated by absorbance at 260 nm and a series of 10-fold dilutions ranging from 10 to 10^8 copies was prepared for standard curves as previously described (23). The amount of bacterial DNA in the samples was estimated from the standard curve.

Real-time PCR was performed using the iQ5 system (Bio-Rad, Hercules, CA, USA). The reaction mixtures contained 2 µl purified DNA from the disk samples, 100 pM primer, and 10 µl 2x iQ SYBR Green Supermix (Bio-Rad). Distilled water was added to a final volume of 20 µl. Thermal cycling conditions were presented in Table 1. PCR data were analyzed using iQ5 Optical System Software (Bio-Rad). All the experiments for quantifying bacterial levels were performed in duplicate and independently repeated five times.

Surface analysis

The SR was analyzed using confocal laser scanning microscopy (LSM 5 Pascal, Carl Zeiss MicroImaging GmbH, Göttingen, Germany). This allows for calculation of the arithmetic mean SR from a mean plane within the sampling area (230 × 230 × 30 µm). The measurements were performed on the 10 specimens of each adhesive.

The SFE was measured by the sessile drop method using deionized distilled water, 1-bromonaphthalene, and formamide as probe liquids as previously described (12). Briefly, a video camera equipped with an image analyzer (Phoenix 300, Surface Electro Optics, Suwon, Korea) was used to visualize the shape of the drop and determined the contact angle. Right and left contact angles of each drop were averaged to give one contact angle per drop. The SFE was calculated by combining contact angle measurements from the interfaced computer according to the previously proposed method (24).

To examine the surface texture of the orthodontic adhesives, SEM was used. Each surface was observed with a magnification set at ×3000 using a S-4700 microscope (Hitachi, Tokyo, Japan).

Statistical analysis

Repeated measures analysis of variance using the Bonferroni correction was used to determine the time-related differences in the bacterial amounts with respect to adhesive type. The Kruskal–Wallis test was used to determine the differences in SR and SFE characteristics among the three adhesive groups. For all analyses, $\alpha < 0.05$ was considered statistically significant.

Results

During primer specificity testing, bacterial genomic DNA showed a specific DNA band around 160 base pair with the universal primer set. For the specific primers, only the target bacteria produced a single DNA fragment corresponding their specific primers and amplified DNA was not detected in other species (data not shown).

Table 2 shows the differences in biofilm composition with respect to adhesive type and incubation time. The results show that both adhesive type and incubation time have significant effects on biofilm composition without interaction effects. There were no significant differences in the adhesion of total bacteria among the tested adhesives (composite = compomer = RMGI, Table 2). However, the composition of oral pathogens in the biofilm was significantly different among the adhesives. The adhesion of MS and *P. gingivalis* was higher with RMGI relative to the other adhesives (composite = compomer < RMGI); however, there was no significant difference in

adhesion of *A. actinomycetemcomitans* among the adhesives (Table 2). Incubation time also significantly influenced the composition of oral pathogens in biofilms. The amount of total bacteria and MS increased from T1 to T2, while *P. gingivalis* and *A. actinomycetemcomitans* significantly decreased from T1 to T2 (Table 2).

There were significant differences in SR among the adhesives. The order of SR from the lowest to highest was composite < compomer < RMGI (Table 3). The difference between composite and RMGI (0.15 μm) was higher than that between composite and compomer (0.03 μm). SEM images showed that the adhesive surface textures were consistent with the SR data. RMGI had larger micro-pores and/or more flaws on its surface relative to composite and compomer (Figure 1). There were significant differences in SFE among the adhesives, but the pattern was different from SR. Compomer had the greatest SFE followed by RMGI and composite (composite < RMGI < compomer, Table 3).

Discussion

This study showed that the composition of oral pathogens in the multi-species biofilm model was significantly influenced by adhesive type. Considering that the same type of adhesives has similar surface characteristics (12), the results of this study can be interpreted as the difference in composite (Transbond XT), compomer (Transbond Plus), and RMGI cement (Multi-Cure). The adhesion level of total bacteria was not significantly different, but the composition of oral pathogens within the biofilm was significantly different among the three adhesives, irrespective of incubation time (Table 2). RMGI significantly enhanced the adhesion of MS and *P. gingivalis* relative to the other adhesives (composite = compomer < RMGI, Table 2). These findings suggest that RMGI offers a favourable environment for enamel demineralization and gingival inflammation by providing favourable niches for oral pathogens. This is partly consistent with the findings of previous single-species adhesion studies demonstrating that MS adhesion to RMGI was significantly greater than MS adhesion to composites or compomers (13, 25).

The composition of oral pathogens in biofilms was also significantly influenced by incubation time. MS binding to the adhesives significantly increased with extended incubation time (T1 < T2, Table 2). However, the adhesion of periodontopathogens including *P. gingivalis* and *A. actinomycetemcomitans* was significantly

Table 2. Compositional changes in multi-species biofilms among various orthodontic adhesives at two time points.

			Significance†	
	Day 1 (T1)	Day 4 (T2)	Time	Adhesive
Total bacteria (Log_{10}/cm^2)				
Transbond XT[a]	6.89 ± 0.28	7.81 ± 0.31	T1 < T2**	Transbond XT = Transbond Plus = Multi-Cure
Transbond Plus[b]	6.86 ± 0.40	7.76 ± 0.35		
Multi-Cure[c]	6.96 ± 0.36	7.89 ± 0.31		
Streptococcus mutans (Log_{10}/cm^2)				
Transbond XT[a]	4.18 ± 0.45	4.57 ± 0.26	T1 < T2**	Transbond XT = Transbond Plus < Multi-Cure*
Transbond Plus[b]	4.26 ± 0.26	4.64 ± 0.25		
Multi-Cure[c]	4.53 ± 0.27	4.89 ± 0.22		
Streptococcus sobrinus (Log_{10}/cm^2)				
Transbond XT[a]	4.13 ± 0.29	4.68 ± 0.28	T1 < T2*	Transbond XT = Transbond Plus < Multi-Cure
Transbond Plus[b]	4.29 ± 0.31	4.73 ± 0.17		
Multi-Cure[c]	4.54 ± 0.21	5.05 ± 0.20		
Porphyromonas gingivalis (Log_{10}/cm^2)				
Transbond XT[a]	3.35 ± 0.62	2.91 ± 0.83	T1 > T2*	Transbond XT = Transbond Plus < Multi-Cure
Transbond Plus[b]	3.29 ± 0.79	3.06 ± 0.82		
Multi-Cure[c]	3.55 ± 0.75	3.55 ± 0.80		
Aggregatibacter actinomycetemcomitans (Log_{10}/cm^2)				
Transbond XT[a]	4.43 ± 0.22	4.34 ± 0.23	T1 > T2*	Transbond XT = Transbond Plus = Multi-Cure
Transbond Plus[b]	4.51 ± 0.28	4.28 ± 0.40		
Multi-Cure[c]	4.57 ± 0.39	4.31 ± 0.64		

†Repeated measures analysis of variance was used to determine significant differences between the two time points using the Bonferroni correction at a significant level of $\alpha < 0.05$; $*P < 0.05$; $**P < 0.001$.

[a]Composite; [b]Compomer; [c]Resin-modified glass-ionomer cement.

Table 3. Surface roughness and surface free energy of orthodontic adhesives used in this study.

	Orthodontic adhesives			
	Transbond XT[a]	Transbond Plus[b]	Multi-Cure[c]	Multiple comparisons†
Surface roughness (μm)	0.10 ± 0.01	0.13 ± 002	0.28 ± 0.07	Transbond XT < Transbond Plus < Multi-Cure*
Surface free energy (mJ/m)	46.35 ± 2.15	53.96 ± 0.66	50.02 ± 1.82	Transbond XT < Multi-Cure < Transbond Plus*

†The Kruskal–Wallis test was used to determine differences among the three groups and multiple comparisons were performed using the Mann–Whitney tests with the Bonferroni correction at a significant level of $\alpha < 0.05$; $*P < 0.001$.

[a]Composite; [b]Compomer; [c]Resin-modified glass-ionomer cement.

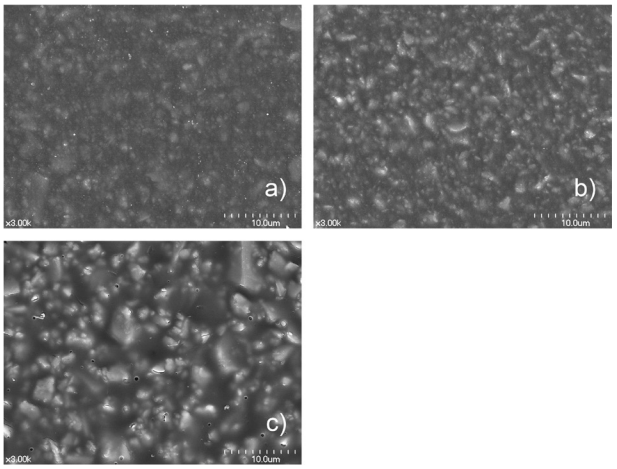

Figure 1. Scanning electron microscopic images of orthodontic adhesives used in this study: (a) Transbond XT (composite); (b) Transbond Plus (compomer); and (c) Multi-Cure (resin-modified glass-ionomer cement).

decreased with time (T1 > T2, Table 2). Because biofilm formation was operated under aerobic conditions in order to mimic an environment around orthodontic adhesives, the adhesion of periodontopathogens may have decreased with extended incubation time due to their anaerobic nature. This is partly supported by the fact the facultative anaerobe, *A. actinomycetemcomitans* grew better than the obligate anaerobe, *P. gingivalis* (Table 2).

In this study, surface characteristics were analyzed to explain the reason for differences in biofilm composition among the adhesives. The results show that RMGI had a higher SR than the other two adhesives (composite < compomer < RMGI, Table 3) due to the presence of larger micro-pores and/or more flaws on the surface (Figure 1).

In contrast to SR, the SFE was highest in compomer than RMGI and composite (composite < RMGI < compomer, Table 3). Differences in SFE might stem from their chemical compositions (12). The setting of RMGI and compomer is initiated with light-activated polymerization followed by an acid-base reaction from the absorption of water (26). The dynamic reaction processes on the surface of RMGI and compomer might be associated with their high SFE. In particular, compomer have lower degree of conversion and acid neutralization extent than RMGI (27), which explain higher SFE of compomer than RMGI.

SR and SFE are reported to significantly influence bacterial adhesion and biofilm formation. Rough surfaces promote biofilm formation by increasing adhesion area and protecting bacteria from external shear forces (8). SFE is positively related to bacterial adhesion due to the important role of polar and van der Waals interactions in bacterial adhesion (28). In this study, however, adhesion of MS was higher in RMGI relative to compomer, in spite of its lower SFE.

The SR of RMGI is about 115 and 180 per cent greater than those of compomer and composite, respectively, while their SFE differences were only 8 and 16 per cent (Table 3). Considering amount of differences in surface characteristics, SR had dominant effect on adhesion of MS to adhesives. Although a direct comparison is not possible, our findings are similar to those of previous studies those reported SR as a governing factor over the influence of SFE determining the *in vivo* supragingival biofilm formation or biofilm formation on composite (8, 29). The reason can be explained in several ways.

Although SFE have impacts on initial adhesion of microorganism (8, 16), they may have more influence on the initial colonizers (including MS in this study) than on the late colonizers. For late colonizers including periodontopathogens (*A. actinomycetemcomitans* in this study), inter-bacterial communication with pre-formed biofilm may be more important than SFE characteristics. A previous study (30) suggested that the differences between SFE characteristics rapidly disappear once the bacteria are allowed to start to grow into a mature biofilm, and this report is supported by our findings.

In addition, the effects of SFE may be smaller than expected in an aqueous condition. During our preliminary study, water contact angles of the MBM coated adhesives were not significantly different among the three adhesives (data not shown). These findings are consistent with previous studies which have shown that differences in SFE between underlying materials become similar after saliva-coating (31, 32).

After the initial adhesion of early colonizers, SR seems to play a leading role in biofilm formation with time (16). Surfaces with higher SR can protect further bacterial colonization and/or accumulation against shear forces better than those with lower SR and eventually help to maintain firm inter-bacterial interactions or binding between bacteria and surfaces (8, 16). In this study, continuous shear force was exerted to simulate the oral environment in the reactor, which may have resulted in the greater effects of SR.

This study indicates that both cariogenic and periodontopathic bacteria can adhere to orthodontic adhesives. In clinical situations, bacteria around orthodontic adhesives grow in aerobic conditions due to their supragingival location. As indicated by the results of our study, patients with poor oral hygiene may be more susceptible to enamel demineralization with time because the adhesion and biofilm formation of MS produce organic acid causing enamel demineralization near the tooth surface. Although periodontopathogens significantly decreased with time (Table 2), the results of the present study demonstrate that a considerable number of periodontopathogens ranging from 10^2 to 10^4 remained adhered to the orthodontic adhesives. Without proper prophylaxis, periodontopathogens may invade periodontal pockets, specifically in the mandibular incisor and/or molar area where the distance between the orthodontic appliance and the gingiva is relatively close. These findings suggest that biofilms around orthodontic adhesives should be considered as one of the risk factors for enamel demineralization and/or gingival inflammation during orthodontic treatment.

This study has limitations. The SR of the adhesives applied on the patients in clinical situation may be different from that of the specimens prepared in the laboratory. In addition, it was difficult to analyze exact relationships between bacterial adhesion and surface characteristics, because SR and SFE influence each other (16). Further *in vivo* studies controlling SR and/or SFE will be needed to investigate the effects of surface characteristics on multi-species biofilm formation.

In spite of the aforementioned limitation, the results of the present study suggest that Multi-Cure, a type of RMGI, may provide a favourable environment for adhesion of oral pathogens (MS and *P. gingivalis*) without differences in adhesion of total bacteria, possibly due to its rough surface. Although RMGI releases fluoride which have cariostatic effect, the fluoride release from RMGI rapidly decreases due to wash out effect (26). Therefore, this *in vitro* study suggests that RMGI may not be a good choice for orthodontic adhesive in patients with poor oral hygiene.

Conclusions

This study was performed to investigate the differences in multi-species biofilm characteristics on three orthodontic adhesives.

This study shows that the composition of oral pathogens in biofilms on orthodontic adhesives significantly differs according to adhesive type and incubation time. Therefore, the null hypothesis of our study was rejected. Adhesion of total bacteria and MS increased with time, whereas the adhesion of periodontopathogens decreased. In particular, RMGI showed greater cariogenic biofilm composition than the other adhesives with extended incubation time, possibly due to its rougher surface than composite or compomer. Our multi-species biofilm model suggests that the use of RMGI adhesive should be carefully considered in orthodontic patients with poor oral hygiene.

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Conflict of interest

None to declare.

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