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**Abstract.** The aim of this study was to evaluate the ecological changes in the biofilm at different stages of maturation using 16S rDNA gene amplicon sequencing and to identify correlations between red/green (R/G) fluorescence ratio and ecological changes. An oral microcosm biofilm was initiated from the saliva of a single donor and grown anaerobically for up to 10 days in basal medium mucin. Quantitative light-induced fluorescence analysis was shown that the R/G ratio of the biofilm increased consistently, but the slope rapidly decreased after six days. The bacterial compositions of 10 species also consistently changed over time. However, there was no significant correlation between each bacteria and red fluorescence. The monitoring of the maturation process of oral microcosm biofilm over 10 days revealed that the R/G ratio and the bacterial composition within biofilm consistently changed. Therefore, the R/G fluorescence ratio of biofilm may be related with its ecological change rather than specific bacteria. © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: [10.1117/1.JBO.21.10.101409](https://doi.org/10.1117/1.JBO.21.10.101409)]

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## 1 Introduction

Oral biofilm or so-called plaque is well known to consist of various microbes, but the exact number of species is not yet known.<sup>1</sup> It has previously been confirmed that there are substantial differences between planktonic bacteria and bacteria in biofilm and that biofilm has a more important role in oral health.<sup>2,3</sup> However, experiments with real oral biofilm are difficult because it is hard to control the human oral environment, and the oral microbiota of the biofilms of each human also exhibit substantial differences.<sup>1</sup> Therefore, an in vitro model that mimics real oral biofilm is needed to understand the complexities of biofilm. Microcosm biofilm models have been suggested to overcome such limitations,<sup>4,5</sup> and some researchers have reported that these in vitro models are similar to real oral biofilms.<sup>6,7</sup>

When an oral biofilm accumulates on the tooth surface, pathogenicity increases and oral disease may be subsequently induced.<sup>3</sup> However, the specific methods for measuring and/or detecting pathogenic changes of biofilm are insufficient, especially in the clinic. As an alternative tool for addressing this problem, quantitative light-induced fluorescence (QLF) has been reported to be capable of detecting the red fluorescence emitted from bacterial porphyrin, which is a metabolic biomolecule that is excited by blue light at 405 nm. There are numerous lines of evidence supporting the finding that mature biofilms emit red fluorescence.<sup>8–11</sup> Although some researchers have demonstrated that the red fluorescence emitted by dental plaque is not an essential factor in cariogenicity, red fluorescence of biofilm indicates that these biofilms were mature.<sup>12,13</sup>

When we observed plaque with QLF, it has been found that biofilms emitting red fluorescence were primarily composed of

anaerobic bacteria that are related not only to caries but also to periodontitis.<sup>13</sup> Moreover, some studies have suggested that increases in red fluorescence according to the maturation of the biofilm are due to ecological changes in the biofilm and not increases in specific species.<sup>10,11,14</sup> Although many previous studies have investigated the fluorescent properties of each oral bacteria species,<sup>8,10</sup> both basic and clinical studies are insufficient for understanding the ecological changes within the biofilm. In our previous study, we monitored the maturation of microcosm biofilms for 10 days and found that the red fluorescence appeared at day 3 and gradually increased until day 7.<sup>15</sup> However, there is still a lack of literature examining the relation between the red fluorescence and the ecological changes in microcosm biofilms during maturation.

Currently, it is quite important to identify the bacteria in mixtures from environmental and clinical sources. However, it is perhaps more important to understand the overall profile of the community composition than it is to simply identify the species. In studies of bacterial taxonomy, the 16S rDNA gene sequences has been by far the most commonly used housekeeping genetic marker.<sup>16</sup> Moreover, 16S rDNA gene sequencing is a powerful tool that has been used to trace the relative abundances of bacteria.<sup>17,18</sup> Therefore, the aim of this study was to investigate ecological changes using a 16S rDNA sequencing approach and to identify the correlations between red fluorescence and ecological changes.

## 2 Materials and Methods

### 2.1 Preparation of the Enamel Discs and Appliances

To make enamel discs, bovine incisors were obtained from a local slaughterhouse. The bovine teeth were collected as anonymous

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by-products of regular slaughtering which was performed to provide the foodstuff for human consumption. Therefore, no ethic approval was needed. A total of 18 bovine incisors without cracks or white spots were selected, and the labial surfaces of the bovine incisors were sectioned (8 mm × 3 mm) using a low-speed saw with a diamond disk. All specimens were embedded in a circular acryl mold, and ground with 600 to 1200 grit abrasive paper (SiC Sand Paper, R&B Inc., Daejeon, Korea) on a water-cooled polishing unit to form a flat enamel surface. After measuring surface hardness with a Vickers microhardness tester (JT Toshi Inc., Tokyo, Japan), all specimens were re-embedded in an acryl mold 1 mm under the mold surface to achieved biofilms of consistent depth.

## 2.2 Microcosm Oral Biofilm Formation

A single human saliva sample was used as an inoculum to form oral microcosm biofilms that consisted of oral microbiota. Approximately 40 mL of stimulated saliva was collected from a single donor (healthy male, age 28) who had refrained from oral hygiene for 24 h. This study was approved by the Ethics committee of the Yonsei Dental Hospital, Korea (02-2012-0030) and the participant provided written informed consent. The saliva was filtered through sterile glass wool (Duksan Chemicals, Ansan, Korea) to remove any debris and diluted in sterile glycerol (final concentration 30%). The mixture of saliva and glycerol was stored at  $-80^{\circ}\text{C}$  until the experimental work. Then 1.5 mL of the prepared saliva was inoculated onto each specimen, and specimens were placed into each of the wells of 24-well microplates (SPL Life Sciences, Pocheon, Korea). After 4 h, the saliva was gently aspirated from the base of the wells, and 1.5 mL of basal medium mucin<sup>7</sup> was added. The plates were then incubated in an anaerobic hood in an atmosphere of 80%  $\text{N}_2$ , 10%  $\text{CO}_2$ , and 10%  $\text{H}_2$  for up to 10 days at  $37^{\circ}\text{C}$  without shaking. The growth medium for each well was replaced daily.

## 2.3 Analyses of the Total and Aciduric Bacteria in the Biofilms

After 1, 2, 3, 7, and 10 days of incubation, the specimens with the biofilms were rinsed in 1.5 mL cysteine peptone water (CPW) to remove the loosely bound bacteria and transferred into tubes with 2 mL of CPW. The biofilms were dispersed by vortexing for 1 min and sonicating for 1 min. The resulting bacterial suspension was serially diluted from  $10^{-3}$  to  $10^{-8}$  in CPW and plated on 5% tryptic soy blood agar for the total microorganism counts and on brain heart infusion medium adjusted to pH 4.8 for the aciduric bacterial counts. All of the plates were incubated in an anaerobic incubator for 72 h, and the numbers of colony-forming units (CFUs) were determined by a single trained operator.

## 2.4 Analyses of the Red Fluorescences of the Biofilms

The red fluorescence values of the biofilms on the tooth specimen were evaluated daily with the quantitative light-induced fluorescence-digital method (QLF-D Biluminator, Inspektor Research Systems BV, Amsterdam, Netherlands) with different specimens for the bacterial counting test. This procedure was carried out under aerobic conditions, and then the specimens were returned to an anaerobic incubator. Fluorescence images

of all enamel specimens were captured with a “Live View”-mode QLF-D system with the following settings: shutter speed, 1/45 s; aperture value, 3.2; and ISO speed, 1600. From each fluorescence image, the red and green values of the biofilm areas on the specimen were measured using an image analyzer (Image-Pro PLUS, Media Cybernetics Inc., Maryland). The red and green values were used to calculate the red/green (R/G) fluorescence ratio to observe the changes in redness over time.<sup>8</sup> All procedures were conducted by a single trained examiner.

## 2.5 16S rDNA Gene-Based Bacterial Analyses

Whole genomic DNA was extracted from the biofilm using a DNeasy Blood & Tissue kit (Qiagen, Chatsworth, California). The isolated DNA was quantified using a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Delaware). A PCR method involving specific primer sets was employed to detect 10 bacteria species (Table 1). PCR was performed according to the manufacturer's recommendation using 20 ng of DNA that was amplified with LightCycler 480 SYBR Green (Roche Diagnostics, Basel, Switzerland). The amplification was performed with a LC480 II (Roche, Basel, Switzerland) using the following parameters: 50 cycles of  $95^{\circ}\text{C}$  for 20 s,  $50^{\circ}\text{C}$  for 20 s, and  $72^{\circ}\text{C}$  for 20 s for *Actinomyces israelii*, *Lactobacillus casei*, *Prevotella intermedia*, *Streptococcus anginosus*, *Streptococcus mitis*, *Treponema denticola*, and *Tannerella forsythia*; and 50 cycles of  $95^{\circ}\text{C}$  for 20 s,  $58^{\circ}\text{C}$  for 20 s, and  $72^{\circ}\text{C}$  for 20 s for *Veillonella* sp., *Porphyromonas gingivalis*, and *Streptococcus mutans*. Melting curve analyses were performed to verify the uniformity of all PCR products. All expression data were normalized using the total bacteria expression.

## 2.6 Statistical Analysis

Each variable related to the characteristics of the biofilm over time was analyzed by repeated-measures ANOVA with post hoc Bonferroni test. Moreover, the correlations of the characteristics of the biofilm with time and changes in red fluorescence were tested with Pearson correlation analysis. The data analyses were conducted using PASW 18.0 (SPSS Inc., Chicago, Illinois). The level of significance was set at  $\alpha = 0.05$ .

## 3 Results

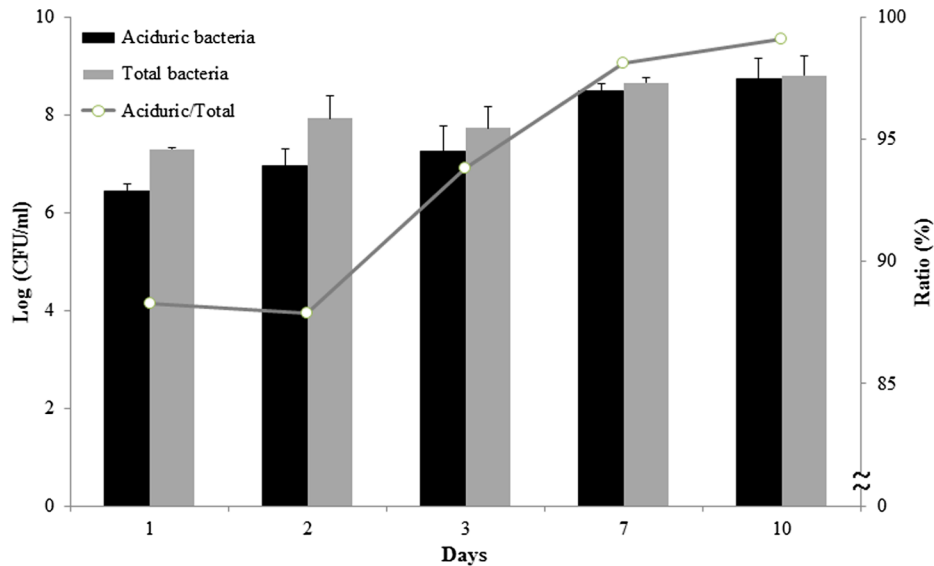
The amounts of total and aciduric of bacteria gradually increased and nearly reached saturation of approximately  $10^9$  CFU/mL by day 7 (Fig. 1). The ratio of the aciduric bacteria amount to the total bacteria amount increased over time from day 1 (88%) to day 10 (97%).

The biofilm on the tooth specimen was becoming redder until day 6, and then this remained similar [Fig. 2(a)]. The R/G ratio was only 0.60 on day 1 but reached a plateau of approximately 2.20 after day 6 [Fig. 2(b)]. Examinations of the green and red intensity values revealed that the red value had barely changed in 10 days, whereas the green value had rapidly decreased until day 6.

Table 2 lists the correlation coefficients of the R/G ratio from the QLF-D and all of the other bacteria. Among these relationships, the R/G ratio was significantly correlated with the total bacteria ( $r = 0.950$ ,  $p = 0.049$ ), aciduric bacteria ( $r = 0.978$ ,  $p = 0.022$ ). However, there was no significant relation between each species and R/G ratio.

**Table 1** Species-specific primers and probes used for real-time PCR.

No.	Bacterial species	Sequence (5' – 3')	Product size (bp)	Ref.
1	<i>Tannerella forsythia</i>	AGCGATGGTAGCAATACCTGTC TTCGCCGGGTTATCCCTC	88	19
2	<i>Porphyromonas gingivalis</i>	TACCCATCGTCGCCTTGGT CGGACTAAAACCGCATACACTTG	126	20
3	<i>Treponema denticola</i>	CTTCCGCAATGGACGAAAGT CAAAGAAGCATTCCCTCTTCTCTTA	114	21
4	<i>Actinomyces israelii</i>	TGAGTAACACGTGAGTAACC CCAAAAACACCACAAAAGTG	125	22
5	<i>Prevotella intermedia</i>	AATACCCGATGTTGTCCACA TTAGCCGGTCTTATTGAA	340	23
6	<i>Streptococcus mutans</i>	GCCTACAGCTCAGAGATGCTATTCT GCCATACACCACTCATGAATTGA	114	19
7	<i>Streptococcus mitis</i>	GAGTCCTGCATCAGCCAAGAG GGATCCACCTTTTCTGCTTGAC	96	24
8	<i>Lactobacillus casei</i>	CTATAAGTAAGCTTTGATCCGGAGATTT CTTCTGCGGGTACTGAGATGT	134	25
9	<i>Veillonella</i> sp.	CCGTGATGGGATGGAACTGC CCTTCGCCACTGGTGTCTTC	102	26
10	<i>Streptococcus anginosus</i>	AGTAGGACGCACAGTTTAT AGCATCTAACATGTGTTAC	155	27

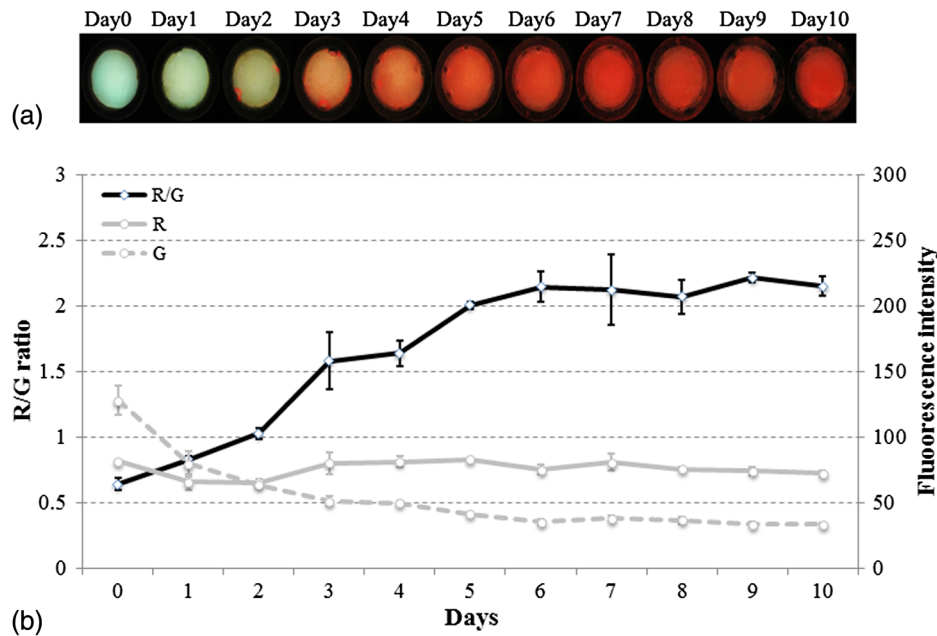
**Fig. 1** Temporal changes in the total (gray bar), aciduric (black bar), and the percent of aciduric bacteria (open circle).

The bacterial compositions of 10 species in the biofilm were compared across each maturation stage. Among these species, *Veillonella* sp. was the most dominant species in the entire biofilm. The ratios of *Veillonella* sp. to the 10 species on days 1, 3, 7, and 10 were 46.8%, 40.3%, 41.8%, and 37.1%, respectively. *L. casei* decreased from day 1 (15.9%) through day 10 (10.0%), and *S. mitis* also decreased from day 1 (10.9%) through day 10 (6.4%). In contrast, *S. anginosus* rapidly increased over time,

and the ratios on days 1, 3, 7, and 10 were 4.6%, 7.1%, 14.2%, and 28.3%, respectively. With the exceptions of these three species, all other species accounted for less than 5% and exhibited no changes in relative abundance (Fig. 3).

#### 4 Discussion

Similar to other previous studies,<sup>11,12,15</sup> we observed that the R/G fluorescence ratio was related to biofilm maturation, although



**Fig. 2** Temporal changes in (a) red fluorescence during dental microcosm biofilms maturation and (b) changes in red, green values and R/G ratio according to time.

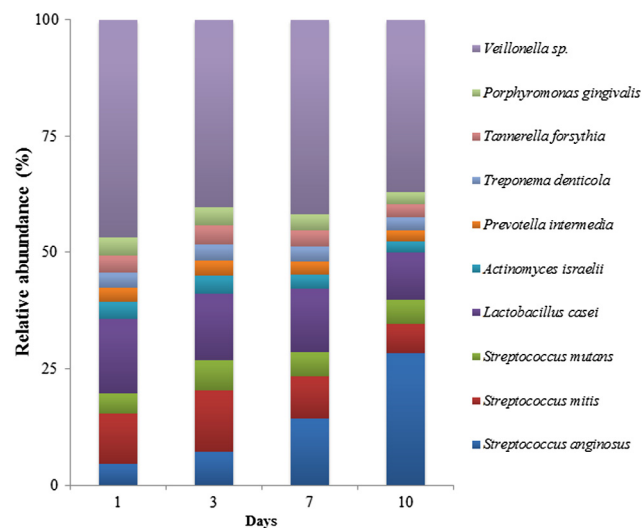
**Table 2** Correlations ( $r$  values) of the red fluorescence intensity (quantified as the R/G ratio) with each bacteria of microcosm biofilms.

R/G	Total	Aciduric	<i>P. intermedia</i>	<i>L. casei</i>	<i>Veillonella</i>	<i>A. israelii</i>	<i>P. gingivalis</i>	<i>S. mitis</i>	<i>S. mutans</i>	<i>T. denticola</i>	<i>T. forsythia</i>	<i>S. anginosus</i>
$r$	0.950 <sup>a</sup>	0.978 <sup>a</sup>	-0.528	-0.812	-0.842	-0.769	-0.733	-0.596	0.403	-0.477	-0.621	0.784
$p$	0.049	0.022	0.472	0.188	0.158	0.231	0.267	0.404	0.597	0.523	0.379	0.216

Note:  $r$  is the Pearson correlation coefficient.

<sup>a</sup> $p < 0.05$ .

the value did not change after six days. In addition, the R/G ratio was strongly correlated with green value rather than red value. Tooth has green fluorescence property but this may be covered by thick biofilm layer and its autofluorescence. In the present study, a relatively low sucrose concentration (0.5%) was applied



**Fig. 3** Temporal shift in the relative abundance of species from 16S rDNA sequencing.

to the microcosm biofilm model to mimic the real oral cavity environment.<sup>28</sup> The change in the red fluorescence from six to ten days was not substantial in our experiments. Volgenant et al.<sup>11</sup> also reported that the red fluorescences of 10- and 14-day biofilms were similar; however, the 17-day mature biofilm emitted a stronger red fluorescence than the earlier biofilms. Although we had no data after 10 days in our results, the bacterial composition within the biofilm could be consistently altered during maturation. Therefore, it can be assumed that the red fluorescence of the biofilm changed in a stepwise and not linear manner.

According to the results of the present study, the amounts of both the total and aciduric bacteria increased over time, and the ratio of aciduric to total bacteria also gradually increased. These results corroborate the extended ecological plaque hypothesis,<sup>29</sup> suggesting that acidogenic and aciduric bacteria should be increased to adapt to the acidic environment of mature biofilm. The conditions inside mature biofilms are relatively anaerobic because oral biofilms are proportional and consists of various bacteria and extracellular matrix.

Recently, Lee et al.<sup>30</sup> reported that the intensity of the red fluorescence of biofilm is correlated with the pH value of the biofilm culture medium at various sucrose concentrations using a microcosm biofilm model. Additionally, in a previous study, we found that the correlation coefficient between the red fluorescence and lesion depth over 10 days was 0.82.<sup>15</sup> Moreover,



similar with our previous study, it has been observed that the red fluorescence strongly relates to the maturation time in the present study. These findings suggest that red fluorescence measurement is an alternative method for evaluating the pathogenic potential of biofilms in the clinic.

Our results from the 16S rDNA gene sequencing revealed that the biofilm microbiota changed in a time-related manner over 10 days. Among the 10 species selected for present study, *Veillonella* sp., streptococci, and lactobacilli had taken possession of more than 90% of the biofilm, and the other species accounted only for small proportions. Specifically, *Veillonella* sp. was the most abundant species of biofilm on all of the days. Similar to *Streptococcus*, *Eubacterium*, and *Actinomyces*, *Veillonella* sp. is typically found in oral biofilms regardless of whether the site is caries-free or caries-active.<sup>31</sup> Although the relationship between *Veillonella* sp. and caries has not yet been clarified, some research suggests that *Veillonella* sp. is involved in the caries process.<sup>32,33</sup> Additionally, Doel et al.<sup>34</sup> deduced that *Veillonella* sp. provides the major contribution in the oral cavity to nitrate reduction, which creates favorable conditions for acid-producing bacteria. *S. anginosus*, which is an acidogenic and aciduric bacteria, rapidly increased during maturation to ultimately account for ~30% of the 10-species complex. It has been reported that *S. anginosus* has relatively strong aciduric properties compared to non-MS species.<sup>35</sup> Moreover, this species is known as a low-pH non-MS species that is similar to *S. gordonii*, *S. oralis*, and *S. mitis*; thus, it could be expected the pH-lowering capacity of biofilm increases with the ratio of low pH non-MS species to the rest of the dental microflora.<sup>36–38</sup> While *S. mitis* increased in proportion until day 3, this species decreased in the later stage of maturation because it could not survive the low pH conditions. According to a previous study, *S. mitis* does not survive well at pH levels below 5.0 without pre-acidification.<sup>39</sup> Similarly, the proportion of *S. mitis* was decreased because the pH of the growth media was under 5.0 in the present study (data not shown). The proportions of *S. mutans* and *L. casei*, which are well-known cariogenic bacteria, did not change much during biofilm maturation. Specifically, the proportion of *S. mutans* (~5%) was maintained across all days. This finding has also been observed in studies because this species is typically found and rapidly increases in the late stages of caries processes, such as deep caries lesions.<sup>40,41</sup> Therefore, these results indicated that the microcosm biofilm model could be used in place of real dental plaque.

Chow et al.<sup>42</sup> reported that *Veillonella* sp. emit red fluorescence under 366-nm light, which could be useful for screening for *Veillonella*. Our pilot study with *V. parvula* also revealed a similar result under 405-nm light (data not shown). Whereas, streptococci and lactobacilli have been found to display green and red fluorescence under 405-nm light.<sup>8,43</sup> Interestingly, there was no red fluorescence in the early stage (day 1 or 2) of the biofilm maturation despite the most abundance species being *Veillonella*. Additionally, it has been reported that red fluorescing bacteria account for less than 1% of the bacteria in dental plaque.<sup>14</sup> The bacterial species that emit red fluorescence are primarily anaerobes, and these species are found in periodontitis and caries lesions. Similarly, *Actinomyces israelii*, which are red fluorescing bacteria, were present in a small proportion (~3%), and this proportion even decreased during maturation in the present study. Periodontal bacteria, such as *P. intermedia*, *P. gingivalis*, *T. denticola*, and *T. forsythia*, are known to be late colonizers in biofilm formation.<sup>44</sup> It has also been revealed

that *P. intermedia* and *P. gingivalis* display strong red fluorescence.<sup>8</sup> Therefore, it can be assumed that these species did not affect the overall red fluorescence of the biofilm because these species exhibited small changes over the 10 days examined in the present study. These results differ from those of previous studies that have suggested that the red fluorescence of biofilms is related to the number of red fluorescent bacteria.<sup>8,13</sup> However, these previous studies only examined single species and provided no data about the quantitative bacterial proportions in the total biofilms. Although we did not determine the exact numbers of each species in the biofilms, it can be assumed that the red fluorescence is related to the ecology of the biofilm not simply the amount of some bacteria. Van der Veen et al.<sup>14</sup> reported that red fluorescing bacteria, such as *Lactobacillus acidophilus*, *Actinomyces odontolyticus*, and *Actinomyces gerencseriae*, account for less than 1% of the total microbiota of dental plaque. Additionally, Thomas et al.<sup>10</sup> also reported that the red fluorescence is greater after 20 weeks than one week in an in situ study that utilized a specimen holder placed in the oral cavity, but there were no correlations between the red fluorescence and the numbers of CFUs of lactobacilli or *A. odontolyticus*. Therefore, the increases in the red fluorescence that occur during biofilm maturation are likely related to changes in the complex ecological system of interacting species rather than increases in the numbers of specific red fluorescing bacterial species. Some researchers have suggested that the correlation of the red fluorescence of biofilm with its maturation is associated with both caries and gingivitis.<sup>30,45</sup> However, the results of the present study have the limitation of being identified in vitro. Therefore, the fluorescence properties of oral biofilm still need to be investigated in clinical situations. Moreover, much additional research on biofilm microbiota and the progression of red fluorescence is required for a more detailed understanding of the molecular mechanisms.

In conclusion, we found that the bacterial composition of biofilm constantly changed in a time-related manner over 10 days and that the red fluorescence of biofilm was related to the maturation process despite displaying no changes after six days. Although the red fluorescence could not exactly explain the maturation stage of biofilm, the red fluorescence of biofilm may be related with its ecological change rather than specific bacteria.

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