Effect of Antibacterial Dental Adhesive on Multispecies Biofilms Formation

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Abstract

Antibacterial adhesives have favorable prospects to inhibit biofilms and secondary caries. The objectives of this study were to investigate the antibacterial effect of dental adhesives containing dimethylaminododecyl methacrylate (DMADDM) on different bacteria in controlled multispecies biofilms and its regulating effect on development of biofilm for the first time. Antibacterial material was synthesized, and *Streptococcus mutans*, *Streptococcus gordonii*, and *Streptococcus sanguinis* were chosen to form multispecies biofilms. Lactic acid assay and pH measurement were conducted to study the acid production of controlled multispecies biofilms. Anthrone method and exopolysaccharide (EPS):bacteria volume ratio measured by confocal laser scanning microscopy were performed to determine the EPS production of biofilms. The colony-forming unit counts, scanning electron microscope imaging, and dead:live volume ratio decided by confocal laser scanning microscopy were used to study the biomass change of controlled multispecies biofilms. The TaqMan real-time polymerase chain reaction and fluorescent in situ hybridization imaging were used to study the proportion change in multispecies biofilms of different groups. The results showed that DMADDM-containing adhesive groups slowed the pH drop and decreased the lactic acid production noticeably, especially lactic acid production in the 5% DMADDM group, which decreased 10- to 30-fold compared with control group ($P < 0.05$). EPS was reduced significantly in 5% DMADDM group ($P < 0.05$). The DMADDM groups reduced the colony-forming unit counts significantly ($P < 0.05$) and had higher dead:live volume ratio in biofilms compared with control group ($P < 0.05$). The proportion of *S. mutans* decreased steadily in DMADDM-containing groups and continually increased in control group, and the biofilm had a more healthy development tendency after the regulation of DMADDM. In conclusion, the adhesives containing DMADDM had remarkable antimicrobial properties to serve as “bioactive” adhesive materials and revealed its potential value for antibiofilm and anticaries clinical applications.

Keywords: dimethylaminododecyl methacrylate, antibacterial material, controlled multispecies biofilm, bonding agent, tooth restoration, caries inhibition

Introduction

Secondary caries is one of the most important factors leading to dental restoration failure (Sakaguchi 2005). Residual bacteria, plaque accumulation on the surface of restorative materials, and microleakage have been considered to be closely related to secondary caries (Mjor and Toffenetti 2000). Numerous antibacterial agents, such as chlorhexidine and quaternary ammonium salt, have been reported to be added into composite resin, glass ionomer cement, primer, or adhesives (Benelli et al. 1993; Imazato et al. 2003; Cheng, Weir, Xu, et al. 2012; Cheng, Weir, Zhang, et al. 2012). As a connector located at the tooth-restoration interface, antibacterial primer or adhesives may be a primary approach to prevent secondary caries.

The antibacterial mechanism of quaternary ammonium salt is widely thought to be “contact killing” (Imazato et al. 2014). MDPB (12-methacryloyloxydodecylpyridinium bromide), a quaternary ammonium salt, was first incorporated into dental resins since the early days of 1994 (Imazato et al. 1994). After that, different quaternary ammonium salts were added into primer and adhesives, and primer or adhesive were also tried to be added into different antibacterial materials, such as MDPB (Imazato et al. 2003; Imazato et al. 2006), methacryloyl ethyl cetyl dimethyl ammonium chloride (Li et al. 2009), quaternary ammonium

dimethacrylate (Cheng, Zhang, et al. 2012), and dimethylaminododecyl methacrylate (DMADDM; Cheng et al. 2013; Wang et al. 2014; Zhou et al. 2014). Those experimental antibacterial adhesive systems have been proved to be effective in killing bacteria or inhibiting *Streptococcus mutans* biofilm, and almost all of those studies focused on single-species biofilms or saliva-based dental plaque biofilms. However, no previous study has investigated the role of antibacterial adhesive on different kinds of

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bacteria in controlled multispecies biofilms. The species composition shift of multispecies biofilms in this study will represent its development tendency to health condition or caries risk. Furthermore, the exopolysaccharides (EPSs) that can contribute to the pathogenesis of dental caries have been seldom studied in previous studies on antibacterial adhesives.

The objectives of this study were to incorporate DMADDM into an adhesive and to investigate, for the first time, the antibacterial effect of novel dental materials on different kinds of bacteria in controlled multispecies biofilms and its regulating effect on biofilm development process. It was hypothesized that (1) adhesive containing DMADDM will inhibit defined multispecies biofilm growth and acid production; (2) the synthesis of EPSs during biofilm development will be influenced; (3) the addition of DMADDM will change the proportion of species composition; and (4) the dual pressure of competition between bacteria and antibacterial agent conditions will lead to the shift of oral flora, from caries risk to health tendency.

Materials and Methods

Synthesis of DMADDM and Specimen Preparation

Clearfil SE Bond (Kuraray Dental, Tokyo, Japan) was used as the parent bonding system. DMADDM was synthesized and verified in previous studies (Cheng et al. 2013; Wang et al. 2014; see Appendix). DMADDM was mixed with SE Bond adhesive, at a DMADDM / (adhesive + DMADDM) mass fraction of 0% (control group), 2.5%, and 5%. The specimens for biofilm experiments were prepared following a previous study (Wang et al. 2014).

Bacteria Inoculation and Biofilm Formation

S. mutans UA159, *Streptococcus gordonii* DL1, and *Streptococcus sanguinis* SK1 (= *S. sanguinis* ATCC 10556) provided by the State Key Laboratory of Oral Diseases (Sichuan University, Chengdu, China) were routinely cultured in brain-heart infusion broth (BHI; Difco, Sparks, MD, USA) at 37 °C anaerobically (90% N₂, 5% CO₂, 5% H₂).

For multispecies biofilm formation, bacterial suspensions were mixed to obtain an inoculum containing a defined microbial population consisting of *S. mutans* (10⁷ colony-forming units [CFUs] / mL), *S. gordonii* (10⁷ CFUs/mL), and *S. sanguinis* (10⁷ CFUs/mL) in 2 mL of BHI with 1% sucrose in 24-well plates. The bacterial concentrations of inoculum were determined according to a previous study (Arthur et al. 2013). For single-species biofilm formation, bacteria were inoculated at a concentration of 10⁷ CFUs/mL in 2 mL of BHI with 1% sucrose in 24-well plates. The bacteria culture medium was changed every 24 h.

Lactic Acid Measurement and pH Measurement

For lactic acid measurement, disks with multispecies biofilms at 16, 48, and 72 h were washed with cysteine peptone water and

transferred to new 24-well plates; buffered peptone water with 0.2% sucrose were added for biofilms to produce acid for 3 h. The buffered peptone water solutions were stored for lactate analysis as described before (Cheng, Weir, Zhang, et al. 2012). The lactic acid production was monitored at the OD_{340nm} with microplate reader (Gene, Hong Kong, China). The standard curves were prepared with a lactic acid standard (Supelco, Bellefonte, PA, USA; Appendix Fig. 1). The 2-mL supernatant of biofilms at 16, 24, 48, and 72 h were used for pH measurement by Orion Dual Star, pH/ISE Benchtop (Thermo Scientific, Waltham, MA, USA).

CFU Counts

For CFU counts, specimens with 16-, 48-, and 72-h biofilms were transferred into Petri dishes with 2 mL of phosphate buffered saline (PBS). Biofilms were harvested by scraping with sterilized blades and sonication/vortexing in PBS buffer. BHI agar plates were used to assess the microorganism viability after serial dilution in PBS.

Polysaccharide Measurement

The water-insoluble glucan of biofilms was determined by the anthrone method as described before (Koo et al. 2003). Briefly, the bacteria in biofilms were collected by sonication/vortexing in PBS buffer, and the precipitate was obtained by centrifugation. Then the precipitate was washed twice with sterile water and resuspended in 4 mL of 0.4M NaOH. After centrifugation, 200 µL of supernatant was mixed with 600 µL of anthrone reagent at 95 °C for 6 min. The absorbance was monitored at the OD_{625nm} with microplate reader (Gene, Hong Kong, China). The standard curves were prepared with a dextran standard (KAYON, Shanghai, China; Appendix Fig. 2)

DNA Isolation and Real-time Polymerase Chain Reaction

Total DNA of biofilms were isolated and purified using a TIANamp Bacteria DNA kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. The bacteria were lysed using enzymatic lysis buffer (20mM Tris-HCl, pH 8.0; 2mM sodium EDTA and 1.2% Triton X-100) containing 25 mg/mL of lysozyme at 37 °C for 1.5 h. The purity and concentration of DNA were detected by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The extracts were stored at -20 °C until use. TaqMan real-time polymerase chain reaction (Life Technologies, Carlsbad, CA, USA) was used to quantify the absolute number of *S. mutans*, *S. gordonii*, and *S. sanguinis* as described by the manufacturer (Takara, Dalian, China; see Appendix).

Biofilm Imaging

For scanning electron microscopy (SEM) imaging, biofilms at different times were washed twice with PBS, fixed with 2.5% glutaraldehyde overnight, and serial dehydrated with ethanol

Figure 1. Acid production of multispecies biofilms. **(A)** The lactic acid production of 16-, 48-, and 72-h multispecies biofilms and **(B)** the supernatant pH of 3 groups—Clearfil SE Bond without dimethylaminododecyl methacrylate (DMADD), Clearfil SE Bond with 2.5% DMADD, and Clearfil SE Bond with 5% DMADD. Data are presented as mean $\pm$ standard deviation. * $P < 0.05$.

(50%, 60%, 70%, 80%, 90%, 95%, and 100%). Then the samples were putter coated with gold for SEM imaging (FEI, Hillsboro, OR, USA).

For live/dead imaging, biofilms were stained following the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). Briefly, the biofilm were stained with 2.5 μ M SYTO9 (Molecular Probes, Invitrogen) and propidium iodide (Molecular Probes) for 15 min. The labeled biofilms were imaged with a DMIRE2 confocal laser scanning microscope (Leica, Wetzlar, Germany) equipped with a 60 $\times$ oil immersion objective lens (Zheng et al. 2013; see Appendix).

For EPS staining, the bacteria were stained with 2.5 μ M SYTO9 (Molecular Probes) for 15 min after biofilms formed, and 2.5 μ M Alexa Fluor 647-labeled dextran conjugate (Molecular Probes) was added at the beginning of biofilm formation (Zheng et al. 2013). The biofilms were imaged with a Leica DMIRE2 confocal laser scanning microscope equipped with a 60 $\times$ oil immersion objective lens as live/dead imaging (see Appendix).

For fluorescent in situ hybridization imaging, biofilms were fixed in 4% paraformaldehyde overnight and investigated by species-specific probes (Zheng et al. 2013). The biofilms were imaged with an FV1000 confocal laser scanning microscope (OLYMPUS, Tokyo, Japan) equipped with a 100 $\times$ oil immersion objective lens (see Appendix).

All 3-dimensional reconstructions of the biofilms were performed with Imaris 7.0.0 (Bitplane, Zürich, Switzerland), and the quantification of live/dead and EPS:bacteria volume ratio was performed with Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) and COMSTAT (<http://www.image-analysis.dk>; Xiao et al. 2012), respectively.

Statistical Analysis

All the experiments repeated at least 3 times independently. One-way analysis of variance was performed to detect

the significant effects of variables, followed by the Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$. Statistical analysis was performed with the SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Compared to control group, 16-, 48-, and 72-h multispecies biofilms on disks with 2.5% and 5% DMADD reduced the lactic acid production significantly ($P < 0.05$; Fig. 1A). Adhesives incorporated with 5% DMADD were more efficient in decreasing lactic acid production versus adhesives with 2.5% DMADD

over time ($P < 0.05$). Incorporation of DMADD retarded the pH drops (Fig. 1B). The pH in control group remained < 4.50 at different time points, which the pH in the 2.5% DMADD group dropped slower versus that in control group, from 6.14 ± 0.57 at 16 h to 4.18 ± 0.09 at 72 h. Moreover, the pH in 5% DMADD group dropped most slowly, staying at about 5.3 at 72 h.

The water-insoluble glucan in the 2.5% and 5% DMADD groups decreased considerably (Fig. 2A; $P < 0.05$). Adhesives with 5% DMADD reduced most obviously compared to the other 2 groups ($P < 0.05$). EPSs were stained red, and bacteria were stained green (Fig. 2B). Biofilms in the control group had a higher EPS:bacteria ratio than those in the other 2 groups ($P < 0.05$; Fig. 2C). There were significant differences only at the 72-h biofilms when the 5% DMADD group was compared with the 2.5% DMADD group.

The SEM images showed that DMADD groups inhibited the development of multispecies biofilms in varying degrees, while the 5% DMADD group had stronger antibacterial activity (Fig. 3A). DMADD groups reduced the CFU counts ($P < 0.05$), and the 5% DMADD group reduced them more remarkably ($P < 0.05$; Fig. 3B). Live bacteria were stained green, and dead bacteria were stained red (Fig. 3C). Yellow color appeared when live and dead bacteria were very near. DMADD groups had much more red staining ($P < 0.05$), indicating a strong antibacterial activity (Fig. 3D).

Real-time polymerase chain reaction results revealed the ratio variation of these bacteria in multispecies biofilms by time with different DMADD content (Fig. 4A). In the group without DMADD, there was an increasing proportion of *S. mutans* in biofilms, and the percentage reached 42.81% at 72 h, while decreasing continuously for both *S. sanguinis* and *S. gordonii* over time. However, the ratio of *S. mutans* steadily dropped in the adhesives containing DMADD, accounting for 5.74% and 2.54% in the 2.5% and 5% DMADD groups, respectively, at 72 h. Unfortunately, the proportions of *S. sanguinis* were also decreasing continually in the both DMADD groups, reaching

14.41% and 0.06% for 2.5% and 5% DMADD, respectively, at 72 h. As for *S. gordonii*, the proportions were increasing steadily in both DMADD groups—79.85% and 97.40%, respectively, at 72 h. In fluorescent in situ hybridization images, *S. mutans* were stained green; *S. sanguinis* were stained red; and *S. gordonii* were stained blue (Fig. 4B). The ratios of *S. mutans* and *S. sanguinis* in DMADD groups were less than those in groups without DMADD. Both *S. mutans* and *S. sanguinis* proportions decreased over time in biofilms.

The SEM images showed that groups with DMADD limited the development of single-species biofilms (Fig. 5A). The groups containing 5% DMADD had better antibacterial effect, and *S. sanguinis* seemed more sensitive to this antibacterial material. The CFU counts of these bacterial showed that *S. mutans* and *S. gordonii* had more CFUs than *S. sanguinis*; also, the groups containing DMADD had fewer CFUs ($P < 0.05$; Fig. 5B).

Discussion

The present study investigated the adhesive containing DMADD on different species of bacteria in the controlled multispecies biofilms and its regulating effect on development of biofilm for the first time. The results indicated that adhesive containing DMADD significantly inhibited multispecies biofilm growth, acid production, and EPS synthesis. Meanwhile, the addition of DMADD changed the proportion of bacteria in multispecies biofilms and the development tendency. Previous studies showed that adhesive materials that are antibacterial or that have remineralization effects could contribute to better prognosis of restorative treatments (Wiegand et al. 2007; Mehdawi et al. 2009). So, it is favorable for adhesives to be “bioactive,” as composite restorations are spliced to the tooth structure with it. Moreover, it is favorable for adhesives to inhibit or kill residual bacteria in the prepared tooth cavity and invading bacteria in tooth restoration interface through marginal leakage (Garcia-Godoy et al. 2010; Reinke et al. 2012; Zhang et al. 2012; Imazato et al. 2014; Li et al. 2014). This study used dental adhesive containing DMADD to investigate its bioactive effect on controlled multispecies biofilm development in vitro.

Figure 2. Extracellular polysaccharides (EPS) of multispecies biofilms. (A) The water-insoluble glucan of each disk from different groups, measured by anthrone method; (B) the 3-dimensional reconstruction of multispecies biofilms (bacteria, stained green; EPS, stained red); (C) the volume of EPS and bacteria, calculated according to 5 random sights of controlled multispecies biofilms. Data are presented as mean $\pm$ standard deviation. * $P < 0.05$. This figure is available in color online at <http://jdr.sagepub.com>.

S. mutans and *Lactobacillus* species have been considered to be associated with dental caries on account of their competence of acid production and acid tolerance (Becker et al. 2002; Takahashi and Nyvad 2011). Numerous studies have focused on the individual role of *S. mutans* single-species biofilms on dental caries (Xu et al. 2011; Meurman et al. 2012; Zhang 2013). However, the cariogenic potential of the microbial consortia, as well as the physiologic interactions among its correspondence, and are not just correlated with the role played by a single strain (Arthur et al. 2013). In this study, controlled multispecies biofilms were used to create a more diverse microbial environment and, consequently, while theoretically possible, to achieve a high degree of reproducibility between experimental runs. *S. sanguinis* and *S. gordonii* are known as non-*mutans* *Streptococci*. *S. sanguinis* is reported as a common inhabitant in the human mouth and is associated with low caries risk (Ge et al. 2008). *S. gordonii* is reported as an early colonizer of the dental plaque biofilm and is associated with sound enamel (Marsh 2009). *S. mutans*, *S. gordonii*, and *S. sanguinis* were chosen for this multispecies biofilms model, and the microflora composition shift will represent its development tendency to health condition or to caries risk. Both the CFU counts and multispecies biofilm images revealed the antibacterial feature of DMADD, which agreed with a previous report (Cheng et al. 2013).

Figure 3. The antibacterial effect of dimethylaminododecyl methacrylate (DMADD) on multispecies biofilms. **(A)** The scanning electron microscopy images multispecies biofilms; **(B)** colony-forming unit counts of biofilms formed on each disk from 3 groups—Clearfil SE Bond without DMADD, Clearfil SE Bond with 2.5% DMADD, and Clearfil SE Bond with 5% DMADD; **(C)** the 3-dimensional reconstruction of multispecies biofilms (live bacteria, stained green; dead cells, stained red); **(D)** the ratio between dead and live bacteria computed in line with 5 random sights of multispecies biofilms. All these data supported the antibacterial effect of DMADD on multispecies biofilms. Data are presented as mean $\pm$ standard deviation. * $P < 0.05$. This figure is available in color online at <http://jdr.sagepub.com>.

The fermentable dietary carbohydrates are the key environmental factors involved in the initiation and development of dental caries (Leme et al. 2006). Biofilms with the exposure to fermentable carbohydrates are responsible for tooth caries (Leme et al. 2006; Hara and Zero 2010; Marsh 2010). Sucrose, one kind of carbohydrate, is a substrate for the synthesis of EPSs and intracellular polysaccharides (Koo et al. 2010). The EPSs can contribute to the pathogenesis of dental caries through at least 6 distinct routes (Koo et al. 2009). However, this important virulence factor has been examined in few previous studies investigating antibacterial dental adhesive.

In this study, DMADD-containing groups significantly reduced both the total water-insoluble glucan and the ratio between EPSs and bacteria when compared with those in control group. EPS production depends on the environment in

which bacteria survive. In this study, when bacteria suffered from the extreme stress, they reduced the EPS synthesis because they needed more energy to survive. The reduction of total water-insoluble glucan might also result from the decrease of bacterial biomass. The lactic acid measurement showed that adhesive containing DMADD noticeably decreased the lactic acid production, especially for the 5% DMADD group, in which lactic acid production was reduced 10- to 30-fold as compared with the control group. The pH measurement results supported the inhibiting effect of DMADD on the acid production; pH dropped more gently in DMADD groups, especially in the 5% DMADD group. Although the multispecies biofilms were cultured in BHI media containing 1% sucrose for 72 h, the pH in the 5% DMADD group was about 5.3, which approximates the critical pH of demineralization, 5.5 (Touger-Decker and Van Loveren 2003).

There were 2 possible reasons to explain it, including the reduced bacteria amount and the deceased proportion of *S. mutans* in biofilm. The proportion of bacteria in multispecies biofilms of different groups evolved dissimilarly over time. In brief, the ratio of *S. mutans* in the control group gradually went up with time, while it had a contrary trend in DMADDM groups. The *S. sanguinis* scale diminished mildly in the control group while dramatically in DMADDM groups. The percentage of *S. gordonii* almost remained unchanged in control group but increased steadily in the experimental groups. *S. mutans* can produce antistreptococcal bacteriocins to suppress *S. sanguinis* and *S. gordonii*; however, *S. sanguinis* and *S. gordonii* produce hydrogen peroxide to compete effectively against *S. mutans* (Kreth et al. 2008). Moreover, bacteria competed with one another for limited resource in biofilms. *S. sanguinis* single-species biofilm development seemed more sensitive to DMADDM when compared with other the 2 species (Fig. 5). So, it implied that dual pressure of both competitions between bacteria and antibacterial agent conditions led to the proportion shift in multispecies biofilms and the change of biofilm development tendency. The adhesives containing DMADDM decreased the ratio of caries-associated bacteria *S. mutans* in multispecies biofilms, which revealed its benefit to dental caries.

DMADDM has a “contact killing” effect on the oral bacteria, but the antibiofilm mechanism of DMADDM is still unclear. If we combine the results of previous study and the present experiment, adhesives containing DMADDM could influence the charge density and surface roughness. DMADDM could kill the early colonizing bacteria directly, and the surface changes could inhibit the development of biofilm indirectly, including influencing the competitions among bacteria during the growth of biofilms.

The biocompatibility of newly synthesized material is a key point for clinical application. Previous study had tested the cytotoxicity of DMADDM using human gingival fibroblasts, which well revealed the biocompatibility of DMADDM (Li et al. 2013). Human dental plaque is a well-recognized example of a natural multispecies bacterial ecosystem but a complex one. Although the multispecies biofilm has been widely used in antibacterial experiments, it can represent the dental plaque only partially. So, more in vivo studies are needed to test the antibiofilm effect of DMADDM.

In conclusion, this study incorporated DMADDM into adhesive to investigate the antibacterial effects on different species

Figure 4. The composition shift of multispecies biofilms. (A) The ratio of *Streptococcus mutans*, *Streptococcus gordonii*, and *Streptococcus sanguinis* in multispecies biofilms, conducted by TaqMan real-time polymerase chain reaction (dimethylaminododecyl methacrylate [DMADDM] content: 0%, 2.5%, 5%); (B) fluorescent in situ hybridization images of multispecies biofilms (*S. mutans*, stained green; *S. sanguinis*, stained red; *S. gordonii*, stained blue). This figure is available in color online at <http://jdr.sagepub.com>.

of bacteria in multispecies biofilms for the first time. The DMADDM reduced the acid production, EPS synthesis, and CFUs of biofilms compared with the control group, which indicated its favorable antibacterial effects. The addition of DMADDM changed the proportion of bacteria in biofilms and made it from caries propensity to healthy tendency. The proportion shift of bacteria was attributed to the dual pressure of both competition among bacteria and antibacterial agent conditions. Above all, the adhesives containing DMADDM with remarkable antimicrobial properties seemed to be a bioactive adhesive material to inhibit biofilm formation and regulate biofilm development tendency to a healthy one, which revealed its potential value for antibiofilm and anticaries clinical applications.

Author Contributions

K. Zhang, S. Wang, L. Cheng, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; X. Zhou, H.H.K. Xu, contributed to conception, design, and data interpretation, drafted and critically revised the manuscript; M.D. Weir, contributed to conception, design, and data interpretation, drafted the manuscript; Y. Ge, M. Li, contributed to design and data acquisition, drafted the manuscript; S. Wang, contributed to design and data analysis, drafted the manuscript; Y. Li, contributed to conception and data analysis, drafted the manuscript; X. Xu, contributed to design and data interpretation, drafted the manuscript; L. Zheng, contributed to conception and data interpretation, drafted the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Figure 5. The antibacterial effect of dimethylaminododecyl methacrylate (DMADDm) on single-species biofilms. **(A)** The scanning electron microscopy images of *Streptococcus mutans*, *Streptococcus gordonii*, and *Streptococcus sanguinis* biofilms; **(B)** colony-forming unit counts of *S. mutans*, *S. gordonii*, and *S. sanguinis* biofilms formed on each disk from different DMADDm content groups. Data are presented as mean $\pm$ standard deviation. * $p < 0.05$.

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