

## **Human *in situ* study of the effect of bis(2-methacryloyloxyethyl) dimethylammonium bromide immobilized in dental composite on controlling mature cariogenic biofilm**

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## Abstract

Cariogenic oral biofilms cause a considerable amount of recurrent dental caries around composite restorations every year, resulting in unprosperous oral health status and expensive restorative treatment for many patients. Quaternary ammonium monomers that can be copolymerized with the current dental resin systems have been increasingly explored for modulation of dental plaque biofilm growth over dental composite surfaces. Here, we investigated the effect of bis(2-methacryloyloxyethyl) dimethylammonium bromide (QADM), against human overlying mature oral biofilms grown intra-orally in human participants for 7 and 14 days, for the first time. Seventeen volunteers wore palatal devices containing composite specimens containing 10% by mass of QADM or a control composite without QADM. After 7 and 14 days, the adherent biofilms were collected for determination of bacterial counts via colony-forming unit (CFU) counts. The biofilm viability, chronological changes, and percentage coverage were also determined by live/dead staining. QADM composites caused a significant inhibition of *S. mutans* biofilm formation for up to seven days. No difference in the CFU values were found for the 14-day period. Our findings suggest that (1) QADM composite was successful in inhibiting 1-3 day biofilms in the oral environment *in vivo*; (2) QADM significantly reduced the portion of *S. mutans* group in a time course where patients at high risk of caries would develop initial enamel carious lesions; and (3) stronger antibiofilm activity is required for the control of mature long-term cariogenic biofilms. These results provide a perspective on the value of integrating bioactive restorative materials with traditional caries management approaches into clinical practice. Contact-killing strategies via dental materials aiming to prevent or at least reduce high numbers of cariogenic bacteria seem to be a promising approach in patients at high risk of recurrence of dental caries around composites.

**Keywords:** Antibacterial, Biofilm, Caries, Dental composite, Quaternary ammonium monomers, Human in situ study.

## INTRODUCTION

In the last decade, dental materials research has intensified attempts to reduce or modulate dental plaque biofilm growth over dental composite surfaces [1]. This is because recurrent caries or caries around restorations (CARS) are identified as one of the major reasons for the failure of composite restorations [2,3]. Replacement rates of failed restorations have been reported to be 37% to 70% with consequences that can seriously compromise the oral health status [4,5]. CARS are frequently located at the gingival margins of the proximal restorations, which are common areas for food impaction [6]. Despite the favorable outcomes of mechanical biofilm removal by brushing; patients consider the cleaning of dental biofilm at the proximal space challenging and very often fail to control biofilm build-up over time.

As dental caries is characterized by continuous mineral loss promoted by organic acids released by bacteria after sugar metabolization, [7] the presence of cariogenic bacteria, mainly streptococci from the mutans group, is a key factor to initiate carious lesions. Cariogenic bacteria are characterized as pathologically shifted species with the ability of generate of large amounts of acid and being able to survive in acidic microenvironments [8]. Hence, the introduction of novel treatment approaches, supplementary to the conventional therapeutic strategies, is considered crucial for the efficient control of CARS. The cariogenic oral biofilm influences the initiation and progression of carious lesions, not just in its primary development but also its recurrence [9]. Reports in the literature have stated a temporal relationship between changes in biofilm composition and enamel demineralization following exposure to sucrose[10,11] An undisturbed dental biofilm exposed to frequent sucrose leads to enamel demineralization after seven days of biofilm accumulation, although changes in the biofilm composition can be observed earlier [10]. As the cariogenic biofilm becomes more mature, some acidogenic and aciduric bacteria become dominant in the biofilm [12].

Another factor of microbiological relevance is that the resin composites facilitate cariogenic biofilm growth [13]. The suggested rationale is based on degradation products from dental monomers such as bisphenol A glycidyl dimethacrylate (BisGMA) and triethylene glycol dimethacrylate (TEGMA), which may help alter the metabolism and promote the proliferation of *Streptococcus mutans* [14]. Therefore, in view of the adverse outcomes in the established cariogenic biofilm control, the synthesis of free radical monomers that have quaternary

ammonium groups in their chemical structures paved the way for a compelling, noninvasive, biofilm-targeted, method that can be used against oral biofilms [15]. Reactive and easily miscible quaternary ammonium monomers have the advantage of copolymerization with the current dental resin systems by covalently bonding with the polymer network. These polymers are referred to as non-leaching antimicrobial or contact-killing agents. The antibacterial action results from the direct contact of the polymer with the microorganisms, with no release of active molecules. Although the exact antimicrobial mechanism of action has not been fully elucidated, it is generally stated that the predominant mode of action is disruption of the cell membrane [16]. This method promises to impart a durable and permanent antibacterial capability to dental composites.

Along with a similar line, studies have been carried out on the synthesis of quaternary ammonium monomers for dental applications. Studies have presented different positions of the functional groups and alkyl chain length for improved balance between mechanical properties, anti-bacterial effect, and biocompatibility [17]. The majority of the synthetic quaternary ammonium monomers have only one methacrylate group and are classified as mono methacrylates. Incorporating a high content of mono methacrylate could compromise the overall cross-linked polymer matrix and, consequently, the mechanical properties [18].

Over the years, several *in-vitro* studies investigated the antibacterial performance bis(2-methacryloyloxyethyl) dimethylammonium bromide, a quaternary ammonium monomer containing two methacrylate groups (QADM)[18]. In these studies, QADM was loaded at 10wt% in different parental formulations such as commercial and experimental adhesive systems [19,20] as well as nanocomposite, [21] rendering great reductions in *S. mutans* and total microorganisms. Overall, these studies achieved a significant reduction of biofilm viability, metabolic activity, lactic acid, and bacterial counts using a 48-h human saliva microcosm biofilm model [22]. The incorporation of QADM also did not compromise the mechanical or bonding performance of the parental materials, and its antibacterial and mechanical properties were long-term and maintained after a one-year follow-up [23].

Although encouraging results were found *in vitro* [19-22], only a few studies used native *in situ* dental plaque to study the effects of quaternary ammonium methacrylate [24,25]. In these studies, bacterial colonization over a short period (hours to 3 days) was assessed. However, response to antibacterial dental composite using QADM to an overlying mature cariogenic

biofilm formed over seven days has not been studied to date. There has been no report of *in situ* studies for longer than three days. Such a longer-term *in situ* study would give meaningful insights in the *in vivo* antibacterial performance of this material in challenging conditions that mimic the clinical scenario of retentive proximal areas where the biofilm could not be removed in high caries-risk patients. Moreover, over a 7-day period, dysbiosis is present due to the proliferation or overgrowth of cariogenic bacteria in a low pH eco-niche and enamel is prone to demineralization.

In light of the evidence available to support quaternary ammonium monomers on initial oral biofilm, the present study evaluated the antibacterial performance of QADM by challenging the effectiveness of the material against *in situ*-formed mature oral biofilms in a relatively long-term study beyond 3 days for the first time. Intact oral biofilms were grown under a cariogenic challenge *in situ* on composites within the oral cavity for 7 and 14 days, respectively. In addition to the determination of bacterial counts, the chronological changes in the biofilm were also visualized by live/dead staining, and the percentages were measured.

## RESULTS

All 17 volunteers completed the study, and no protocol deviation was identified. Treatment compliance was satisfactory. The mean and standard deviation values of colony forming unit (CFU) of biofilms collected at 7 and 14-day are plotted in Fig. 2A-B. The QADM composite had a significant effect on the viability of *S. mutans* at the 7-day period ( $p=0.0303$ ). This effect corresponds to a 43% reduction of both solutions compared with the control. The effect of QADM composite on the viability of total streptococci, lactobacilli and total microorganisms of the *in situ* biofilms showed a slight reduction in CFU counts in the same period. However, there was no statistical significance between the groups ( $p > 0.5$ ). At the 14-day period, the microbiological composition of biofilms formed on restoration was statistically similar for all evaluated conditions ( $p > 0.5$ ).

Figure 3 displays the variable percentage of mutans streptococci related to total streptococci (MS/TS) showed the statistically significant difference between the tested groups ( $p=0.0385$ ) at the 7-day period (Fig. 3A). However, the percentage of mutans streptococci related

to total microorganisms was similar at the same period. These variables have shown no difference at the 14-day period (Fig. 3B).

Figure 4 shows (A-D and F-I) live/dead staining images of biofilms grown on the QADM and control composites at the 1<sup>st</sup>, 3<sup>rd</sup>, 7<sup>th</sup> and 14<sup>th</sup> days. Biofilms grown on the control composite at the 1<sup>st</sup> and 3<sup>rd</sup> days were primarily alive with continuous green staining (4A-B). Widespread bacterial cell killing was more pronounced on the 1<sup>st</sup> and 3<sup>rd</sup> day-biofilm accumulation for QADM composite (4E-F). At the 7<sup>th</sup> and 14<sup>th</sup> day, the overly mature biofilm structure was compact with innumerable layers presenting a mushroom-like configuration with channels in the outer layer (Fig. 4C-D for control and Fig. 4G-H for QADM). Complete coverage of the composite surface is observed after seven days in the oral cavity. No significant difference between control and QADM can be observed.

In Fig. 5, image analyses indicated that the living cells grown over control composites accounted for  $93 \pm 3\%$  ( $\pm$ SD) and  $93 \pm 7\%$  ( $\pm$ SD) of the total biofilm cells for the 1<sup>st</sup> and 3<sup>rd</sup> day-biofilm, respectively. In contrast, lower percentages of living cells ( $46 \pm 8\%$  for the 1<sup>st</sup> day and  $37 \pm 3\%$  for the 3<sup>rd</sup> day) were determined on QADM composite for the same period where the remaining cells were inactive and dead.

## DISCUSSION

Bacterial attachment to dental composite surfaces and subsequent cariogenic biofilm formation are a complex process [26]. It is controlled by the interplay among biological factors, such as the bacterial ability to rapidly convert dietary sugars to acid, lower the pH, and demineralize the tooth structure, patient-related factors, and physicochemical factors such as surface topography, surface charge and surface energy of the dental materials [27,28]. Typical treatment methods for biofilm-mediated recurrence of caries lesions around the composite restorations involves operative replacement of the composites, which incurs additional health care costs and additional loss of tooth structures.

To avoid the formation of biofilms over dental materials, an attractive alternative and complementary method to dental caries management is the use of biomaterials that possess antibacterial surfaces [27]. Resin-based materials that today are widely used clinically present no antibacterial activity. The new contact-active antibacterial material is effective to prevent biofilm formation by killing bacteria [29]. Additionally, it can reduce bacteria amounts in the

surrounding microenvironment thereby significantly the potential for extending the material's service life.

The performance of quaternary ammonium monomers for dental applications has been intensely investigated *in vitro* in the past years with the overall positive outcome. Investigations have shown immediate and robust antibacterial effect (more than 3 log reductions) against oral microorganisms [21,23,25]. QADM presents a greater affinity for polymerization due to its di-functional monomers and effectiveness in producing active surfaces with higher densities of immobilized antimicrobial agents [30,31]. For the specific quaternary ammonium used in our study, the previous *in vitro* studies have pointed out the reduction of 68% and 79% in biofilm CFU counts and lactic acid production, respectively, on cured primers specimens [20].

The present study represents the first study on a human *in vivo* effect of antibacterial dental resins for relatively long periods of biofilms growing more than three days. For this *in situ* study, we challenged the antibacterial performance of a dual methacrylate-group-QADM incorporated in composites against overly mature oral biofilms formed inside the oral cavity for up to 14 days for the first time. The rationale for it was to investigate the effect of this type of monomer in an acidogenic biofilm structure capable of causing substantial mineral loss and deep lesions on the enamel surface. This approach has high clinical relevance since anti-caries therapies aimed at controlling the assembly of cariogenic biofilms should contribute with its bioactivity for prevention of the onset of early carious lesions clinically known as white spots.

The data revealed that QADM has compromised the *S. mutans* group biofilm accumulation at seven days period formation. This result is particularly interesting since *S. mutans* group presents the two most common species constantly linked to caries formation, *S. mutans* and *S. sobrinus*. [32]. The cariogenic potential of *S. mutans* is determined genetically being accentuated when sucrose is available [33]. Sucrose-mediated biofilm formation created spatial organizations as expressed by a complex network of microcolonies, which modulate the development of compartmentalized acidic microenvironments across the 3D biofilm architecture [34]. Furthermore, within the 3D biofilm, *S. mutans* displays properties that are dramatically distinct from their planktonic counterparts, including much higher resistance to antibacterial approaches, which make the biofilm much more difficult to kill than planktonic bacteria [32].

Although our research has revealed the inhibitory effect of QADM on *S. mutans* biofilms, the exact mechanism of this inhibition is still unclear. The antibacterial efficacy can be related to

the contact-killing mechanism of quaternization of the amino groups of QADM available on the bottom layer of the biofilm adjacent to composite. The negatively charged counterions that stabilize the bacterial membrane are displaced by the positively charged cationic  $N^+$  sites in the chemical structure of the quaternary ammonium-based resin. Indeed, the live/dead images obtained at the initial period of biofilm formation showed the presence of a higher proportion of nonviable bacteria (red-orange color areas). Accordingly, previous studies have highlighted similar viability of 3D biofilms growing on resin-based materials containing quaternary ammonium monomers [35,36]. Furthermore, Beyth and co-workers have suggested an intracellularly mediated death program, in which, the bacterial lysis promoted by the presence of quaternary ammonium on the resin surface may function as a stressful condition triggering programmed cell death to the bacteria further away in the biofilm [25,37].

Subsequently, no expressive microbial reduction results on CFU values or microorganism proportions were observed for *S. mutans*, and the others evaluated culture media after the 14-day period. These results point out the challenge faced by anti-caries approaches against mature biofilms. The bacterial adhesion processes under *in vivo* and *in vitro* conditions differ considerably [30]. Bacteria in biofilms are far less sensitive to antibacterial agents because of the exopolymeric matrix, extracellular polysaccharides, specific gene expression, and metabolic activity; factors that protect antibacterial therapies to reach target bacteria [38]. The live-dead images of biofilms show a well-developed dense and compact EPS-matrix, and the presence of bacterial cell clusters or microcolonies (Fig. 4C-D for control and Fig. 4H-I for QADM). The relative alteration of the proportion dead/life found at 7-14-day images are related to the uneven spatial distribution of vital and dead microorganisms found in matured and thick dental biofilms with decreased vitality towards the outer layers [38]. Tawakoli et al. [39] also supported the high variability of the live/dead distribution, and the CFU counts as challenges found in *situ* biofilm models. Recently, new investigations have started to emerge using nanoparticles to improve penetration of therapeutics agents into the biofilm matrix of oral cariogenic biofilm [40]

Another aspect to consider is the spatial arrangement, charge density and counter anion of the quaternary ammonium monomers and their antibacterial activity [29]. Previous studies have designed antibacterial monomers containing an eight-carbon or longer chain and have correlated this with significant increased significant antibacterial activity *in vitro* [41]. Similarly, higher surfaces charge density has been associated with an improved antibacterial performance *in vitro*.



These monomers need be investigated for *in vitro* to a human *in vivo* translation of their antibacterial performance [42]. Future studies are warranted to investigate this further, especially whether the charge density can be reflected as a relevant factor for the antibacterial effect of these new quaternary ammonium monomers.

In summary, the findings in this paper demonstrate that QADM composite at 10% can promote a substantial and valuable bacterial reduction of *S. mutans* biofilm. This was achieved not only in the initial days of contact but also reaching a 7d-period, a time course where patients at high risk of caries would develop initial enamel carious lesions. However, dental caries results from interactions over time. An undisturbed cariogenic biofilm well-established on the composite surface over long periods is extremely hard to eradicate. Its inhibition should not rely only on contact with an antibacterial surface. Removing/disturbing biofilm from all tooth and composite surfaces and reducing sugar intake within three days is expected to control carious lesion development.

Concomitant and multitargeting strategies are needed against mature long-term cariogenic biofilms. The results of the present study provide a perspective on the value of integrating bioactive restorative materials with traditional caries management approaches into clinical practice. Contact-killing strategies via dental materials aiming to prevent or at least reduce high numbers of cariogenic bacteria seem to be a promising approach to help patients at high risk of recurrence of dental caries around composites.

## **MATERIALS AND METHODS**

### ***Study design and participants***

This study involved a prospective, randomized, single-blind, split-mouth *in-situ* design conducted according to the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The region's ethical committee (protocol # 1232012) has also approved it. Seventeen healthy volunteers of both genders, aged from 21 to 36 years, accepted to participate in this study fulfilling the required criteria. Inclusion criteria were normal salivary flow rate, a good general and oral health with no active caries lesions or periodontal treatment needs, ability to comply with the experimental protocol, not have used antibiotics during the three months before the study and not using fixed or removable orthodontic

devices. Exclusion criteria were failing to use the device according to the established protocol, taking medication interfering with saliva flow rate or containing antimicrobial agents. The sample size was determined by a power analysis and based on previous data [25]. Seventeen volunteers were recruited: 16 for the study and one volunteer to allow temporal visualization of the biofilm formation by live/dead staining. After being screened, the volunteers were verbally informed about the study aim and procedures and received written information and the informed consent form. At the next appointment, maxillary alginate impressions were made to the fabrication of the palatal devices.

During the experimental period, each volunteer used a removable acrylic custom-made palatal device containing the tested materials as shown in Fig. 1A. Seven days before the experiment beginning (washout period) and during the whole experiment, the volunteers were asked to use a standard toothbrush and non-fluoridated paste. Each acrylic palatal device enclosing four composite specimens (5 x 5 x 2 mm); two specimens for control composite and two specimens to QADM composite. To promote plaque accumulation and to protect it from disarrangement, recessions were created by placing the surface of the composite specimens about 1 mm below the covered plastic mesh (Fig. 1A-detail) [43].

To divert any possible carry-across effect, the sequence in which the experimental units were assigned in the palatal device took into consideration that antibacterial dental materials should be placed in one side of the palatal appliance and, consequently, control materials on the opposite side (Fig. 1A). The split-mouth experimental design is a practical approach for testing the effects of various agents on the composition of dental plaque [44]. Within each side of the palatal device, the positions of the specimens were randomly determined, according to a computer-generated randomization list [45]. The outcome variables evaluated were colony forming unit counts for total microorganisms, total streptococci, mutans streptococci, and lactobacilli on the specimens.

### ***Specimen preparation***

The light-curable composite was made by blending a monomer resin consisting of BisGMA (bisphenol-glycidyl dimethacrylate) and TEGDMA (triethylene glycol dimethacrylate) at 1:1 ratio (all by mass) with 0.2% camphorquinone and 0.8% ethyl 4-N, N-dimethylaminobenzoate. As reinforcement co-fillers, barium-boroaluminosilicate glass particles

with a median diameter of 1.4  $\mu\text{m}$  (Caulk/ Dentsply, Milford, DE, USA) were silanized with 4% 3-methacryloxypropyltrimethoxysilane and 2% n-propylamine [21]. The fillers were mixed with the resin at a total filler mass fraction of 60% to form a cohesive paste.

The synthesis of bis(2-methacryloyloxyethyl) dimethylammonium bromide via a modified Menshutkin reaction was previously described [18], and it is summarized in Fig. 1B. Briefly, 10 mmol of 2-(N, N-dimethylamino)ethyl methacrylate (DMAEMA; Sigma-Aldrich, St Louis, MO, USA) and 10 mmol of 2-bromoethyl methacrylate (BEMA; Monomer-Polymer Labs, Trevose, PA, USA) were combined with 3 g of ethanol in a closed vial. After stirring at 60 °C for 24 h for the reaction to complete, the solvent was removed via evaporation under vacuum. This process yielded QADM as a clear and viscous liquid. QADM was mixed with the BisGMA-TEGDMA resin at a QADM mass fraction of 10%. A preliminary study has shown that this mass fraction yielded strong antibacterial properties without compromising the resin's mechanical properties [21].

Thirty-six light-curable composite specimens were fabricated for the experimental composition (QADM at 10wt.%) each, and a further 36 specimens without antimicrobial monomer served as control. The composite was inserted and light-activated for 20 sec using a Light Emitting Diode (Radii-cal, SDI Limited Victoria, Australia; standard curing mode, irradiance output provided of 689  $\text{mW}/\text{cm}^2$ ). The specimens were mounted in standardized sample chambers inside the device with an anterior-posterior position using body impression material (Aquasil Ultra, Dentsply DeTray GmbH, Konstanz, Germany) as demonstrated in Fig. 1A.

### Clinical phase

Audiovisual orientation and written instructions of the *in situ* protocol were given to the volunteers to assure their adhesion and avoid protocol deviation during the study. To provide a cariogenic challenge during the clinical phase, the application of a 20% sucrose solution extra-orally on the restored specimens was performed by the volunteers, eight times per day at predetermined times. According to previous studies, the sucrose was gently dried after 5 min, and the device was reinserted into the mouth [42,44]. No restriction was made about the volunteer's diet, but they were instructed to avoid F-rich food containing bioavailable F, such as black tea, but they drank fluoridated water [about 0.7 ppm fluoride].

### **Microbiological and biochemical analysis**

On the 7<sup>th</sup> and 14<sup>th</sup> examination days, the subjects refrained from eating, drinking, and tooth cleaning 12 h after the last application of the sucrose solution and dentifrice before presenting at the clinic. On the 7<sup>th</sup> day, the device was removed from the mouth, and the biofilm and one enamel disc, respectively, from each side, were carefully removed and collected (Fig. 1B). Then, the device with the remaining discs was reinserted into the mouth. On the 14<sup>th</sup> day, a similar process was performed to collect the two residual biofilms from the specimens. After the collection, the biofilm was processed for analysis. Firstly, it was weighed ( $\pm 1$  mg) in pre-weighed microcentrifuge tubes and agitated during a 2 min period in a Disrupter Genie Cell Disruptor (Precision Solutions, Rice Lake, WI, USA). An aliquot of 50  $\mu$ L of the sonicated suspension was diluted in 0.9% NaCl and serial decimal dilutions were inoculated in triplicate by the drop-counting technique in the following culture media: (1) Mitis salivarius agar containing 20% sucrose, to determine TS, and in mitis salivarius agar plus 0.2 bacitracin/mL, to determine MS; (2) Rogosa agar supplemented with 0.13% glacial acetic acid to assess the number of CFU of lactobacilli (LB); and (3) brain heart infusion enhanced with 5% sterile defibrinated sheep blood agar plates were used to determine total microorganisms (TM). The plates were incubated in 10% CO<sub>2</sub> at 37 °C for 48 h. The CFU were counted, and the results were expressed as CFU/mg biofilm wet weight, the percentage of MS in relation to TM, and the percentage of MS in relation to TS.

### ***Live/dead assay***

To visualize the microorganisms during the initial phase of formation as well during the experimental periods, one volunteer used a palatal device containing eight composite specimens: four specimens for control composite and four specimens for QADM composite. One specimen of each group was removed at the 1<sup>st</sup>, 3<sup>rd</sup>, 7<sup>th</sup> and 14<sup>th</sup> days. The specimens were immediately washed with phosphate buffered saline (PBS) and stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, USA) to qualify the bacterial cell viability. This assay employs two nucleic acid stains: the green-fluorescent SYTO<sup>®</sup>9 stain and the red-fluorescent

propidium iodide stain [39]. These stains differ in their ability to penetrate healthy bacterial cells. When used alone, SYTO 9 stain labels both live and dead bacteria.

In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red. A volume of 100  $\mu$ L of the previously described fluorescence dyes was pipetted onto the specimens and incubated in a dark chamber for 15 min. The biofilms grown over the specimens were then examined using an epifluorescence microscope (TE2000-U, Nikon, Melville, NY, USA) at a magnification of 100x. Images (n=4) were acquired and analyzed (NIS Elements software, Nikon Instruments Inc, Melville, NY, USA) for quantification of live (green fluorescence) and dead (red fluorescence) bacteria.

### Statistical Analysis

The assumptions of equality of variances and normal distribution of errors were checked for all the response variables tested and those that did not satisfy these assumptions were transformed using Box-Cox power transformation [42]. To determine the differences between test and control values in the *in situ* experiment, the viable bacteria counts, percent MS/TS and percent MS/TM were submitted to a two sample independent Student's t-test. The significance level was set at  $\alpha = 0.05$ . The statistical appraisal was computed with SPSS for Windows XP 17.0 (SPSS Inc., Chicago, IL, USA).

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### Conflict of interest statement

The authors declare that there is no conflict of interest pertaining to the data presented in this article.

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## Legends

**Figure 1.** **A.** *In situ* palatal devices used by 16 volunteers. Each device containing four slabs: 2 filled with QADM composite on one side, and two filled with control composite on the other side in this *in situ* study. The biofilm is collected from the surface of specimens on 7<sup>th</sup> and 14<sup>th</sup> day, respectively. Top left is a magnified description showing details of biofilm formation over the composite specimens inside the device. **B.** Synthesis route of Bis(2-methacryloyloxyethyl) dimethylammonium bromide monomer via Menshutkin reaction. Details for dual polymerizable groups and bacterial terminal.

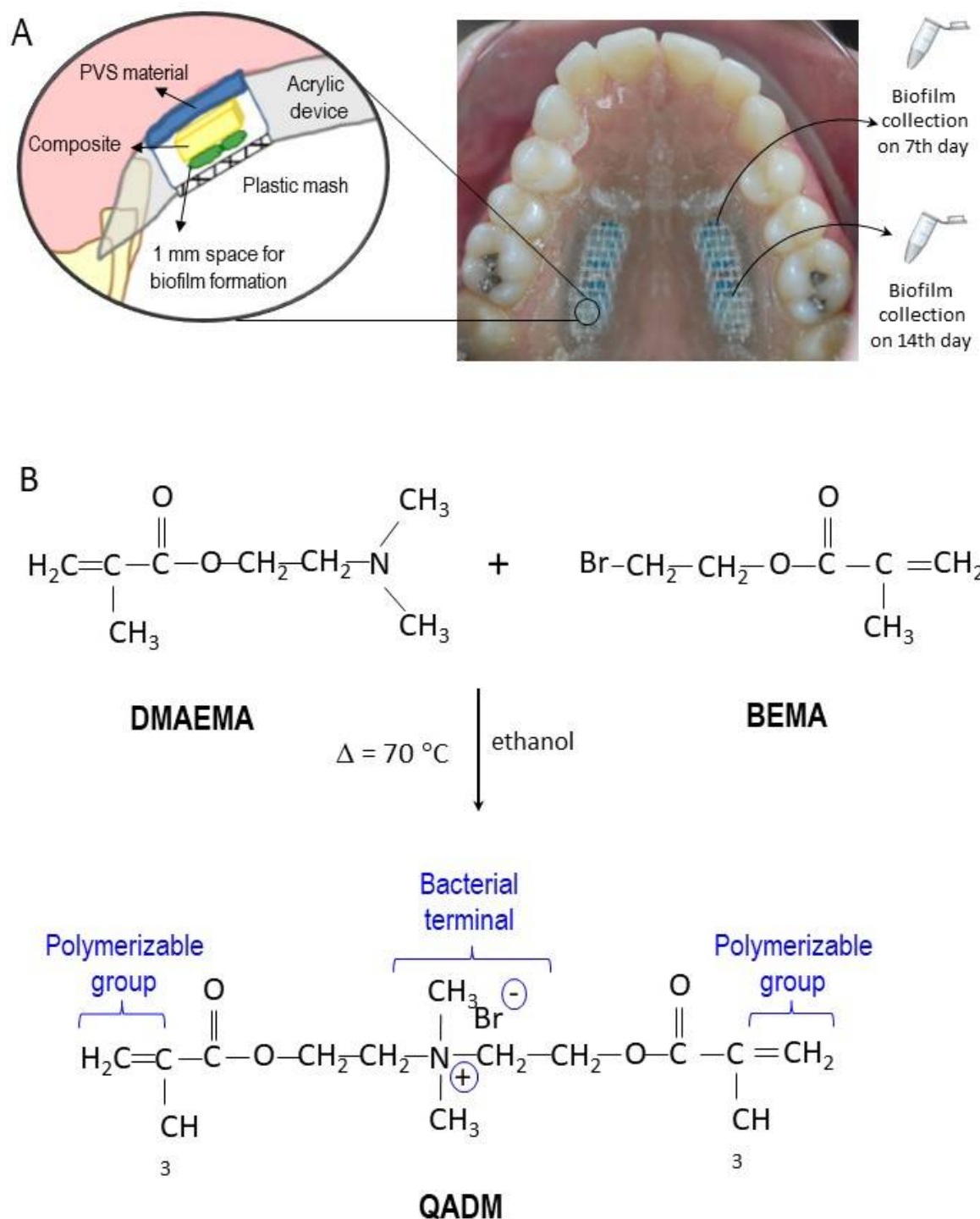
**Figure 2.** **A.** Colony-forming unit (CFU) counts for the viability of mutans streptococci, total streptococci, lactobacilli and total microorganisms present in biofilms formed *in situ* after 7-day and **B.** after the 14-day period. Error bars represent the standard deviation of mean and data followed by different letters differ statistically ( $p < 0.05$ ). The reduction in CFU counts from biofilms adherent on the QADM composites was significantly different from control for *Streptococcus mutans* after 7 days of growth. After 14 days, no further reduction was observed for *S. mutans*.

**Figure 3.** **A.** The percentage of mutans streptococci related to total streptococci (MS/TS) and percentage of mutans streptococci related to total microorganisms (MS/TM) present in biofilms formed *in situ* after 7-day and **B.** 14-day period. The MS/TS was greatly reduced for biofilms adherent on the QADM composite in relation to control at the 7-day period. Error bars represent SD and data followed by different letters differ statistically ( $p < 0.05$ ).

**Figure 4.** Fig. 4 shows (A-H) live/dead staining images of biofilms grown on the QADM and control composites at 1<sup>st</sup>, 3<sup>rd</sup>, 7<sup>th</sup> and 14<sup>th</sup> day period. Biofilms grown on the control composite at 1<sup>st</sup> and 3<sup>rd</sup> day were primarily alive with continuous green staining (4-A-B). Widespread cell killing of bacteria was more pronounced on the 1<sup>st</sup> and 3<sup>rd</sup> day-biofilm accumulation for QADM composite (4-F-G). At 7<sup>th</sup> and 14<sup>th</sup> day, the overly mature biofilm structure is compact with in numerous layers presenting a mushroom-like configuration with channels in the outer layer (Fig. 4C-D for control and Fig.4 H-I for QADM).

**Figure 5.** Fig. 5 expresses the relative percentage of live/dead bacterial cell found for the biofilm grown over the control (5A) and QADM (5B) composite, respectively. An increase in the dead percentage is observed for the biofilm grown over the QADM composite during the 1<sup>st</sup> and 3<sup>rd</sup> day.

## Legends



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filled with QADM composite on one side, and two filled with control composite on the other side in this in situ study. The biofilm is collected from the surface of specimens on 7<sup>th</sup> and 14<sup>th</sup> day, respectively. Top left is a magnified description showing details of biofilm formation over the composite specimens inside the device. **B.** Synthesis route of Bis(2-methacryloyloxyethyl) dimethylammonium bromide monomer via Menshutkin reaction. Details for dual polymerizable groups and bacterial terminal.

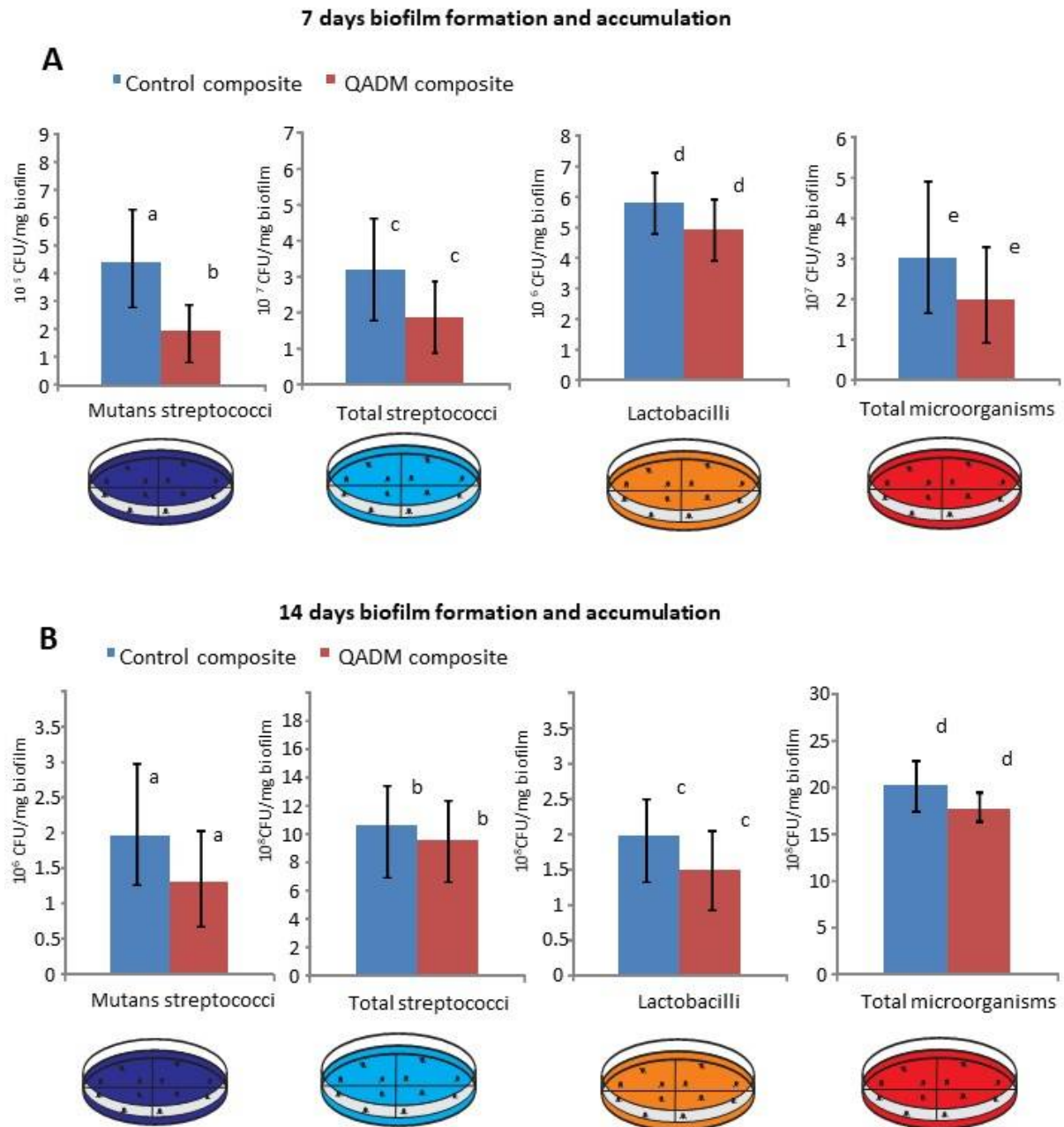
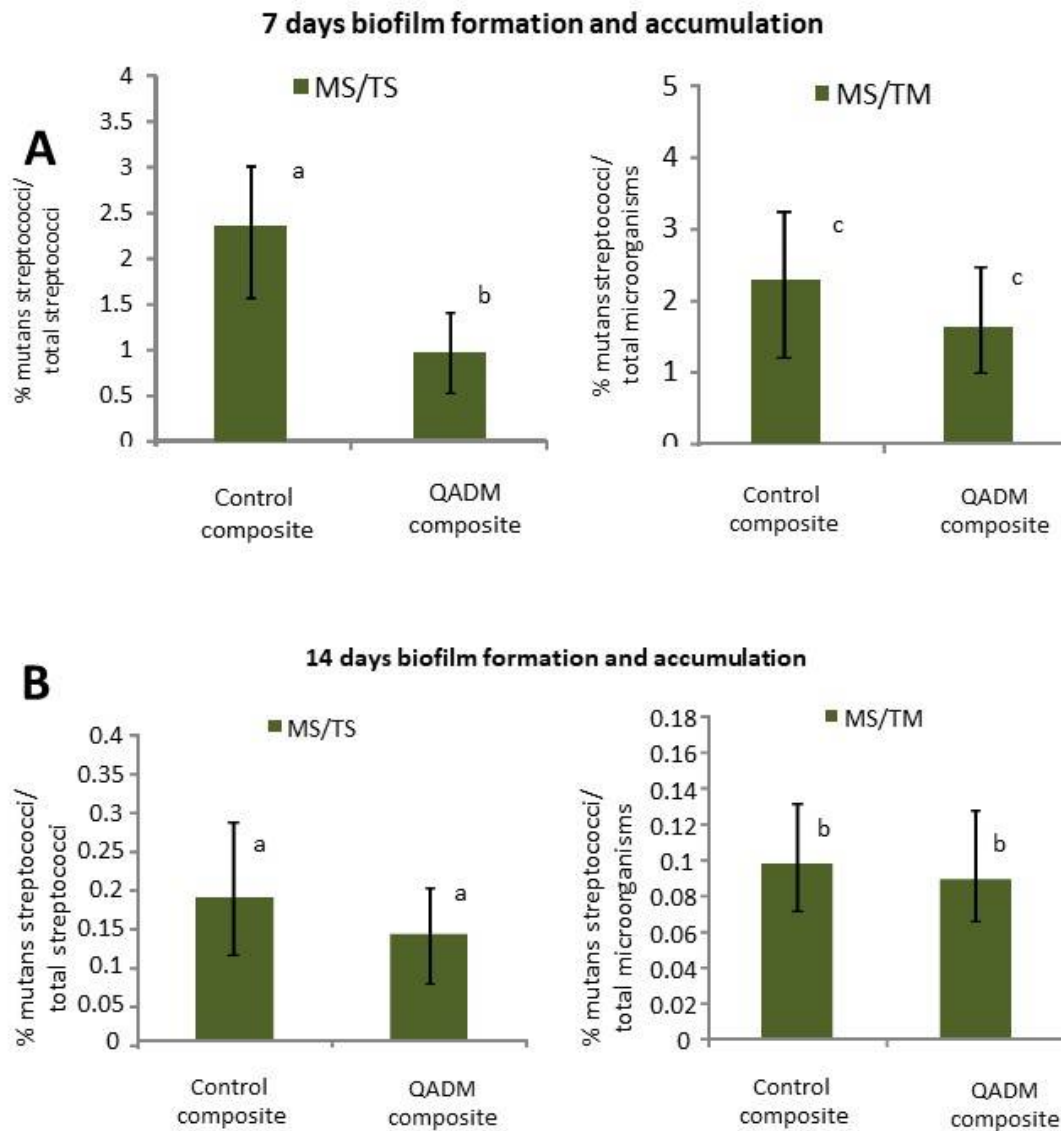


Fig.2

**Figure 2. A.** Colony-forming unit (CFU) counts for the viability of mutans streptococci, total

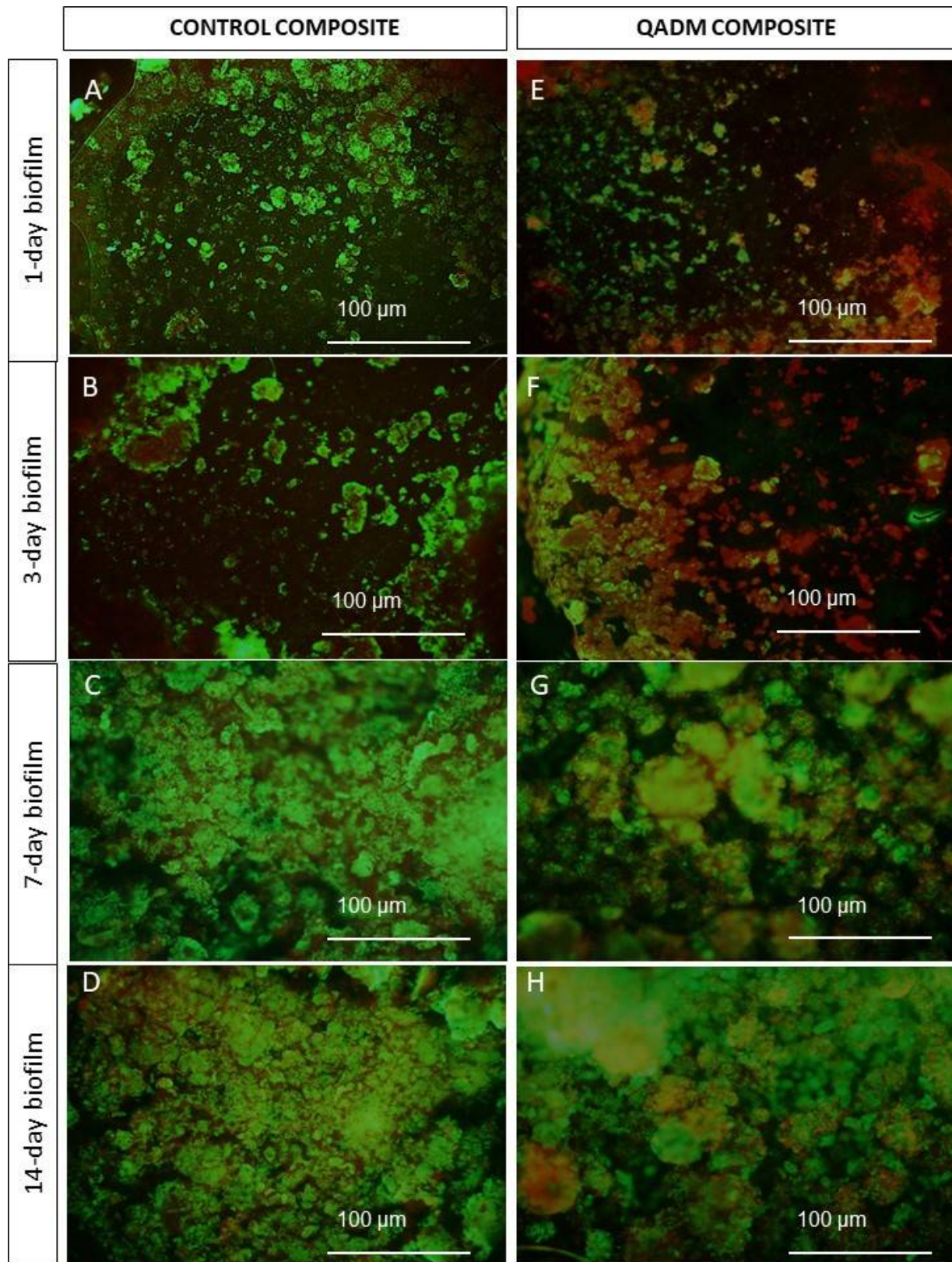


streptococci, lactobacilli and total microorganisms present in biofilms formed in situ after 7-day and **B.** after the 14-day period. Error bars represent the standard deviation of mean and data followed by different letters differ statistically ( $p < 0.05$ ). The reduction in CFU counts from biofilms adherent on the QADM composites was significantly different from control for *Streptococcus mutans* after 7 days of growth. After 14 days, no further reduction was observed for *S. mutans*.



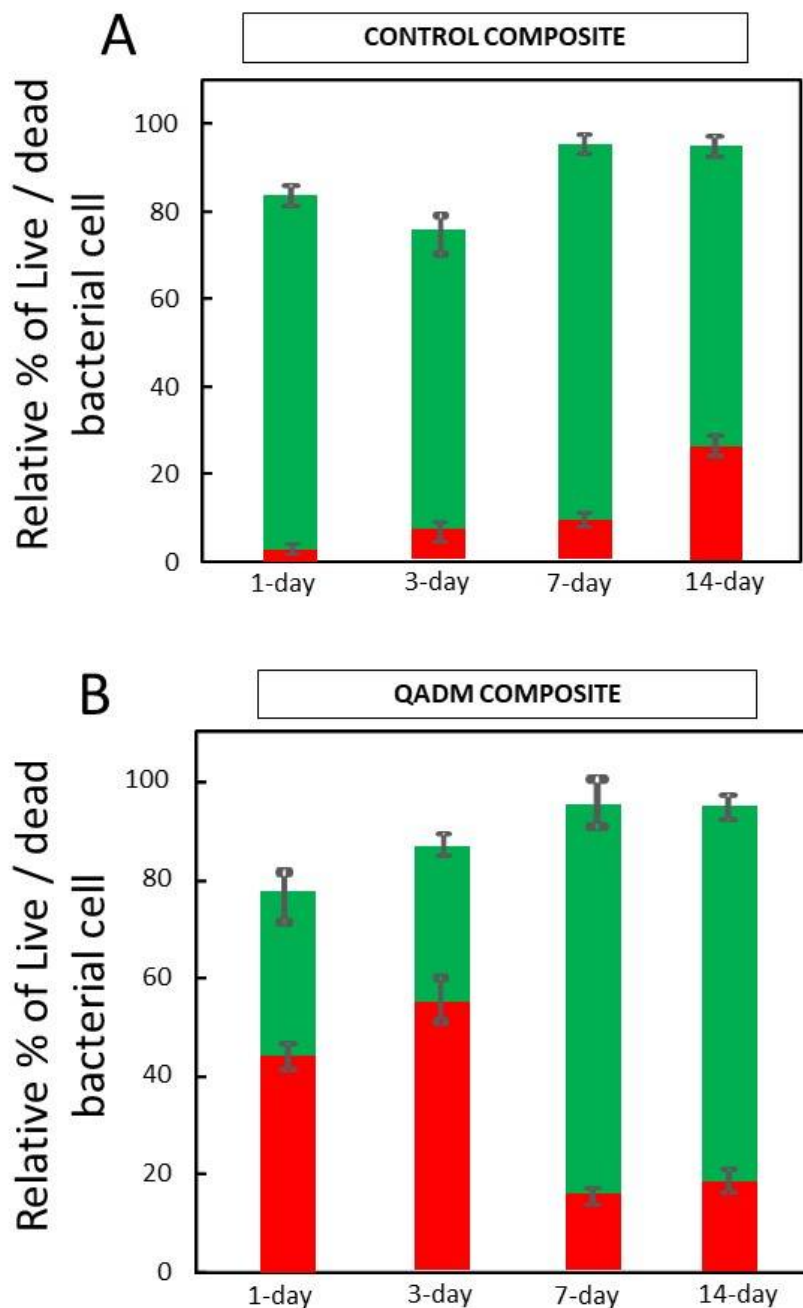
**Figure 3.** A. The percentage of mutans streptococci related to total streptococci (MS/TS) and

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