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Quaternary ammonium silane-based antibacterial and anti-proteolytic cavity cleanser

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ABSTRACT

Objective. Secondary caries and degradation of hybrid layers are two major challenges in achieving durable resin–dentin bonds. The objectives of the present study were to investigate the effects of a 2% quaternary ammonium silane (QAS) cavity cleanser on bacteria impregnated into dentin blocks and the gelatinolytic activity of the hybrid layers.

Methods. Microtensile bond strength was first performed to evaluate if the 2% QAS cavity cleanser adversely affected bond strength. For antibacterial testing, *Streptococcus mutans* and *Actinomyces naeslundii* were impregnated into dentin blocks, respectively, prior to the application of the cavity cleanser. Live/dead bacterial staining and colony-forming unit (CFU) counts were performed to evaluate their antibacterial effects. Gelatinolytic activity within the hybrid layers was directly examined using *in-situ* zymography. A double-fluorescence technique was used to examine interfacial permeability immediately after bonding.

Results. The cavity cleanser did not adversely affect the bond strength of the adhesives tested ($p > 0.05$). Antibacterial testing indicated that 2% QAS significantly killed impregnated bacteria within the dentin blocks compared with control group ($p < 0.05$), which was comparable with the antibacterial activity of 2% chlorhexidine ($p > 0.05$). Hybrid layers pretreated with 2% QAS showed significant decrease in enzyme activity compared with control group. With the use of 2% QAS, relatively lower interfacial permeability was observed, compared with control group and 2% chlorhexidine ($p < 0.05$).

Significance. The present study developed a 2% QAS cavity cleanser that possesses combined antimicrobial and anti-proteolytic activities to extend the longevity of resin–dentin bonds.

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1. Introduction

Although considerable advancements have been achieved in dentin bonding over the past decade, the durability of tooth-colored plastic fillings remains a clinically formidable issue that has not been satisfactorily addressed. There are two major challenges in achieving durable resin–dentin bonds. Secondary caries has been thought to be one of the primary challenges [1]. The minimal intervention approach is currently recommended for the treatment of deep carious lesions, to preserve tooth structure and avoid damage to the dental pulp complex [2,3]. Nevertheless, viable bacteria are inadvertently retained within the remaining hard tissues following conservative removal of the caries-infected dentin, resulting in secondary caries and failure of the restoration over time [4,5]. Microleakage of bacteria through interfacial gaps generated from polymerization shrinkage of methacrylate-based resin composites also leads to secondary caries and restoration failure [6,7].

Degradation of hybrid layers, caused by hydrolysis of hydrophilic adhesive resin [8,9] and enzymatic degradation of the exposed collagen fibrils in an aqueous environment [9,10], is the other important contributor to the poor durability of resin–dentin bonds. The acid etching step of contemporary etch-and-rinse dentin bonding techniques exposes endogenous dentin proteases such as matrix metalloproteinases (MMPs) and cysteine cathepsins that are normally embedded within the collagen matrix by apatite crystallites; once exposed, these proteases are activated by the mildly acidic resin monomers present in dentin adhesives [11–13]. The activated collagen-bound proteases induce progressive degradation of denuded collagen fibrils within the hybrid layers, resulting in deterioration of the interfacial bonds over time [12].

Because of these issues, the use of a cavity cleanser that possesses combined antimicrobial and anti-proteolytic effects prior to restorative procedures is highly beneficial in preventing the development of secondary caries and degradation of hybrid layers. Chlorhexidine is commonly used as an effective agent to disinfect dentin cavities [14]. This is attributed to its broad spectrum antimicrobial [15] and anti-proteolytic properties [8,16]. Nevertheless, chlorhexidine is water-soluble and only binds electrostatically to the demineralized dentin collagen matrix [17,18]. Chlorhexidine eventually desorbs from the denuded collagen matrix and slowly leaches out of the hybrid layers over time, thus compromising its long-lasting effectiveness [19].

A quaternary ammonium silane (QAS; codenamed K21; $C_{92}H_{204}Cl_4N_4O_{12}Si_5$; CAS number 1566577-36-3) has been synthesized *via* sol–gel reaction, by reacting 3-(triethoxysilyl)-propyldimethyloctadecyl ammonium chloride with tetraethoxysilane as the anchoring unit, using a molar ratio of 4:1 [20,21]. The use of TEOS as anchoring unit enables a three-dimensional organically-modified silicate network to be formed by condensation of additional tetra- and triethoxysilane molecules with remaining silanol groups within the molecule [22]. When it is applied to acid-etched dentin, this three-dimensional silicate network can progressively condense within the dentinal substrate to provide relatively long-term antimicrobial and anti-enzymatic effectiveness [21]. Although the free QAS molecule possesses potent inhibitory effect on dentin proteases [20], it is not known whether a QAS-containing cavity cleanser has inhibitory effects against bacteria impregnated within dentin, and whether it is effective against endogenous dentin proteases, after the cavity cleanser is applied to acid-etched dentin.

Accordingly, the objectives of the present study were to investigate the effect of a 2% QAS-containing cavity cleanser against bacteria impregnated into human dentin blocks, and to evaluate the gelatinolytic activity of the resin–dentin interface using *in-situ* zymography and functional enzyme activity assays. The hypotheses tested were: (1) pretreatment of dentin surface with QAS cavity cleanser has no adverse effect on dentin tensile bond strength; (2) the QAS cavity cleanser has no effect in inhibiting bacteria impregnated into dentin blocks; (3) hybrid layers pretreated with QAS cavity cleanser are less affected by endogenous dentin proteases, and (4) QAS cavity cleanser has inhibitory effects on soluble MMP-9 and cathepsin K activities.

2. Materials and methods

The 2% QAS cavity cleanser was purchased from KHG fiteBac Technology (Marietta, GA, USA). The chemical formula of QAS is shown in Fig. 1. The cavity cleanser consisted of QAS dissolved in ethanol.

2.1. Bond strength testing

2.1.1. Tooth preparation

Thirty-six extracted intact, caries-free human third molars were collected after the donors' informed consent was obtained under a protocol approved by the Human Assurance Committee of the Augusta University. The extracted teeth were stored in 0.9% (w/v) NaCl containing 0.02% NaN_3 at 4 °C for no more than one month. The roots of the teeth were removed 2–3 mm below the cementoenamel junction using a slow-speed diamond-impregnated saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) with water cooling. The occlusal third of each tooth crown was cut perpendicular to the longitudinal axis of the tooth to create a flat mid-coronal dentin surface. The exposed dentin surface was polished wet with 600-grit silicon carbide paper under running water to create a standardized smear layer.

2.1.2. Bonding procedures

Each exposed dentin surface was acid-etched with 37% phosphoric acid (Uni-Etch, Bisco Inc., Schaumburg, IL, USA) for 15 s, rinsed with deionized water for 15 s and gently air-dried. The specimens were randomly assigned to two groups according to the adhesive employed: Prime & Bond® NT™ (PB, Dentsply DeTrey, Konstanz, Germany) and Adper™ Single Bond Plus (SBP, 3M ESPE, St. Paul, MN, USA). The compositions of the adhesives are shown in Table 1. Specimens from each adhesive group were further randomly allocated to one of the following three subgroups for dentin pretreatment with 2% QAS cavity cleanser (KHG fiteBac Technology), 2% chlorhexidine cavity cleanser (CHX, Bisco Inc.) or deionized water (control)

Table 1 – Composition of dental adhesives tested in the present study.

Adhesive	Composition
Primer & Bond NT™	Di- and trimethacrylate resins
	PENTA
	Nanofillers-amorphous silicon dioxide
	Photoinitiators
	Stabilizers
	Cetylamine hydrofluoride
	Acetone
Adper™ Single Bond Plus	Bis-GMA
	HEMA
	Glycerol 1,3-dimethacrylate
	Diurethane dimethacrylates
	Photoinitiators
	Copolymer of polyacrylic and polyitaconic acids
	Ethanol
	Water

Abbreviations: Bis-GMA: bisphenol A glycidyl dimethacrylate; HEMA: 2-hydroxyethyl methacrylate; PENTA: dipentaerythritol penta acrylate monophosphate.

[N = 6]. The acid-etched dentin surfaces were pretreated with the respective cavity cleanser or deionized water using a disposable brush tip for 20 s and then gently air-dried [21]. The adhesives were applied and light-cured for 15 s using a light emission diode curing unit (Elipar S10, 3M ESPE) according to manufacturer's instructions. Resin composite build-ups were constructed with four 1-mm increments that were light-cured for 60 s each. The bonded specimens were stored in deionized water at 37 °C for 24 h.

2.1.3. *Bond strength evaluation*

After 24 h of water-storage, the bonded teeth were vertically sectioned into 0.9 mm thick composite-dentin slabs with a slow-speed diamond-impregnated saw. The slabs were further sectioned into 0.9 mm × 0.9 mm beams. Each beam was attached vertically to a Geraldeli testing jig with cyanoacrylate adhesive (Zapit, Dental Ventures of North America, Corona, CA, USA) and stressed to failure under tension with a universal testing machine at a crosshead speed of 1 mm/min [23]. After testing, the cross-sectional area of each beam at the site of failure was measured with a pair of digital calipers (Fisher Scientific, Pittsburg, PA, USA) to calculate the tensile bond strength.

2.1.4. *Failure mode*

The dentin side of the fractured beams were examined with a stereoscopical microscope to determine the failure mode. Failure modes were classified as (i) adhesive failure; (ii) mixed failure; (iii) cohesive failure in resin composite and (iv) cohesive failure in dentin.

2.2. *Antibacterial activities*

2.2.1. *Bacteria culture and impregnation into dentin blocks*

Streptococcus mutans (ATCC 700610) and *Actinomyces naeslundii* (ATCC 12104) strains were used to measure the antibacterial

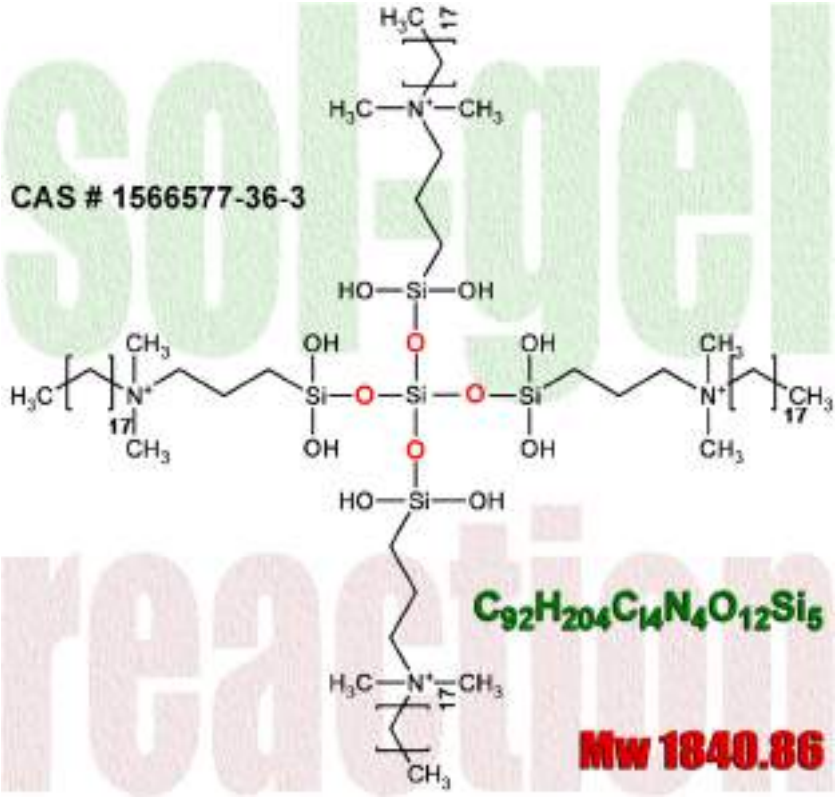

Fig. 1 – The idealized chemical formula of QAS, the sol–gel reaction product between 3-(triethoxysilyl)-propyldimethyloctadecyl ammonium chloride and tetraethoxysilane. Mw: molecular weight.

activities of two cavity cleansers (2% QAS and 2% chlorhexidine cavity cleanser). *Streptococcus mutans* was cultured in Brain Heart Infusion (BHI) broth at 37 °C with 5% CO_2 aerobically. *Actinomyces naeslundii* was incubated in BHI under anaerobic conditions at 37 °C (5% CO_2, 90% N_2, 5% H_2). After culturing for 24 h in BHI broth at 37 °C, the bacteria were diluted in BHI to a final density of 1.0×10^{10} colony-forming units (CFU) per mL. Bacteria density was measured by spectrophotometry (Beckman Coulter, Inc., Indianapolis, IN, USA) at an optical density at 600 nm.

Dentin blocks ($4 \times 4 \times 0.8$ mm) were cut at a distance of 1 mm away from the deepest pulpal horn using a water-cooled slow-speed diamond-impregnated saw, with one surface facing the pulp chamber and the other dentin surface facing the occlusal enamel [24]. The exposed dentin surfaces were wet-polished with 1200-grit silicon carbide paper, acid-etched with 37% phosphoric acid for 15 s to remove the smear layer, and thoroughly rinsed with deionized water for 60 s [25]. The dentin blocks were sterilized with ethylene oxide for 12 h according to the manufacturer's specifications and degassed for 7 days to remove the ethylene oxide. Each block was immersed in 2 μL of *S. mutans* or *A. naeslundii* suspension (1.0×10^{10} CFU/mL) for 10 min to simulate bacteria colonization on dentin [26].

2.2.2. *Live/dead bacterial staining*

Each of the two cavity cleansers was applied to the dentin surface and left undisturbed for 20 s. Ten microliter of cavity cleanser was used for each dentin block. The dentin blocks from each of the three groups (control, 2% QAS and 2% CHX; N = 6) were stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA). Live bacteria were stained with the SYTO 9 nucleic acid stain component of the kit to produce green fluorescence. Dead bacteria were stained with propidium iodide, an intercalating agent that does not penetrate the membrane of live cells, to produce red fluorescence. A confocal laser scanning microscope (CLSM, LSM 780, Carl Zeiss, Oberkochen, Germany) was used to acquire the images using a 20× objective lens, with the channels set at excitation/emission maxima 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide.

2.2.3. *CFU counts*

A 2 μL aliquot of 24-h *S. mutans* or *A. naeslundii* suspension (1.0×10^{10} CFU/mL) was placed on each dentin block for 10 min to enable the bacteria to impregnate the dentinal tubules. Then, 10 μL of each cavity cleanser was applied to the dentin surfaces. The dentin blocks were used for measuring viable CFU using the sonication method [27]. After cavity cleanser application, dentin blocks impregnated with *S. mutans* or *A. naeslundii* were placed in polyethylene centrifuge tubes with 2 mL of cysteine peptone water. The tubes were vortexed at maximum speed for 20 s and sonicated at a frequency of 40 kHz for 5 min to harvest bacteria. The harvested bacterial suspensions were plated on BHI agar plates using a serial dilution method. After incubating the agar plates for 3 days, the number of colonies was counted. Six dentin blocks were tested for each cavity cleanser (N = 6).

2.3. *In-situ zymography*

Ten teeth in each cavity cleanser group were used for *in-situ* zymography of the resin–dentin interface. One grain of tetramethylrhodamine B isothiocyanate (excitation/emission: 540/625 nm; MilliporeSigma, St. Louis, MO, USA) was dissolved in three drops of adhesive (Prime & Bond® NT™) to produce a homogeneous mixture in the dark [28]. After acid-etching with 37% phosphoric acid for 15 s, the dentin surfaces were pretreated with the respective cavity cleanser for 20 s and gently air-dried. The dyed adhesive was applied and a 2-mm thick layer of resin composite was placed over the bonded dentin. After storage in deionized water at 37 °C for 24 h, a 1 mm-thick slab containing the resin–dentin interface was vertically cut from the center of each bonded specimen.

Each bonded slab was affixed to a microscope slide and polished to obtain an approximately 50-μm thick specimen. The EnzChek™ Gelatinase/Collagenase Assay Kit (E-12055, Molecular Probes) was employed for *in-situ* zymography. Briefly, 50 μL of the self-quenched fluorescent gelatin mixture was placed on top of each slab and covered with a glass cover slip. The glass slides were incubated in the dark, in a 100% humidity chamber at 37 °C for 48 h. Hydrolysis of the self-quenched fluorescein-conjugated gelatin, caused by endogenous gelatinolytic enzyme activity within the hybrid layer, was evaluated with confocal laser scanning microscopy (CLSM) at excitation/emission wavelength of 488/530 nm (LSM 780, Carl Zeiss, Oberkochen, Germany). Green fluorescence was imaged together with the red fluorescence released by the adhesive using different channels of the two-photon CLSM. Optical sections of 85 μm thick were acquired from different focal planes for each slab. The stacked images were analyzed, quantified, and processed with ZEN 2010 software (Carl Zeiss). Quantification of the green fluorescence intensity emitted by the hydrolyzed fluorescein-conjugated gelatin (N = 6) was calculated with Image-Pro Plus 6.0 (Media Cybernetics, Inc., Silver Spring, MD, USA). Gelatinolytic activity was expressed as the percentage of the green fluorescence within the hybrid layer.

2.4. *Inhibition of soluble rhMMP-9 and cathepsin K*

The inhibitory effects of two cavity cleansers (2% QAS and 2% CHX) on soluble rhMMP-9 and cathepsin K were evaluated using purified recombinant human (rh) MMP-9 (AS-55576), the Sensolyte Generic MMP assay kit (AS-72095) and Sensolyte 520 cathepsin K assay kits (AS-72171) (all from Sensolyte, AnaSpec Inc., Fremont, CA, USA). The MMP assay kit comprises an intact thiopeptolide that is cleaved by specific MMPs to release a sulfhydryl group that can react with Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) to form a colored product (2-nitro-5-thiobenzoic acid). The latter can be monitored at 412 nm using a microplate reader. The cathepsin K assay kit contains a QXL™ 520/Hilyte Fluor™ 488 FRET peptide substrate for specific enzymes. When the FRET substrate is cleaved by active cathepsin K, it releases HiLyte Fluor™ 488, the fluorescence of which can be detected at 490/520 nm (excitation/emission) using a fluorescence microplate reader. Each experiment was performed in sextuplicate (N = 6).

2.5. Permeability of bonded interfaces

Five teeth from each of the cavity cleanser groups were used for permeability evaluation of the resin–dentin interfaces. Dentin blocks were cut at a distance of 2.5 ± 0.1 mm away from the deepest pulpal horn. One microliter of a yellow fluorescent dye (Alexa Fluor™ 532, excitation/emission: 532/553 nm; ThermoFisher Scientific, Waltham, MA, USA) was dissolved in 3 drops of adhesive (Prime & Bond® NT™) to prepare a homogeneous fluorescent adhesive in the dark. Each dentin segment to be bonded was fixed to a perforated Plexiglass block using cyanocrylate glue. The assembly was connected *via* an 18-gauge stainless steel tube to a polyethylene tubing. The latter was attached to a column of 0.1% blue fluorescent dye solution (Alexa Fluor™ 405, excitation/emission: 401/421 nm; ThermoFisher Scientific) oriented 20 cm above the Plexiglass block to simulate pulpal pressure of non-inflamed human dental pulps. This generated water pressure through the dentinal tubules during pretreatment with the cavity cleanser, bonding and resin composite build-up. The set-up was left in the dark for 4 h to enable water to continue permeate the resin–dentin boned interface.

After completion of the water permeation process, the bonded tooth was removed from the Plexiglass block and cut vertically to obtain a 1 mm thick slab containing the water-perfused resin–dentin interface. Each bonded slab was affixed to a microscope slide with cyanocrylate glue and polished to obtain an approximately 50 μm thick section. Each specimen was visualized with CLSM equipped with a 40× oil immersion objective lens. Blue fluorescence was imaged together with the yellow fluorescence released by the adhesive using different channels of the two-photon CLSM. Optical sections (85 μm thick) were acquired from different focal planes for each slab. Stacked images obtained from six specimens per group (N = 6) were analyzed, quantified, and processed with ZEN 2010 software (Carl Zeiss). Quantification of the blue fluorescence within and above the hybrid layer was calculated with Image-Pro Plus 6.0 (Media Cybernetics, Inc., Silver Spring, MD, USA) to represent the relative permeability of the corresponding resin–dentin interface.

2.6. Statistical analyses

Data derived from bond strength testing, antimicrobial activity evaluation, *in-situ* zymography and interfacial permeability were respectively examined for their normality (Shapiro-Wilk test) and equal variance (modified Levene test) assumptions prior to the use of parametric statistical methods. For bond strength testing, results obtained from the control, 2% QAS and 2% CHX groups were analyzed with two-factor analysis of variance, to examine the effects of "adhesives" and "disinfectants", as well as the interaction of those two factors on bond strength results. *Post-hoc* comparisons were conducted using the Holm–Sidak procedure. For the other parameters, results obtained from the control, 2% QAS and 2% CHX groups were analyzed with one-factor analysis of variance and Holm–Sidak *post-hoc* comparison procedures. Statistical significance was pre-set at $\alpha = 0.05$.

Table 2 – Means ± standard deviations of the tensile bond strength of dentin created with the two different adhesives pretreated with different cavity cleansers.

Adhesive	Cavity cleanser	Bond strength (MPa)
Primer & Bond NT™	Control	38.4 ± 5.4 ^{Aa}
	2% QAS	35.5 ± 5.7 ^{Aa}
	2% CHX	35.8 ± 7.0 ^{Aa}
Adper™ Single Bond Plus	Control	36.2 ± 6.4 ^{Ab}
	2% QAS	33.4 ± 4.2 ^{Ab}
	2% CHX	33.5 ± 5.3 ^{Ab}

For comparison within each adhesive (Prime & Bond NT™ or Adper™ Single Bond Plus), bond strength values with the same upper case letters within the vertical column for each cavity cleanser are not significantly different ($p > 0.05$).
For comparison of the three cavity cleanser, bond strength values with the same lower case letters within the same adhesive (Prime & Bond NT™ or Adper™ Single Bond Plus) are not significantly different ($p > 0.05$).

3. Results

3.1. Tensile bond strength

Bond strength for each group after 24 h of incubation is shown in Table 2. Two-way ANOVA demonstrated that the factors, "adhesives" and "disinfectants" did not significantly affect bond strength to dentin ($p > 0.05$). The interaction between "adhesive" and "disinfectant" was also not significant ($p > 0.05$).

For both adhesives PB and SBP, no significant difference in bond strength was found among the control (deionized water), 2% QAS and 2% CHX groups ($p > 0.05$). The use of 2% QAS cavity cleanser before adhesive application did not adversely affect the dentin bond strength of either adhesive.

Failure mode distribution in two adhesives groups is shown in Table 3. Generally, bonds with low strength values tended

Table 3 – Percentage distribution of failure modes.

Failure mode	Prime & Bond NT™			Adper™ Single Bond Plus		
	Control	2% QAS	2% CHX	Control	2% QAS	2% CHX
A	12	14	7	15	11	13
M	41	43	46	38	44	40
CD	3	2	4	4	2	3
CC	4	1	3	3	3	4

Failure mode. A: adhesive; CDL: cohesive failure in dentin; CC: cohesive failure in resin composite; M: mixed failure.

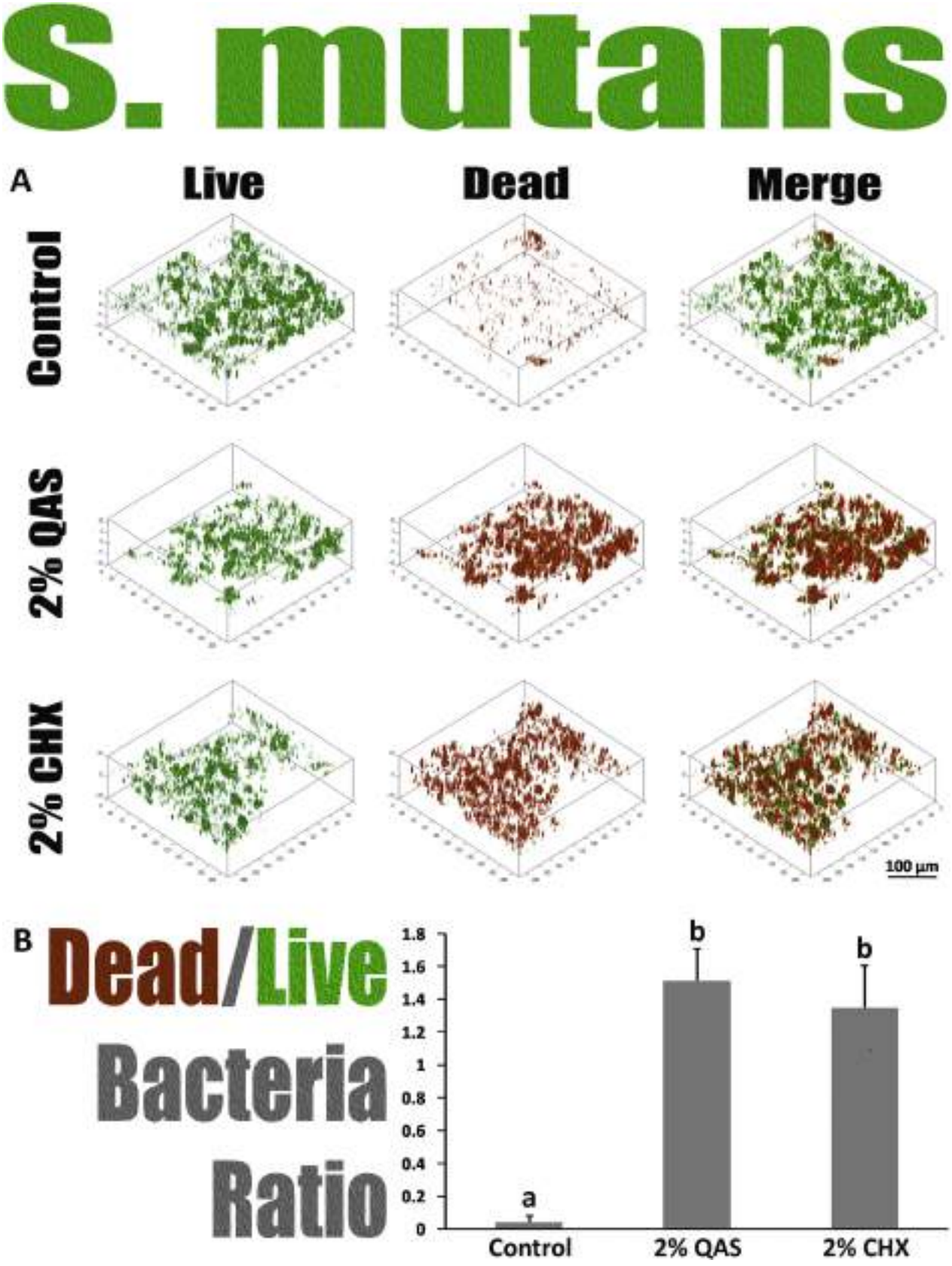

Fig. 2 – A. Representative 3-D profiles of *S. mutans* impregnated in dentin blocks after application of deionized water (control), and 2% QAS or 2% CHX as cavity cleansers. B. Bar chart of the dead/live bacteria ratio of the three groups based on analysis of the live-dead staining profiles of the dentin blocks. Values are means and standard deviations (N = 6). Columns labeled with different letters are significantly different ($p < 0.05$).

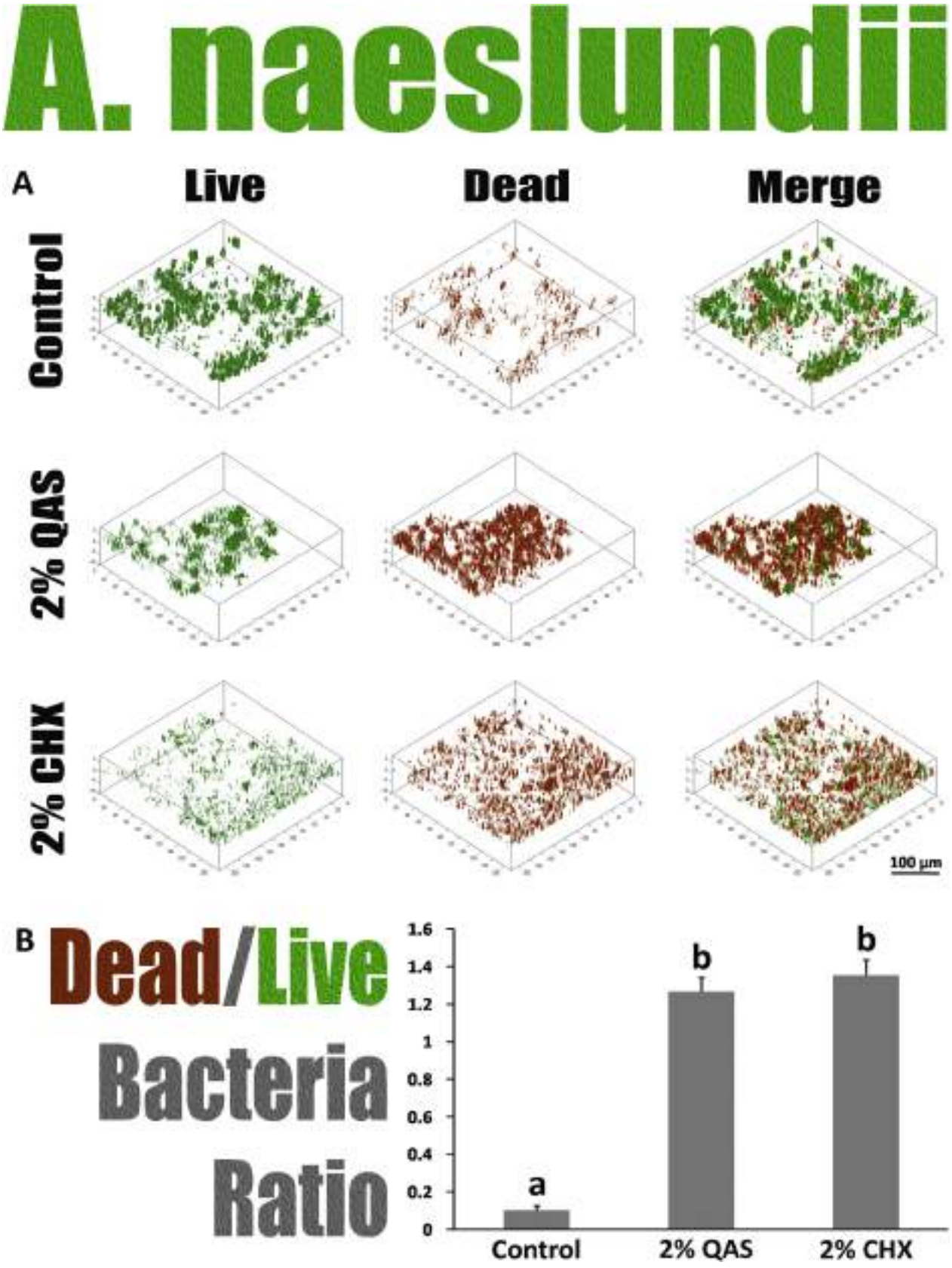

Fig. 3 – A. Representative 3-D profiles of *A. naeslundii* impregnated in dentin blocks after application of deionized water (control), and 2% QAS or 2% CHX as cavity cleansers. B. Bar chart of the dead/live bacteria ratio of the three groups based on analysis of the live-dead staining profiles of the dentin blocks. Values are means and standard deviations (N = 6). Columns labeled with different letters are significantly different ($p < 0.05$).

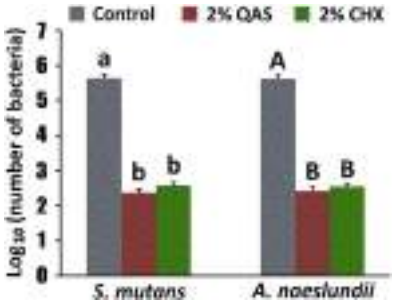

Fig. 4 – CFU counts of *S. mutans* or *A. naeslundii* impregnated in dentin blocks for the deionized water control and the two cavity cleansers groups. Values are mean and standard deviations (N = 6). For each bacterium strain, columns labeled with different letters are significantly different ($p < 0.05$).

to fail within the adhesive (*i.e.* adhesive failure). The failure modes determined for all test beams showed a clear prevalence of mixed failures.

3.2. *Antibacterial activities*

Representative three-dimensional plots of live and dead bacteria distribution in *S. mutans* and *A. naeslundii*-impregnated dentin after placement of the two cavity cleansers are shown in Figs. 2 and 3. In the control dentin group (without cavity cleanser), images of *S. mutans* and *A. naeslundii* showed primarily live bacteria, with small amounts of dead bacteria. In contrast, bacteria in the 2% QAS and 2% CHX dentin groups consisted primarily of dead bacteria (Figs. 2 A and 3 A). The ratios between dead and live bacteria were significantly higher than that in the control group ($p < 0.05$) (Figs. 2 B and 3 B), indicating that 2% QAS and 2% CHX pretreated dentin possessed antimicrobial activity. The antibacterial effect of 2% QAS was not significant different from 2% CHX.

Fig. 4 shows the CFU counts of *S. mutans* or *A. naeslundii* impregnated in dentin blocks for the two cavity cleansers groups (mean ± SD; N = 6). For both *S. mutans* and *A. naeslundii*, control dentin blocks without cavity cleanser had the highest CFU. Dentin blocks treated with the 2% QAS and 2% CHX cavity cleansers significantly reduced the CFU by three orders of magnitude, compared to the control group without cavity cleanser ($p < 0.05$). There was no significant difference in CFU between the two cavity cleansers. These results indicated that 2% QAS cavity cleanser had inhibition effect on bacteria residing within the dentinal tubules of the dentin blocks.

3.3. *Inhibition of MMP-9 and cathepsin K*

The single-fluorescence *in-situ* zymography technique developed by Mazzoni et al. [29] enables identification of proteolytic activity directly within the hybrid layer. In the present study, a double-fluorescence *in-situ* zymography technique was used to enable the locations of the dentin adhesive and the activated endogenous gelatinase enzymes to be detected simultaneously with CLSM. Representative CLSM images of the three groups are shown in Fig. 5 A. The relative percentages of green fluorescence intensity within the hybrid layers that are indicative of *in-situ* gelatinolytic activities are depicted in Fig. 5B. For the control specimens pretreated with deionized water, the dentin slabs showed intense green fluorescence within the hybrid layers after 48 h of incubation, with an intensity value of 90.0 ± 5.5%, which is indicative of extensive hydrolysis of the fluorescence-conjugated gelatin within the hybrid layers. In contrast, weak green fluorescence was detected within the hybrid layers in both the 2% QAS (14.0 ± 4.9%) and 2% CHX (19.2 ± 4.3%) groups. These values were significantly lower than that of the control group ($p < 0.05$). No significant difference was found between the 2% QAS group and the 2% CHX group ($p > 0.05$). Intratubular gelatinolytic activities [29] are thought to be derived from the proteins that regulate peritubular dentin formation [30], or by precipitation of dentinal fluid-derived matrix metalloproteinases during laboratory specimen preparation [31]. These intratubular gelatinolytic activities were not taken into account during quantification of the gelatinolytic activities in the present work.

The inhibitory effects of 2% QAS and 2% CHX on soluble rhMMP-9 and cathepsin K are represented in Fig. 6. The relative percentages of rhMMP-9 and cathepsin K inhibition by the kit inhibitor control, GM6001, were 99.45 ± 0.7% and 77.52 ± 1.21%, respectively. Over 90% of soluble rhMMP-9 was inhibited by 2% QAS (91.47 ± 4.03%) and 2% CHX (93.00% ± 3.05%); these values were not significantly different from the kit inhibitor control ($p > 0.05$). For cathepsin K, the extent of inhibition was 71.17 ± 5.30% for 2% QAS and 71.91 ± 5.86% for 2% CHX. There were no significant differences compared with the control group (76.23 ± 2.28%). For 2% QAS and 2% CHX group, no significant differences in both rhMMP-9 and cathepsin K inhibition were found between the two cavity cleansers ($p > 0.05$).

3.4. *Water permeability of bonded interfaces*

A double-fluorescence technique was employed to enable the locations of the dentin adhesive and the water permeation to be detected simultaneously with CLSM. The fluorescence representative images (separate channels; yellow for adhesive, blue for water permeability) of the permeability of resin–dentin interface are shown in Fig. 7 A. The relative percentages of interfacial permeability are presented in Fig. 7B. For the specimens pretreated with deionized water and 2% CHX, areas of water permeation could be observed throughout the hybrid layer, as well as the adhesive layer, reaching 94.7 ± 3.0% and 92.2 ± 4.3% permeability, respectively. In contrast, when 2% QAS was applied on the acid-etched dentin, only the hybrid layer is completely permeated by water, with a relative permeability of 53.7 ± 4.8%. This permeability value was significantly lower than that of control and 2% CHX groups ($p < 0.05$).

4. Discussion

The use of tetraethoxysilane as anchoring unit for QAS synthesis enables a three-dimensional, organically-modified silicate network to be formed, by hydrolysis of remnant silanol groups (Fig. 1) and subsequent condensation of tetra- and triethoxysi-

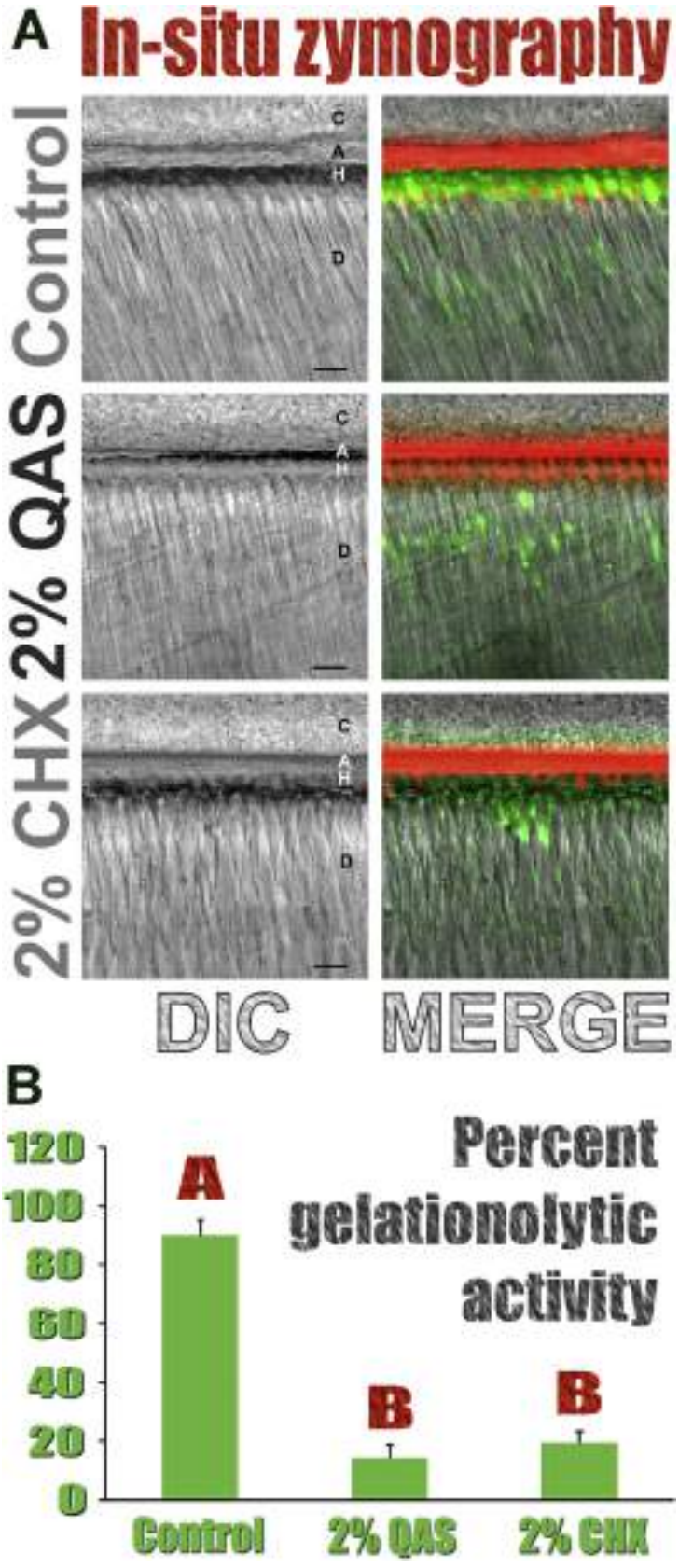

Fig. 5 – A. Representative CLSM images of *in-situ* zymography performed in resin–dentin interfaces pre-treated with the deionized water control, 2% QAS cavity cleanser or the 2% CHX cavity cleanser prior to adhesive application. Bars = 10 μm. Left column: differential interference contrast (DIC) images of the resin–dentin interfaces. Right column: superimposition of the DIC mages over image of merged channels; red for adhesive and green for endogenous dentin gelatinase activity.

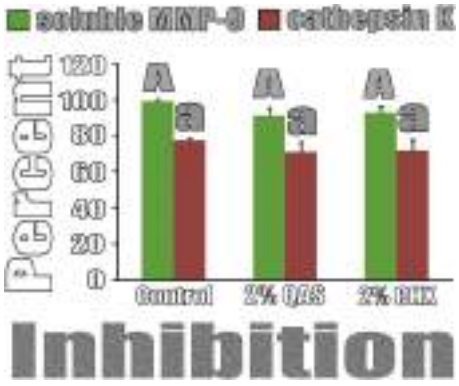

Fig. 6 – Inhibitory effects of 2% QAS and 2% CHX on soluble rhMMP-9 and cathepsin K compared with the deionized water control. The relative percentages of rhMMP-9 and cathepsin K inhibition were compared against the kit inhibitor control, GM6001. Values are means and standard deviations (N = 6). For each proteolytic enzyme, columns labeled with letters of the same case are not significantly different (p > 0.05).

lane molecules to form additional Si–O–Si linkages. Unlike the condensation reaction of methoxysilanes which produces methanol as a toxic by-product [32], sol–gel reaction between ethoxysilanes produces ethanol as the condensation product. This enables QAS to be used for intraoral application without purification to removal methanol.

Cavity disinfectants have been reported to adversely affect dentin bond strength [33,34]. The bond strength of both commercially available adhesives to dentin were not significantly affected by both cavity cleansers. Hence, the first null hypothesis that "pretreatment of dentin surface with QAS cavity cleanser has no adverse effect on dentin tensile bond strength" cannot be rejected. This may be attributed to the formation of an interpenetrating network between the condensing polysiloxane network and the methacrylate resin network during polymerization of the adhesive resin blends [35,36]. Because of its long hydrocarbon chains, QAS increases the hydrophobicity of acid-etched dentin by changing its surface energy. This, in turn, leads to better wetting of the demineralized dentin matrix and improved infiltration of the adhesive monomers [37]. In addition, water from the dentinal tubules is consumed during hydrolysis of the silanol groups in the QAS [38]. This reduces residual water within the demineralized dentin, which facilitates resin infiltration and improves adhesive polymerization [39,40]. These factors may have contributed to good bond strength associated with the use of the experimental QAS cavity cleanser.

Streptococcus mutans and *A. naeslundii* species are cariogenic oral pathogens associated with secondary caries [41]. After cavity preparation, residual bacteria often exist within the dentinal tubules. To simulate bacteria colonization of the dentinal tubules, dentin blocks were acid-etched with 37% phosphoric acid to render the orifices of dentinal tubules patent. Incubation of bacterial suspension on the smear later-depleted dentin facilitated bacterial impregnation. When applied on the bacteria-impregnated dentin blocks, the cavity cleansers readily flowed into dentinal tubules to kill the intratubular bacteria. From the results of antibacterial activities evaluation, the antibacterial effect of 2% QAS was comparable with that of 2% CHX (Figs. 2–4). Unlike 2% CHX, unreacted QAS monomers may continue to form siloxane bridges within the silicate network to continue exert antibacterial activity after adhesive application [42]. Thus, the second null hypothesis that "the QAS cavity cleanser has no effect in inhibiting bacteria impregnated into dentin blocks" cannot be rejected. With four positively-charged quaternary ammonium arms and four long, lipophilic C_{18} alkyl chains [43], QAS attaches to bacteria with negatively-charged cell walls *via* electrostatic interaction. Penetration of bacterial cell walls and membranes by its long C_{18} alkyl chains causes leakage of cytoplasmic components and subsequently cell death [44].

The hybrid layer remains the weakest link within the resin–dentin interface created in tooth-colored restorations. In the present work, double-fluorescence *in-situ* zymography was employed to enable the location of the dentin adhesive and areas within the hybrid layer with activated endogenous gelatinolytic activity to be detected simultaneously by CLSM. Although previous studies have utilized telopeptide fragments from degrading collagen fibrils and dry mass loss to evaluate endogenous enzymes activity [20,45], similar techniques were not employed in the present work because of those were indirect measurement methods. The control groups of present study showed extensive gelatinolytic activity within the hybrid layers created with the etch-and-rinse technique, as indicated by the intense green fluorescence. In contrast, specimens pretreated with 2% QAS cavity cleanser displayed only weak gelatinolytic activity within the hybrid layers after incubation for 48 h, with significantly lower green fluorescence distribution compared with the control (Fig. 5). In addition, quantitative assay of rhMMP-9 and cathepsin K inhibition by 2% QAS showed that the results were comparable with the kit inhibitor control (Fig. 6). Thus, the third hypothesis that "hybrid layers pretreated with QAS cavity cleanser are less affected by endogenous dentin proteases" and the fourth hypothesis that "QAS cavity cleanser has inhibitory effects on soluble MMP-9 and cathepsin K activities" are validated by those experiments.

Collagenolytic and gelatinolytic activities have been documented in hybrid layers created by both etch-and-rinse and self-etch dentin adhesive systems [9,46,47]. The results of the present work also confirm these findings using *in-situ* zymography, with obvious gelatinolytic activity within the hybrid layers in the control groups. Endogenous dentin proteases become exposed and activated during the acid-etching and adhesive placement steps of conventional bonding procedures [12,48], which contributes to the degradation of exposed col-

B. Bar chart comparing the percent gelatinolytic activities within hybrid layers created in the three groups. Values are means and standard deviations (N = 6). Columns labeled with different letters are significantly different (p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

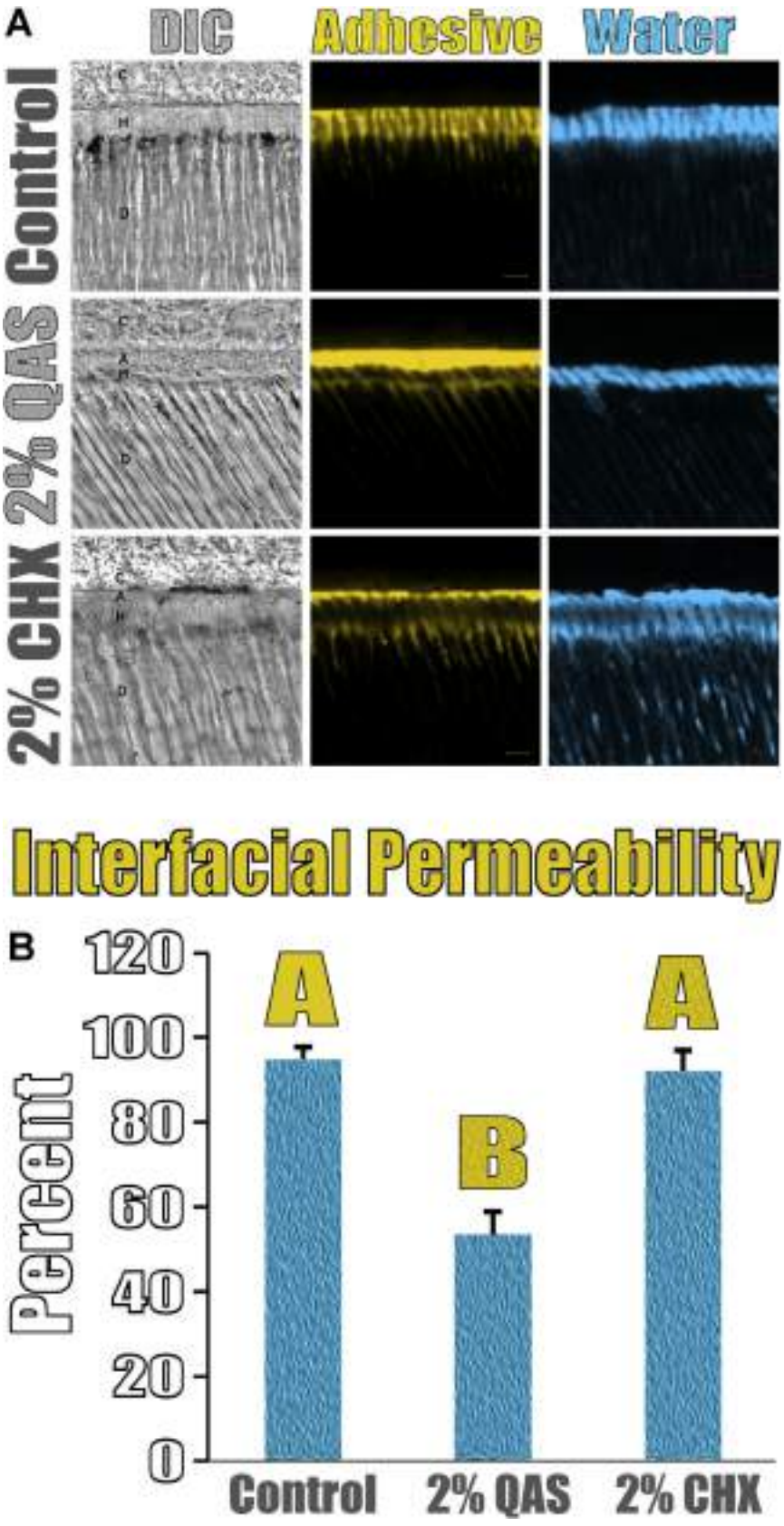

Fig. 7 – A. Representative CLSM images illustrating permeability of resin–dentin interfaces to simulated 20 cm water pressure in the control, 2% QAS and 2% CHX groups. Bars = 10 μm. Left column: differential interference contrast (DIC) image of the resin–dentin interfaces. Middle column: adhesive fluorescence; Right column: blue fluorescent-dye containing water that permeated the resin–dentin interface. B. Bar chart comparing the relative permeability of the resin–dentin interfaces in

lagen fibrils within the hybrid layers, and is thought to be important contributors to the poor durability of resin–dentin bonds.

Several factors may have contributed to the inhibitory effect of proteolytic enzymes by QAS. The catalytic domains of MMPs contain cysteine-rich repeats, including glutamic acid residues with negative charges [19]. The four positively-charged quaternary ammonium groups of QAS tested in the present study may bind to the negatively-charged glutamic acid residues *via* electrostatic interaction [19,49]. This non-specific binding may have altered the configuration of the catalytic site of the MMPs, sterically blocking their active site, thus adversely affecting MMP activity [49]. Additionally, the chloride counterions of QAS may bind to cations such as Ca^{2+} and Zn^{2+} that are necessary for MMPs activation, which is also conducive to inhibiting MMP activity [20]. The catalytic sites of cysteine cathepsins contain cysteine, histidine and aspartane residues. Cysteine can form a catalytic thiolate-imidazolium ion pair, which acts as a nucleophile for attack on the carbonyl carbon atom of the scissile peptide bond [45]. The cationic QAS is likely to show similar electrostatic binding to block the active sites of cathepsin K, thus inhibiting its activity [45]. The aforementioned factors may all have contributed to protecting the demineralized dentin collagen matrix from enzymatic degradation.

Theoretically, the resin–dentin interface should be impermeable to water permeation from the pulpal side in order to achieve a fluid-tight seal. This is paramount for preserving the integrity of resin–dentin bonds. A silver tracer has often been used to examine interfacial nanoleakage [50]. Because of the ambiguity of this tracing technique in microleakage and nanoleakage studies [51,52], a double-fluorescence CLSM technique was performed to evaluate interfacial permeability after bonding. From the present interfacial permeability results, relatively lower interfacial permeability was observed with the use of 2% QAS cavity disinfectant compared with control group and 2% CHX group (Fig. 7). This may be attributed to the progressive condensation of three-dimensional organically-modified silicate network around or within the methacrylate network. This may have resulted in occlusion of hybrid layers and dentinal tubules that are not completely infiltrated by dentin adhesives, thus reducing dentin permeability [21].

An issue associated with the application of the QAS cavity cleanser is the temporal hydrolytic stability of the siloxane bridges within the molecule after condensation. Water sorption is responsible for hydrolysis and degradation of resin components. Sorption of water molecules into a polymerized resinous network provides a route for leaching of water-soluble unreacted resin monomers and salivary and bacterial enzyme-degraded resin oligomers over time [53]. The chemical structure of the QAS shown in Fig. 1 likely represents a statistical average based on the sol–gel condensation process, where some structures will have multiple QAS arms and others will have none. The present QAS formulation is solubilized in ethanol. It is not known whether incompletely condensed QAMS within the polymer matrix would be expected to be released after aging in water or saliva. Using thermogravimetric analysis, the authors have examined the stability of the 30% QAS dissolved in ethanol before aging and after aging at 37 °C at 100% relative humidity for 6 months in the dark. Because of the minimal differences between the two thermograms (Supplementary Information), it may be rationalized that the 2% QAS cavity cleanser formulation is relatively stable. Understandably, thermogravimetric analysis is not the best way to examine hydrolytic stability. Hence, ongoing work is being performed to examine the stability of resin–dentin bonds created using the 2% QAS cavity cleanser after thermomechanical cycling and long-term aging.

5. Conclusion

Within the limitations of the present study, it may be concluded that 2% QAS cavity cleanser possesses combined antimicrobial and anti-proteolytic activities, without adversely affecting dentin bond strength. This indicates that the 2% QAS cavity cleanser may play a role in eliminating secondary caries, preventing hybrid layer degradation, and extending the longevity of resin–dentin interfacial bonds over time. Future studies are required to validate whether the antibacterial and anti-proteolytic effects of 2% QAS cavity cleanser are maintained after aging. This would provide more convincing evidence to justify the use of the 2% QAS cavity cleanser as an alternative to 2% CHX as a cavity disinfectant.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.dental.2018.10.001.

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