



Biofilm matrix-mediated localization potentiates antimicrobial activity in cariogenic biofilms



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The extracellular polymeric substance (EPS) matrix serves as a structural scaffold for biofilm organization, contributing to structural integrity and antimicrobial tolerance by modulating the penetration and distribution of antimicrobial agents. However, whether this EPS matrix can also potentiate antimicrobial localization and activity remains poorly understood. Here, we investigated the role of the EPS matrix in erythrosine B (EB)-mediated killing of *Streptococcus mutans* within mature biofilms following brief topical exposure. Biofilms formed on saliva-coated hydroxyapatite disks were exposed to EB and evaluated for bactericidal activity, glycolytic acid production, and EB spatial distribution. EB markedly reduced viable bacteria in mature biofilms and suppressed glycolytic acid production. Confocal imaging revealed preferential localization of EB within EPS-rich regions of intact biofilms, whereas selective enzymatic disruption of the glucan-rich matrix, particularly following glucanohydrolases treatment, markedly reduced EB retention. Consistently, EB-mediated bactericidal activity was attenuated in biofilms grown under glucan-deficient conditions, in which sucrose-dependent glucan-rich matrix formation was limited. Together, these findings support a matrix-dependent antimicrobial model in which glucan-rich biofilm matrix promotes EB localization and retention, thereby enhancing bactericidal activity in mature biofilms. This work challenges the conventional view of the biofilm matrix solely as a protective barrier and highlights its broader role as a functional determinant of antimicrobial performance in mature biofilms.

Biofilms represent highly structured microbial communities embedded within a self-produced extracellular polymeric substance (EPS) matrix that regulates their architecture, function, and resilience^{1–3}. This matrix provides mechanical stability while creating spatially heterogeneous microenvironments that regulate nutrient diffusion, metabolic activity, and tolerance to external stresses, including antimicrobial agents^{2–4}. Consequently, the EPS matrix has long been regarded as a primary barrier limiting antimicrobial efficacy, particularly in mature biofilms where its density and complexity are maximized^{3,5,6}. However, emerging evidence suggests that the matrix is not merely a passive protective scaffold but may actively influence the localization, retention, and functional activity of exogenous compounds within biofilm communities^{2,4,7}. Despite this, whether the EPS matrix can modulate or even potentiate antimicrobial efficacy remains poorly understood.

Dental caries is a biofilm-mediated disease driven by ecological shifts within dental plaque that favor acidogenic and aciduric microbial communities^{8–10}. Among cariogenic bacteria, *Streptococcus mutans* is a key

contributor owing to its ability to form structured biofilms, synthesize extracellular glucans from dietary sucrose, generate organic acids, and thrive under acidic conditions^{11,12}. The pathogenic potential of *S. mutans* biofilms is strongly influenced by dietary sucrose, which fuels acid production and drives the synthesis of EPS, particularly glucan-rich matrix components^{12–14}. These matrix structures promote bacterial adhesion, accumulation, and spatial organization, thereby supporting the development of dense, EPS-rich biofilms that sustain localized acidic microenvironments^{15–17}. Thus, mature *S. mutans* biofilms provide a relevant model for investigating how EPS-rich architecture shapes antimicrobial performance in established biofilm communities.

The EPS-rich architecture of mature biofilms presents a major challenge for conventional antimicrobial approaches by stabilizing embedded communities and limiting diffusion through the matrix^{3,6,7}. This restricted transport contributes to the increased antimicrobial tolerance of sessile bacteria compared with planktonic or developing populations^{5,6}. Although

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chlorhexidine (CHX) is widely used for chemical plaque control, its short-contact bactericidal performance can be limited in established EPS-rich biofilms, where matrix organization may restrict antimicrobial distribution and activity^{18–22}. These limitations highlight the need for short-contact antimicrobial strategies that operate within EPS-structured communities rather than relying solely on matrix penetration.

Erythrosine B (EB) is a xanthene dye widely used as a plaque-disclosing agent and has also been reported to exhibit antimicrobial activity, including as a photosensitizer in antimicrobial photodynamic therapy^{23–27}. However, despite its established clinical use and antimicrobial potential, the light-independent activity of EB within EPS-rich *S. mutans* biofilms remains poorly understood, particularly during brief exposures relevant to topical oral care applications²⁷. Given the central role of the EPS matrix in shaping biofilm architecture and antimicrobial tolerance, it also remains unclear whether matrix-associated interactions influence the localization and functional activity of EB within established biofilms relative to conventional antimicrobials such as CHX^{15,17}.

In this study, we tested whether EPS matrix integrity regulates EB localization and bactericidal activity in mature *S. mutans* biofilms under a single 10-min topical treatment. Using biofilms formed on saliva-coated hydroxyapatite disks, we evaluated the effect of EB on bacterial viability, glycolytic acid production, and spatial distribution across the biofilm, together with cytocompatibility in oral-derived cells. Matrix dependency was further examined using enzymatic disruption of the glucan matrix and glucan-deficient biofilm conditions. Our findings provide evidence for a matrix-dependent antimicrobial mechanism and offer insight into how biofilm EPS architecture can be leveraged to improve the efficacy of short-contact treatments against mature biofilms.

Results

EB compromises bacterial viability in mature *S. mutans* biofilms following a brief topical exposure

EB inhibited *S. mutans* growth in a dose-dependent manner under both planktonic (glucose) and biofilm-promoting (sucrose) conditions (Supplementary Fig. 1). Minimal effects were observed at low EB concentrations (0.00125–0.01 mg/mL), whereas 0.1 mg/mL significantly reduced bacterial accumulation and 1 mg/mL nearly inhibited bacterial growth, comparable to CHX. Under sucrose conditions, EB ≥ 0.1 mg/mL also markedly suppressed bacterial accumulation. This was accompanied by reduced biofilm biomass (Supplementary Fig. 1c), indicating that EB inhibits both planktonic proliferation and early biofilm formation in a 96-well plate assay. In a saliva-coated hydroxyapatite (sHA) disc model, a single 10-min topical exposure to EB (0.1 mg/mL) markedly reduced bacterial viability in pre-formed *S. mutans* biofilms (Fig. 1a). Confocal imaging analysis for live/dead cells (depicted in green and red, respectively) within the biofilms revealed predominantly viable cells in the vehicle-treated (phosphate buffer containing 20% (v/v) ethanol) control group, whereas EB-treated biofilms exhibited extensive red fluorescence distributed throughout the biofilm structure, indicating widespread membrane-compromised cells. In contrast, CHX-treated biofilms retained substantial green fluorescence after the 10-min treatment, indicating a more limited effect on membrane integrity than EB (Fig. 1b). Apparent overlap of green and red signals, particularly in CHX-treated biofilms, may reflect fluorescence originating from cells located at different depths within the biofilm and projected onto the same x-y plane; therefore, such overlap was not interpreted as dual staining of individual cells. Fluorescence-based quantification confirmed a significant decrease in the proportion of live cells following EB treatment compared with both control and CHX groups (Fig. 1c). Consistently, viable cell counting showed a multi-log reduction in viable bacteria within biofilm after EB exposure relative to the vehicle-treated control ($P < 0.05$), whereas CHX treatment did not show a significant reduction under the same conditions (Fig. 1d). In addition, single 10-min topical exposure to EB visibly disclosed the 24-h biofilm formed on sHA disks, consistent with its established role as a plaque-disclosing agent, whereas biofilms in the control and CHX groups were barely distinguishable under the same conditions (Fig. 1e). Collectively,

these data demonstrate that brief topical exposure to EB is sufficient to substantially compromise bacterial viability in mature *S. mutans* biofilms while enabling visual biofilm disclosure.

EB reduces the glycolytic acid production in mature *S. mutans* biofilms

Glycolytic pH-drop analysis of *S. mutans* biofilms (Fig. 2a) demonstrated rapid acidification in the vehicle-treated control, characterized by a steep initial decline followed by stabilization at a low pH plateau (Fig. 2b). EB reduced overall glycolytic acid production across all tested concentrations. During the first 30 min, representing the most active acidogenic phase, EB achieved 95.0%, 99.4%, and 99.9% inhibition of H⁺ production at 0.1, 1, and 10 mg/mL, respectively, relative to the control. Biofilms treated with 1 and 10 mg/mL EB maintained near-neutral pH values throughout the 120-min assay, while 0.1 mg/mL reduced acidification and sustained higher pH levels than the vehicle-treated biofilm.

CHX (0.12%) exhibited comparable early inhibition (99.8%) and a similarly stable pH trajectory, whereas NaF (250 ppm F[−]) showed limited reduction (23.5%) and an acidification profile closely resembling the control. Because EB treatment also markedly reduced viable bacterial recovery under the same single 10-min topical treatment, the diminished pH drop is interpreted as a reduction in the overall glycolytic acid production of the treated biofilm, rather than as evidence of reduced glycolytic activity on a per-cell basis. Biofilm-free sHA control subjected to the same EB treatment and glucose-challenge procedure showed no appreciable pH change, indicating that the observed effect was not attributable to residual EB in the measurement.

EPS matrix integrity governs Erythrosine B retention in *S. mutans* biofilms

To determine whether EB retention reflects biofilm-specific matrix properties, dye retention was quantified on HA disks across four surface conditions (Fig. 3a). EB was not detectably retained on plain HA disks and was retained only weakly on saliva-coated HA disks, indicating that neither the HA substrate nor the salivary pellicle substantially drove dye accumulation (Fig. 3b). In contrast, 24-h preformed *S. mutans* biofilms retained markedly higher levels of EB than saliva-coated HA disks ($P < 0.0001$; Fig. 3b). Enzymatic disruption of the glucan matrix with dextranase and mutanase significantly attenuated this retention ($P < 0.0001$; Fig. 3b), supporting a functional link between EPS matrix integrity and dye accumulation. These quantitative differences were corroborated by representative disc photographs, which showed intense pink staining on intact biofilms, markedly reduced staining following glucan-matrix disruption, and minimal staining on abiotic control surfaces (Fig. 3c). Collectively, these findings identify EPS matrix integrity as a key determinant of EB retention and support a matrix-associated mechanism of EB accumulation in mature *S. mutans* biofilms.

Glucan-rich matrix architecture contributes to EB localization and retention in mature biofilms

To determine whether the EPS matrix influences EB distribution within mature biofilms, confocal imaging was performed in 24-h-old *S. mutans* biofilms (Fig. 4a). In biofilms with intact-EPS architecture, EB-associated fluorescence was extensively distributed throughout matrix-rich regions surrounding bacterial microcolonies, appearing integrated within the extracellular scaffold rather than confined to discrete cellular compartments. In contrast, enzymatic degradation of the glucan-rich matrix markedly reduced EB-associated fluorescence and disrupted its spatial continuity across the biofilm structure. Quantitative image analysis further confirmed a significant decrease in mean EB fluorescence intensity following matrix disruption compared with intact biofilms ($P < 0.05$) (Fig. 4b and Supplementary Fig. 2). Independent EPS staining verified effective matrix degradation, as enzyme-treated biofilms exhibited a profound reduction in EPS fluorescence relative to control and intact groups ($P < 0.0001$), while no difference was observed between vehicle-treated and intact conditions (Fig. 4c). Together, these findings indicate that the

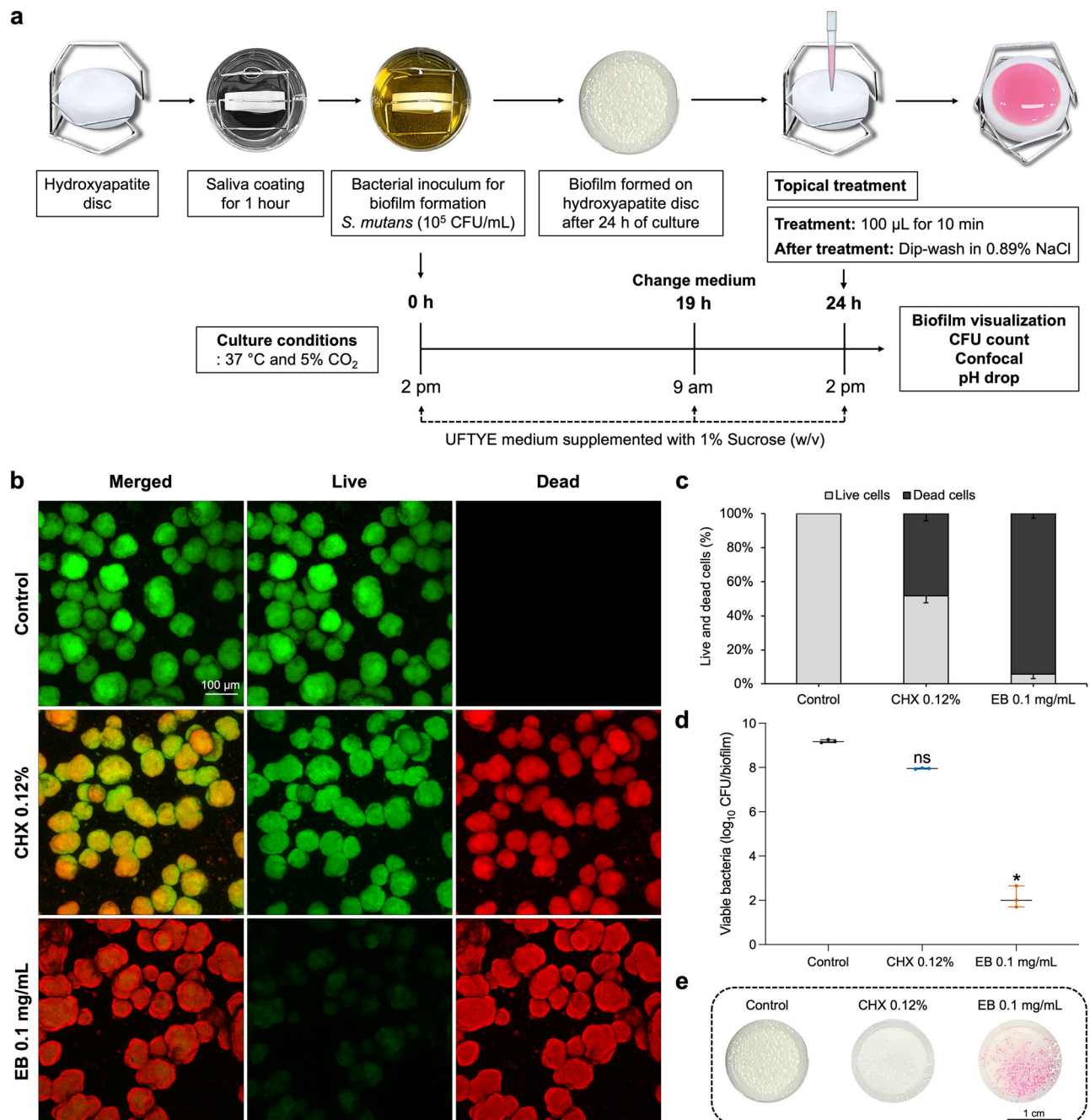


Fig. 1 | Single-exposure erythrosine B reduces the viability of mature *S. mutans* biofilms. Preformed 24-h *S. mutans* biofilms on sHA disks were treated once with EB (0.1 mg/mL), (CHX 0.12%), or the corresponding vehicle control for 10 min.

a Experimental scheme for single topical treatment. **b** Representative confocal z-stack projections of biofilms stained with SYTO9 and propidium iodide (PI), showing viable-cell-associated fluorescence (green) and membrane-compromised-cell-associated fluorescence (red). Scale bar = 100 µm. **c** Relative proportions of SYTO9 and PI-associated bacterial biovolume quantified from the corresponding

confocal image stacks. **d** Viable biofilm-associated bacteria determined by CFU enumeration. CFU values are expressed as log₁₀ CFU/biofilm and are shown as individual biological replicates with the median and interquartile range ($n = 3$). Statistical significance was assessed using the Kruskal–Wallis test followed by Dunn’s multiple-comparisons test, with each treatment group compared with the vehicle-treated control. ns not significant; * $P < 0.05$. **e** Biofilm visualization of 24-h *S. mutans* biofilms on sHA disks after a single 10-min treatment with vehicle control, CHX (0.12%), or EB (0.1 mg/mL). Scale bar = 1 cm.

organization and integrity of the glucan-rich matrix influence the post-wash distribution of EB within mature biofilms. To further evaluate the spatial relationship between EB and EPS signals, higher-magnification confocal z-stacks were analyzed using Coloc 2. EB and EPS fluorescence showed positive spatial association (Supplementary Table 1), with Pearson’s correlation coefficient of 0.672 ± 0.073 . Costes-thresholded Manders’ coefficients were $tM1 = 0.804 \pm 0.087$ and $tM2 = 0.794 \pm 0.083$, further supporting EB localization within EPS-rich biofilm regions.

Time-dependent confocal imaging further showed that EB-associated fluorescence was weak after 1 min of EB exposure but increased after 5 and 10 min, indicating progressive EB accumulation within biofilm during exposure (Supplementary Fig. 3). Consistent with this imaging pattern, CFU analysis showed exposure-time-dependent bacterial killing, with the 10-min EB exposure producing the strongest reduction in viable counts under the tested conditions ($P < 0.0001$) (Supplementary Fig. 4). Together, these findings support EPS-associated EB localization and retention, and antimicrobial activity in mature *S. mutans* biofilms.

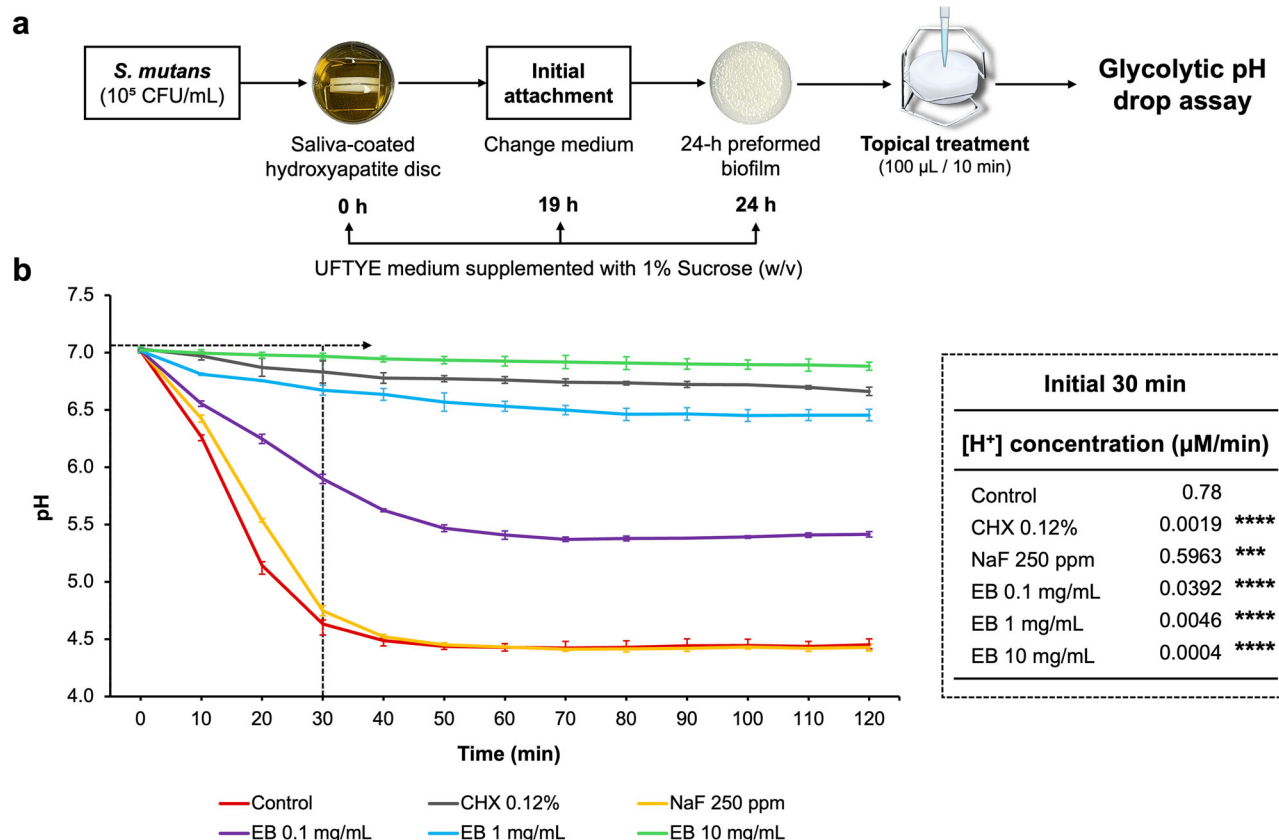


Fig. 2 | A single 10-min topical exposure to erythrosine B reduces the glycolytic acid production capacity of mature *S. mutans* biofilms. **a** Experimental workflow for glycolytic pH-drop assay. Preformed 24-h *S. mutans* biofilms on sHA disks were subjected to a single topical treatment with EB (0.1, 1, or 10 mg/mL), CHX (0.12%), or NaF (250 ppm F⁻), or the corresponding vehicle control for 10 min. Following treatment, the biofilms were washed, depleted of carbohydrate reserves, and

subjected to glucose challenge. **b** Biofilm pH profiles recorded over 120 min following glucose addition, and the corresponding net H⁺ production rates calculated during the initial 30 min of the assay. Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple-comparisons test, comparing each treatment group with the vehicle-treated control *** $P < 0.001$; **** $P < 0.0001$.

Glucan-rich matrix structures contribute to EB retention

To further define the matrix components contributing to EB retention, mature *S. mutans* biofilms were treated with proteinase K, dextranase and/or mutanase prior to EB exposure (Fig. 5a). DAPI associated fluorescence showed relatively limited variation in bacterial biomass among the treatment groups (Fig. 5b). In contrast, EB-associated fluorescence decreased after glucanohydrolase treatment, with stronger reduction observed after mutanase or combined dextranase-mutanase treatment (Fig. 5c). Proteinase K treatment did not completely abolish EB-associated fluorescence, suggesting that proteinase-degradable matrix components are not the sole determinant of EB retention. Biofilms treated with the heat-inactivated dextranase-mutanase retained EB-associated fluorescence comparable to that of the EB-treated control, indicating that the reduction observed with the active enzyme mixture was attributable to enzymatic matrix disruption rather than nonspecific effects of the enzyme treatments (Fig. 5c).

Consistent with these changes, EPS-associated fluorescence was markedly reduced following active glucanohydrolase treatment, confirming disruption of the glucan-rich matrix (Fig. 5d). DAPI-normalized EB fluorescence analysis showed that the reduction in EB signal was not dependent on loss of bacterial biomass (Fig. 5e). Because mutanase targets α -1,3-glucan-rich, relatively water-insoluble matrix structure, these findings suggest that mutanase-degradable α -1,3-glucan-rich matrix contributes substantially to EB retention. Neither the sodium acetate (NaOAc) nor the phosphate-buffered saline (PBS)-only control significantly altered EPS-associated fluorescence compared with the intact control (Supplementary Fig. 5).

Bactericidal activity under distinct matrix and growth conditions

Bacterial survival was quantified by CFU enumeration under distinct biofilm matrix and growth conditions, with biofilms receiving the corresponding vehicle (20% ethanol in PBS) for 10 min serving as controls (Fig. 6a). In biofilms formed in 1% sucrose, exposure to EB (0.1 mg/mL) resulted in a significant multi-log reduction in CFU compared with the corresponding buffer-treated control (** $P < 0.01$; Fig. 6b). In contrast, when the glucan-rich matrix was enzymatically disrupted prior to EB exposure, CFU counts following treatment were not significantly different from those of the control group (Fig. 6c), indicating marked attenuation of EB-mediated killing following matrix disruption.

EB-mediated killing was also attenuated in glucose/fructose-grown biofilms, in which formation of the sucrose-dependent glucan-rich matrix is limited; EB treatment did not significantly reduce viable counts relative to the corresponding control under these growth conditions (Fig. 6d). Because glucose/fructose-grown biofilms differ from sucrose-grown biofilms in both matrix development and carbohydrate-dependent bacterial physiology, this comparison cannot attribute the difference in EB susceptibility to matrix abundance as the sole determinant of EB susceptibility. Rather, together with the enzymatic matrix-disruption experiment, these findings support an association between glucan-rich biofilm architecture and the bactericidal activity of EB. To determine whether the influence of glucan-rich matrix on antimicrobial activity was specific to EB, CHX was evaluated under the same biofilm matrix conditions. In contrast to EB, CHX showed greater bactericidal activity following disruption of the glucan-rich matrix and in glucose/fructose-grown biofilms (Supplementary Fig. 6).

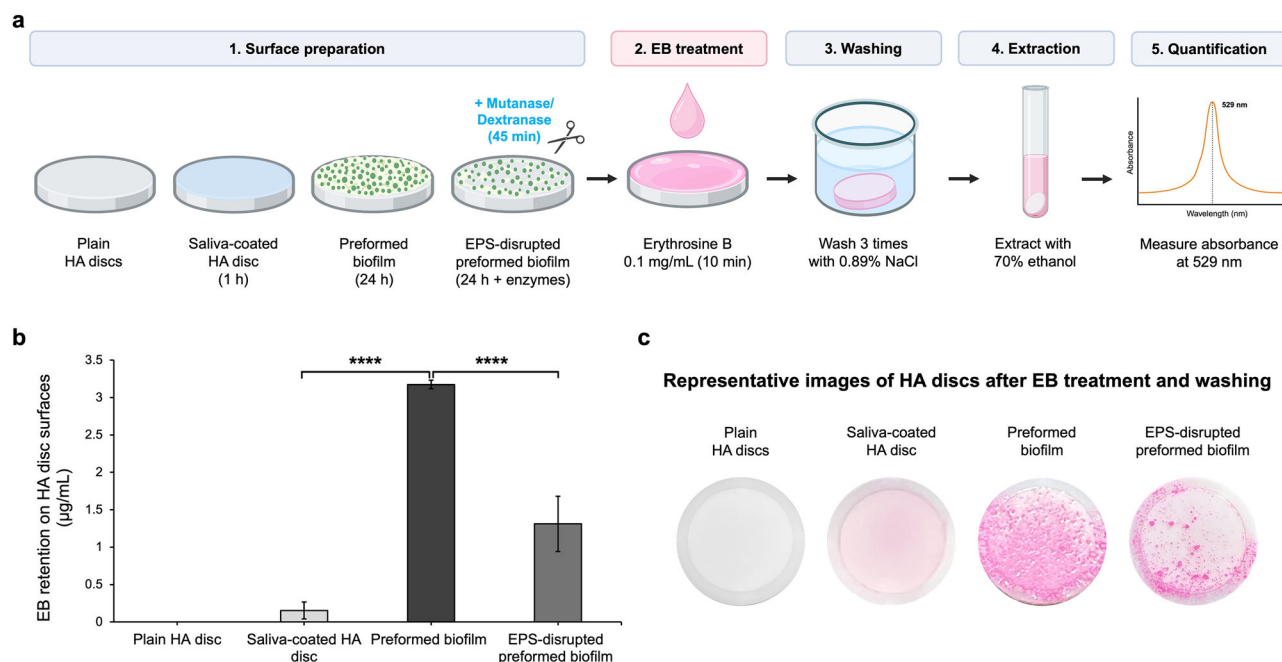


Fig. 3 | Erythrosine B retention is enhanced by the intact biofilm matrix.

a Schematic overview of the EB retention assay applied to four HA disc surface conditions: plain HA discs, saliva-coated HA discs, 24-h preformed *S. mutans* biofilms, and EPS-disrupted preformed biofilms. Created in BioRender. Kim (2026) <https://BioRender.com/456sx26>. **b** Quantification of EB retained on HA disc

surfaces following EB treatment and washing with 0.89% NaCl. Data are presented as mean $\pm$ SD from three independent experiments ($n = 3$). Statistical significance was determined by one-way ANOVA followed by Šidák's multiple-comparisons test for selected preplanned comparisons. **** $P < 0.0001$. **c** Representative photographs of HA discs after EB treatment and washing under each surface condition.

Quantitative imaging assessment of matrix-dependent bactericidal activity

To further determine whether glucan-rich matrix integrity influences EB-mediated bactericidal efficacy, *S. mutans* biofilms were treated with EB (0.1 mg/mL) under intact or enzymatically disrupted matrix conditions, and the results were compared with the corresponding vehicle-treated controls (Fig. 7a). In control biofilms, SYTO9-associated green fluorescence predominated, consistent with a high proportion of membrane-intact cells within the established biofilm architecture. Following EB exposure, biofilms with an intact-EPS matrix exhibited extensive propidium iodide (PI)-associated red fluorescence throughout the biofilm structure (Fig. 7b), together with a marked reduction in the viable-cell-associated signal, consistent with pronounced membrane damage.

In contrast, prior enzymatic disruption of the glucan-rich matrix substantially attenuated this effect. Matrix-disrupted biofilms retained a much higher proportion of viable-cell-associated fluorescence following EB exposure than biofilms with an intact matrix. Quantitative analysis showed that total bacterial biovolume remained comparable among the control, EB-treated intact matrix, and matrix-disrupted groups. However, total biofilm biovolume and the EPS-to-bacterial biovolume ratio were markedly reduced following dextranase-mutanase treatment (Fig. 7c–e). Consistent with these findings, supplementary analysis showed reductions in EPS biovolume, EPS thickness, and overall biofilm thickness after matrix disruption, while bacterial thickness remained relatively preserved (Supplementary Fig. 7). Thus, enzymatic treatment primarily altered the matrix-rich structural components rather than producing a comparable loss of bacterial biomass. Despite this preservation of the bacterial biomass, matrix-disrupted biofilms showed a higher viable bacterial fraction after EB treatment than biofilms with an intact matrix (Fig. 7f), indicating reduced EB-mediated killing. Collectively, these imaging findings support the contribution of glucan-rich matrix integrity to the full bactericidal activity of EB in mature *S. mutans* biofilms.

Enzyme treatment alone did not appreciably alter the LIVE/DEAD fluorescence profile compared with the corresponding control, indicating that glucanohydrolase treatment under these conditions did not produce

substantial bacterial membrane damage (Supplementary Fig. 8). LIVE/DEAD confocal imaging further supported the contrasting effects of glucan-rich matrix disruption on EB and CHX activity. In CHX-treated biofilms, glucan-matrix disruption increased PI-associated fluorescence and a corresponding reduction in SYTO9-associated signal compared with the biofilms with intact matrix (Supplementary Fig. 8). The accompanying loss of EPS-associated fluorescence confirmed disruption of the glucan-rich matrix. Together, these observations indicate that glucan-rich matrix disruption attenuates EB-mediated killing but enhances CHX-mediated killing, supporting an agent-dependent influence of biofilm matrix architecture on antimicrobial activity.

Cytocompatibility of erythrosine B in oral-derived cell lines

To assess the cytocompatibility of EB at the tested concentration, human gingival fibroblasts and human oral keratinocytes were exposed to EB (0.1 mg/mL) for 24 or 48 h, with CHX included as a positive control. EB did not reduce metabolic activity in the tested cell lines at either time point compared with vehicle-treated controls (Supplementary Fig. 9). In contrast, CHX significantly decreased metabolic activity at both tested concentrations and at both time points in both cell types. Because these conditions represent prolonged exposure rather than topical application, they were considered to be a conservative assessment of cytocompatibility.

Immediately following a single 10-min exposure, the metabolic activity of EB-treated cells remained comparable to that of the vehicle-treated control, whereas both concentrations of CHX markedly reduced cellular metabolic activity. A similar pattern was observed during the subsequent recovery period, with EB-treated cells maintaining metabolic activity comparable to the control, while substantial reductions persisted following CHX exposure (Fig. 8a, b).

LIVE/DEAD confocal imaging performed immediately after the 10-min exposure provided an independent assessment of cell viability (Fig. 8c, d). Vehicle-control and EB-treated cells showed predominantly calcein-positive fluorescence, with little to no detectable ethidium homodimer-1 (EthD-1)-positive membrane-compromised cells. In contrast, cells exposed to either 0.012% or 0.12% CHX exhibited

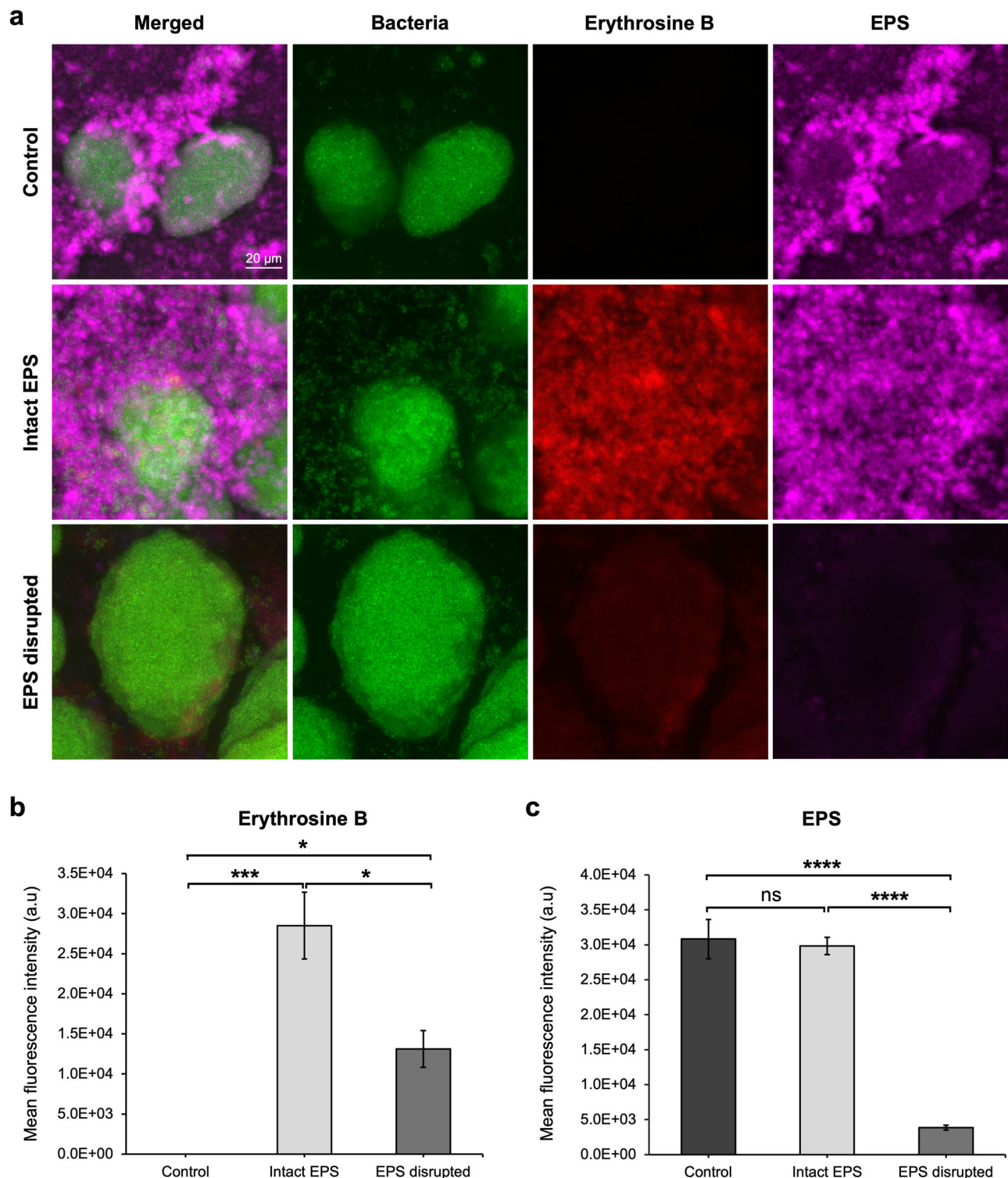


Fig. 4 | Glucan-rich matrix architecture contributes to erythrosine B localization and retention in mature *S. mutans* biofilms. **a** Representative confocal 3D reconstructions generated from z-stacks of vehicle-treated control biofilms, EB-treated biofilms with an intact matrix, and biofilms subjected to dextranase-mutanase matrix disruption. DAPI-stained bacterial biomass is shown in green (pseudocolor), the Alexa Fluor 647-dextran-labeled glucan-rich EPS matrix in magenta, and EB-associated fluorescence in red. Scale bar, 20 μ m. **b** Quantification of mean EB-associated fluorescence intensity. **c** Quantification of mean EPS fluorescence intensity. Data are presented as mean $\pm$ SD ($n = 3$ independent biological

replicates). Quantitative EB-EPS spatial association was additionally assessed from confocal z-stacks using Pearson's correlation coefficient and Costes-thresholded Manders' coefficients. EB was assigned as Channel 1 and EPS as Channel 2; therefore, tM1 represents the EB fluorescence overlapping EPS-positive regions, whereas tM2 represents the EPS fluorescence overlapping EB-positive regions (Supplementary Table 1). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparisons test. ns not significant; * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$.

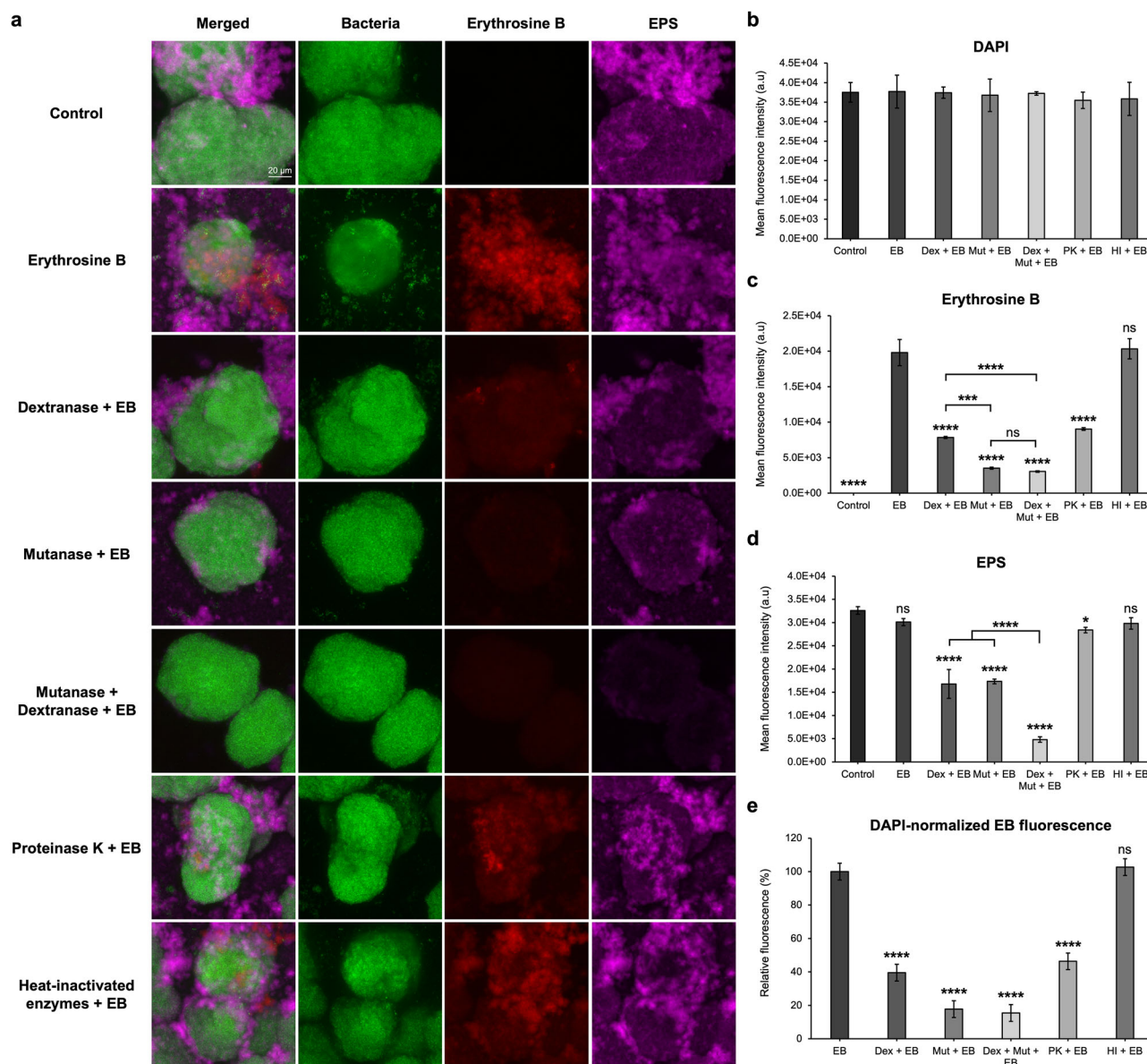


Fig. 5 | Selective disruption of glucan-rich matrix architecture reduces erythrosine B retention in mature *S. mutans* biofilms. **a** Representative confocal 3D reconstruction generated from z-stacks acquired at 5× scan zoom. Mature 24-h *S. mutans* biofilms were subjected to vehicle control, EB alone, dextranase, mutanase, combined dextranase-mutanase, proteinase K, and heat-inactivated dextranase and mutanase. Following enzymatic pretreatment, biofilms were exposed to EB (0.1 mg/mL) for 10 min. Biofilms were subsequently washed under standardized conditions to remove unbound EB. DAPI-stained bacterial biomass is shown in green (pseudocolor), EB-associated fluorescence in red, and Alexa Fluor 647-dextran labeled glucan-rich EPS matrix in magenta. Scale bar, 20 μm. **b–e** Quantitative analysis was performed using independently acquired full-field confocal stacks. Mean fluorescence intensity (arbitrary units, a.u.) was quantified for **b** DAPI-labeled bacteria, **c** EB-associated fluorescence, and **d** EPS-associated fluorescence. **e** EB-associated

fluorescence was normalized to the corresponding DAPI fluorescence for each biological replicate and expressed relative to the EB-treated intact-matrix group, which was set to 100%. Data are presented as mean ± SD ($n = 3$ independent biological replicates). Differences among groups were assessed using one-way ANOVA, followed by multiple-comparisons tests. For DAPI and EPS-associated fluorescence, Dunnett's test was used to compare each treatment group with the vehicle-treated control. For EB-associated fluorescence and DAPI-normalized EB fluorescence, Dunnett's test was used to compare each group with the EB-treated intact-matrix group. Šidák's multiple-comparisons test was used for pairwise comparisons between the three glucanohydrolase-treated groups for EB fluorescence and for comparisons of the glucanohydrolase combined treatment with each single-glucanohydrolase enzyme treatment for EPS fluorescence. ns not significant; * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$.

extensive EthD-1-positive staining with a marked loss of viable-cell-associated fluorescence, indicating pronounced membrane damage. Together, the metabolic and membrane integrity-based assessments showed that EB did not measurably compromise oral cell viability under the tested conditions, whereas CHX exhibited substantial cytotoxicity at the test concentrations.

Discussion

This study identifies a matrix-dependent bactericidal mechanism in mature *S. mutans* biofilms, in which glucan-rich biofilm architecture governs EB localization, retention, and antimicrobial activity. Following a single 10-min topical exposure and washing, EB markedly reduced recoverable viable bacteria, induced widespread membrane damage, and suppressed glycolytic acidification in preformed biofilms. The time-course results further showed that EB localization and bactericidal killing were exposure-time-dependent, supporting the use of the 10-min condition as an experimentally defined

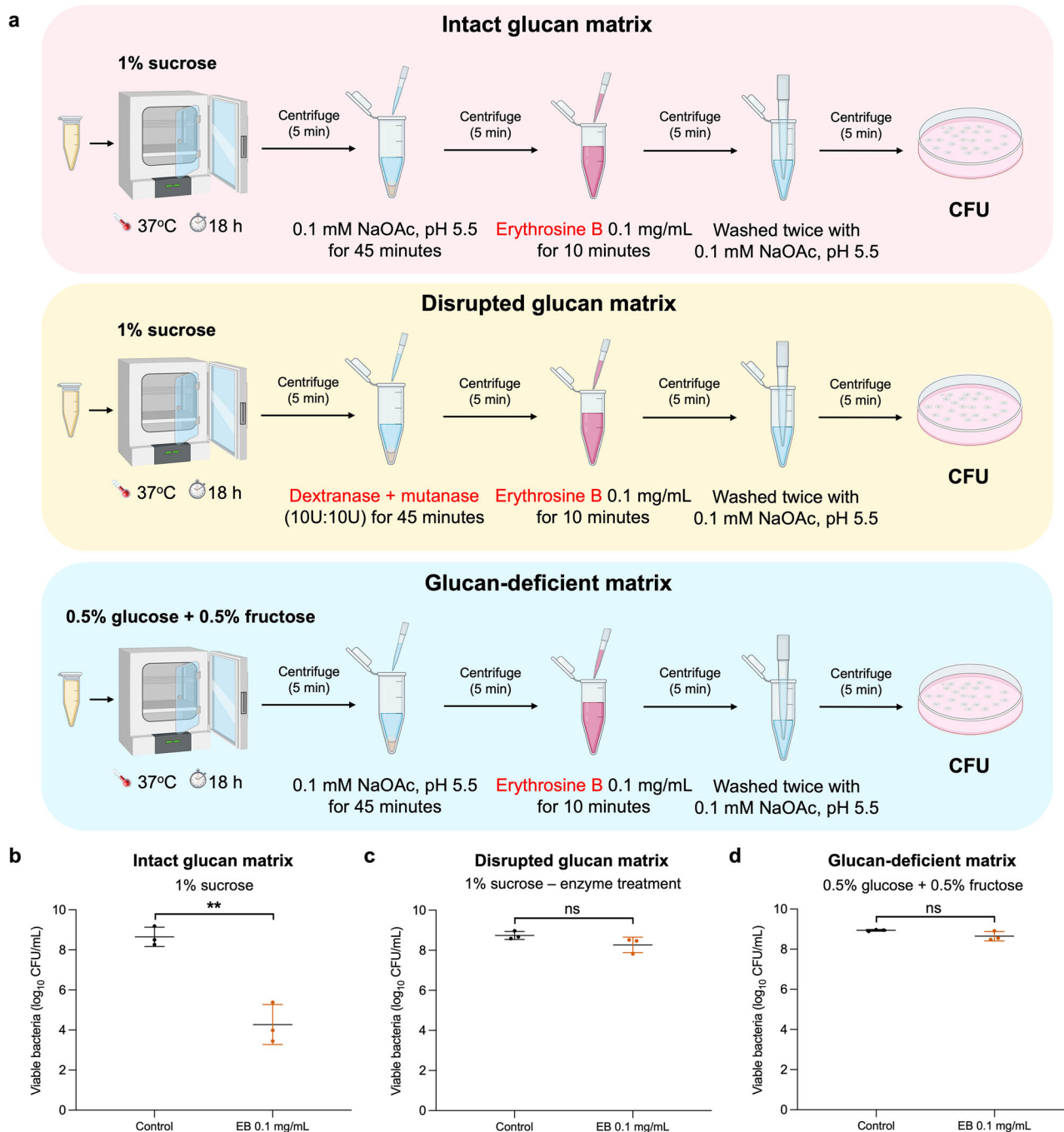


Fig. 6 | Influence of biofilm matrix integrity and growth conditions on the bactericidal activity of erythrosine B. a Experimental scheme illustrating 18-h biofilm formation under three EPS conditions: sucrose-grown biofilms with an intact glucan-rich matrix (1% sucrose), sucrose-grown biofilms subjected to enzymatic matrix disruption, and glucose/fructose-grown biofilms with limited formation of the sucrose-dependent glucan-rich matrix. Biofilms were subsequently exposed to EB (0.1 mg/mL) or the corresponding vehicle control for 10 min before CFU enumeration. Created in BioRender. Kim (2026) <https://BioRender.com/>

bm7rp8j. **b** Recoverable viable bacteria in sucrose-grown biofilms with an intact matrix. **c** Recoverable viable bacteria after enzymatic disruption of the glucan-rich matrix. **d** Recoverable viable bacteria in glucose/fructose-grown biofilms. Data are shown as individual biological replicates with mean $\pm$ SD ($n = 3$). CFU values were $\log_{10}$ -transformed prior to statistical analysis. Within each experimental condition, vehicle-treated and EB-treated biofilms were compared using an unpaired two-tailed Student's *t*-test. ns not significant; ** $P < 0.01$.

exposure period that enabled robust EB accumulation or retention alongside antimicrobial activity under the tested conditions.

Mature biofilms are generally less susceptible to antimicrobial treatment, because the extracellular matrix can restrict agent penetration and promote physiological heterogeneity, a phenomenon observed across antibacterial and antifungal biofilm systems^{5,6,28–31}. Consistent with this biofilm-associated tolerance, CHX exhibited limited bactericidal activity

against mature *S. mutans* biofilms under single 10-min topical exposure^{15,20–22}. In contrast, EB produced a substantial reduction in recoverable viable bacteria and markedly reduced the overall acidification capacity of mature biofilms, a key virulence trait associated with *S. mutans* cariogenicity^{12,32}. Because EB treatment also substantially reduced viable bacterial counts, the attenuated pH drop likely reflects the reduction in viable bacterial biomass rather than a specific inhibition of glycolytic activity

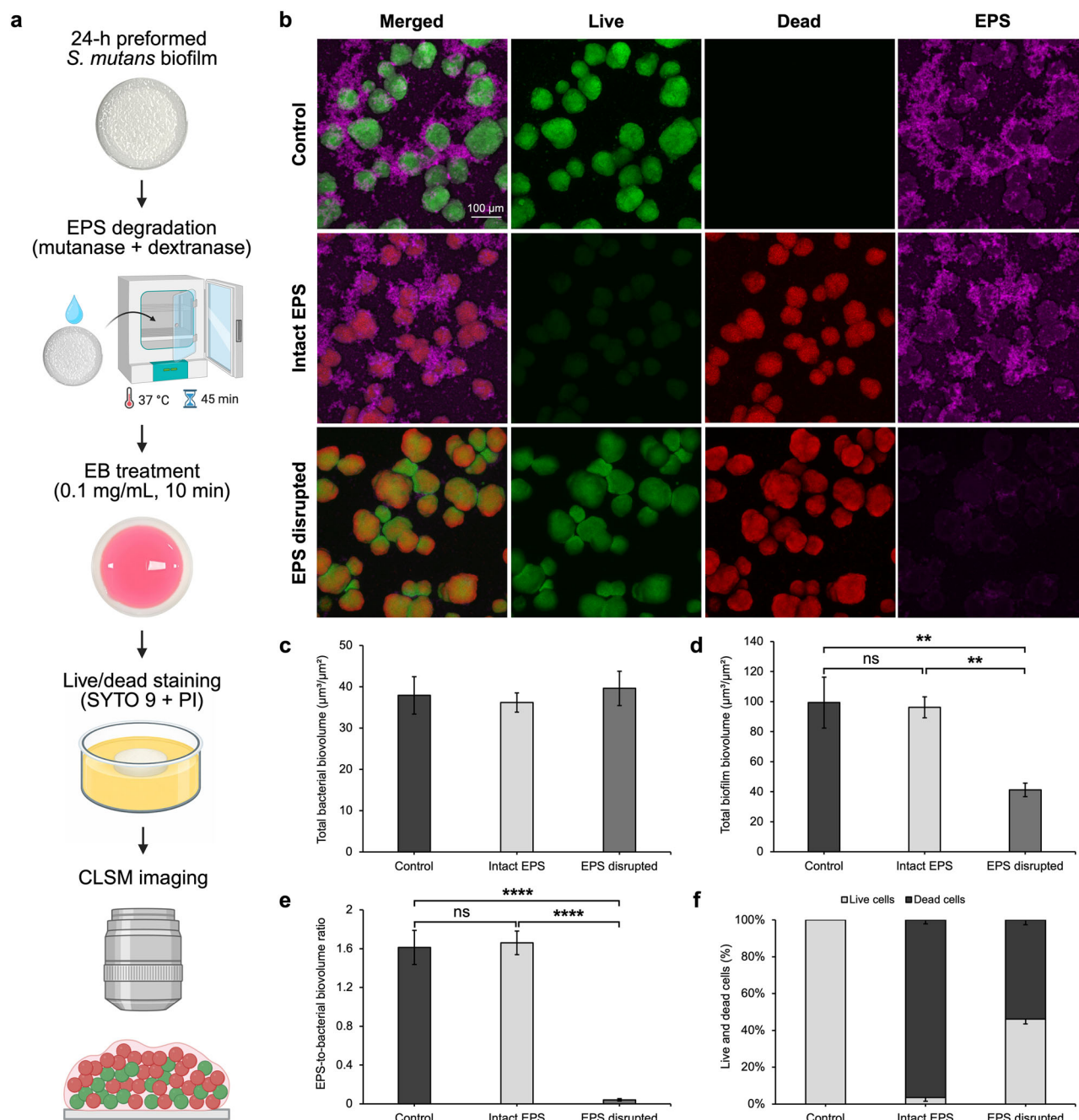


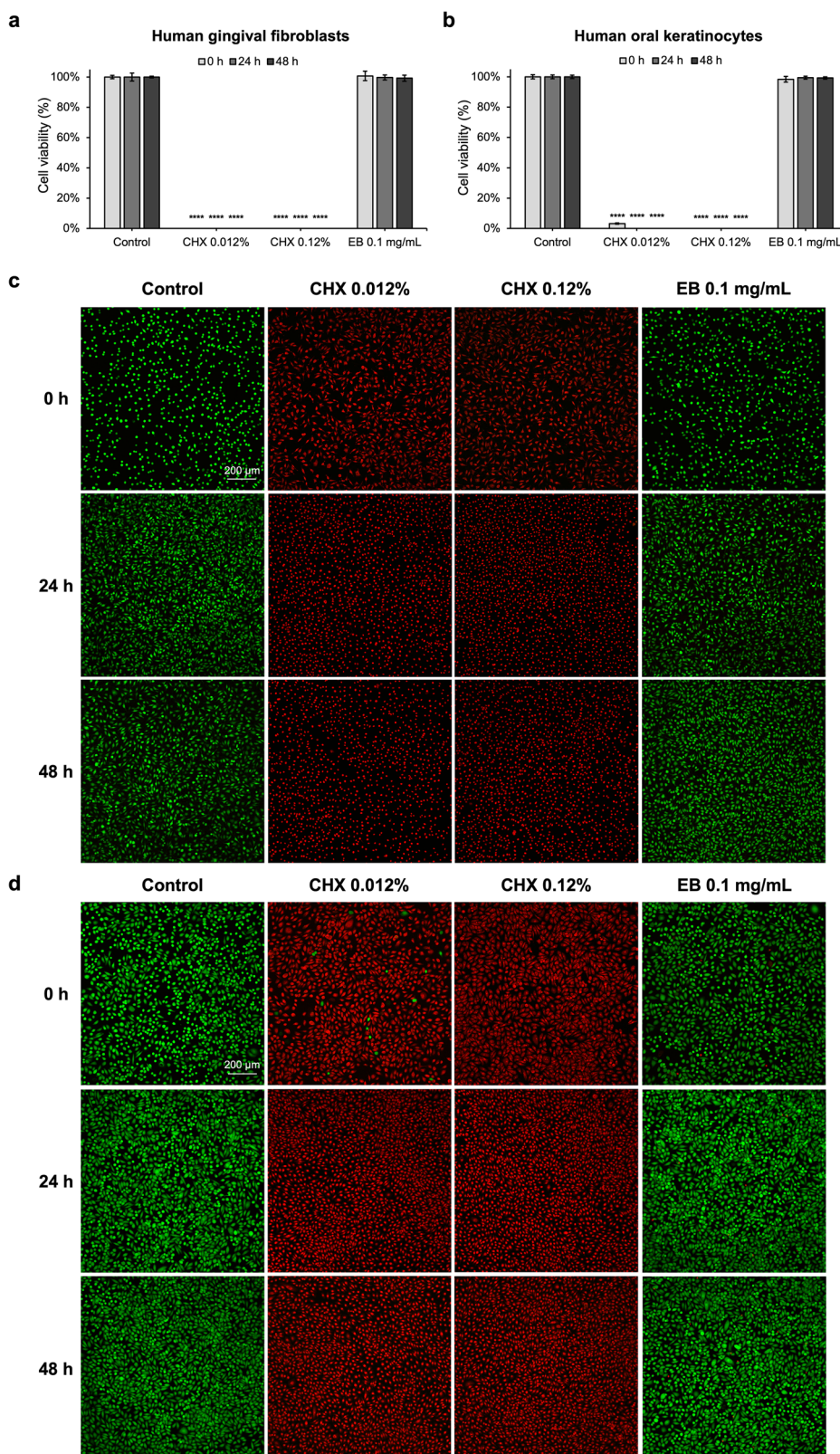
Fig. 7 | Glucan-rich EPS matrix disruption attenuates erythrosine B-mediated killing in *S. mutans* biofilms. **a** Experimental scheme illustrating how EPS disruption modulated EB-mediated killing in preformed 24-h *S. mutans* biofilms. For EPS disruption, biofilms were incubated with mutanase and dextranase in 0.1 mM NaOAc buffer (pH 5.5) at 37 °C for 45 min, while non-enzyme-treated biofilms received the same buffer alone under identical conditions. The intact-EPS and EPS-disrupted biofilms were then exposed to EB (0.1 mg/mL) for 10 min, whereas the control biofilms remained untreated, followed by LIVE/DEAD staining and confocal imaging. Created in BioRender. Kim (2026) <https://BioRender.com/y4wqc6f>. **b** Representative confocal z-stack projections showing viable-cell-associated fluorescence (SYTO9, green), membrane-compromised-cell-associated fluorescence (PI, red), and Alexa Fluor 647-dextran labeled glucan-rich matrix (magenta) in vehicle-

treated control, EB-treated intact-matrix, and EB-treated matrix-disrupted biofilms. Scale bar = 100 μm . **c** Total bacterial biovolume calculated from SYTO9- and PI-signals, respectively. **d** Total biofilm biovolume. **e** EPS-to-bacterial biovolume ratio, calculated by normalizing EPS biovolume to the corresponding bacterial biovolume. **f** Relative proportions of SYTO9- and PI-associated bacterial biovolume, expressed as percentages of total bacterial biovolume. Data are presented as mean $\pm$ SD of three independent biological replicates ($n = 3$). Differences among control, intact EPS, and EPS-disrupted groups were analyzed by one-way ANOVA followed by Tukey's multiple-comparisons test. ns not significant, ** $P < 0.01$, **** $P < 0.0001$. Statistical annotations are not shown for panels in which none of the pairwise comparisons reached statistical significance.

on a per-cell basis. These findings therefore demonstrate that EB simultaneously reduces bacterial survival and the overall acidogenic output of the treated biofilm, supporting the importance of assessing both viability and function-based endpoints when evaluating antibiofilm efficacy^{33,34}.

A central finding of this study is that the glucan-rich biofilm matrix influenced EB localization, retention, and antimicrobial activity. This observation should not be interpreted as evidence that EB diffuses more efficiently than CHX. Rather, our finding supports a model in which EB enters the hydrated biofilm matrix during topical exposure and

Fig. 8 | Cytocompatibility of erythrosine B following a single 10-min exposure in human oral cells. Human gingival fibroblasts and human oral keratinocytes were exposed to EB (0.1 mg/mL), CHX (0.012% or 0.12%), or the corresponding vehicle control for 10 min, followed by removal of the treatment solution, washing, and recovery in fresh culture medium. MTT-based metabolic activity of human gingival fibroblasts (a) and human oral keratinocytes (b) immediately after treatment and after 24 and 48 h of recovery. MTT-based metabolic activity was expressed relative to the corresponding vehicle-treated control. Cell viability was normalized to the corresponding control at each time point and expressed as a percentage. Data are presented as mean $\pm$ SD ($n = 4$). Statistical significance was assessed by two-way ANOVA followed by Dunnett's multiple-comparisons test, comparing each treatment group with the corresponding control at each time point (0, 24, and 48 h). **** $P < 0.0001$. Representative LIVE/DEAD confocal images of human gingival fibroblasts (c) and human oral keratinocytes (d) immediately after the 10-min exposure (0 h) and after 24 and 48 h of recovery. Viable cells are shown by Calcein-AM-associated green fluorescence, whereas membrane-compromised cells are shown by ethidium homodimer-1-associated red fluorescence. Scale bar = 200 μ m. Data are presented as mean $\pm$ SD ($n = 4$ independent biological replicates).



subsequently remains associated with EPS-rich microenvironments after removal of the external treatment solution. This distinction between accumulation and post-wash retention is important because a compound may enter the biofilm but be rapidly removed during washing, whereas another compound may remain locally associated with matrix-rich domains and thereby maintain prolonged contact with embedded bacterial cells.

The time-course experiments support this interpretation. EB-associated fluorescence increased progressively with exposure time, consistent with time-dependent accumulation within the biofilm, while the persistence of fluorescence after washing indicated post-exposure retention. In parallel, the CFU data showed exposure-time-dependent reduction in recoverable viable bacteria, with the 10-min condition producing the

greatest bacterial effect under the tested conditions. Together, these findings establish a functional association between progressive EB accumulation or retention within the biofilm and increased antimicrobial activity. Importantly, these effects were strongly influenced by biofilm matrix integrity. Enzymatic disruption of the glucan-rich matrix substantially reduced EB accumulation and antimicrobial activity. Consistent with this interpretation, EB-mediated killing was also attenuated in glucose/fructose-grown non-sucrose biofilm, where sucrose-dependent glucan-rich matrix is limited. Together, these findings suggest that the structural glucan-rich matrix can influence antimicrobial performance of matrix-targeting agents, extending the conventional view of the biofilm matrix as solely a barrier to antimicrobial penetration (Fig. 9)^{2,3,28}.

The selective enzymatic matrix-disruption experiments suggest that EB retention depends on the organization of the glucan-rich matrix rather than on total biofilm biomass or protein-containing matrix components alone. In *S. mutans* biofilms, α -1,3-glucan-rich, relatively water-insoluble matrix contributes to the rigid EPS scaffold that stabilizes microcolonies and creates matrix-rich domains¹⁵. Disruption of this insoluble glucan-rich architecture would be expected to reduce physical confinement, increase dye washout, and weaken local EB residence within the matrix. Therefore, the reduced EB signal after mutanase-mediated matrix disruption is best interpreted as evidence that α -1,3-glucan-rich matrix integrity contributes to EB retention. However, this does not establish direct binding of EB to α -1,3-glucan or identify a specific molecular binding partner, as enzymatic degradation may also alter matrix porosity, biofilm architecture, and associated matrix components¹⁵. Rather, the data support a matrix-associated accumulation model in which the chemically structured and spatially organized glucan-rich environment contributes to the distribution and persistence of exogenous compounds within mature biofilms^{2,4,35}. It is also possible that confinement within the matrix alters the local molecular environment or aggregation state of EB, which could influence its antimicrobial activity. However, changes in these properties of EB with EPS were not examined in the present study, and this possibility therefore remains to be tested experimentally.

The distinct behavior of EB and CHX may be partly explained by differences in their physicochemical properties. CHX is a cationic amphiphilic bisbiguanide and may interact strongly with negatively charged

bacterial surfaces and matrix components³⁶. Such electrostatic interactions may promote local association and sequestration, potentially limiting distribution within the hydrated matrix. In contrast, EB is an anionic xanthene dye, widely used as a plaque-disclosing compound and antimicrobial photosensitizer^{24,37}. Its anionic character may result in interactions with bacterial surfaces and matrix components that differ from those of cationic CHX, potentially contributing to its distinct distribution within the biofilm. Following its accumulation within the matrix, the aromatic and iodinated structures of EB may support weak, reversible non-electrostatic interactions with organic matrix components, thereby promoting post-wash retention. CHX was used as a functional comparator in the viability and glycolytic pH-drop experiments, but CHX localization and retention were not directly measured in the mechanistic imaging experiments. Therefore, although the distinct responses of EB and CHX to glucan-matrix disruption are experimentally supported, the physicochemical mechanisms underlying these differences remain a working hypothesis based on their known molecular properties and reported mode of action³⁶.

The contrasting response of CHX further highlights an agent-dependent role of the biofilm matrix in antimicrobial activity. Glucan-matrix disruption increased CHX-mediated killing, consistent with its conventional role as a protective barrier, whereas the same disruption reduced EB retention and bactericidal activity. These findings suggest an alternative strategy in which matrix-retentive antimicrobials exploit, rather than overcome, glucan-rich biofilm architecture to enhance local antimicrobial activity. Such an approach may be particularly relevant to mature, cariogenic biofilms, where abundant glucan-rich matrix contributes to biofilm persistence and antimicrobial tolerance.

These observations have broader implications for the conventional view of the biofilm matrix in antimicrobial therapy. The dominant paradigm in antibiofilm therapy has focused on disrupting, degrading, or bypassing the EPS matrix to improve antimicrobial penetration^{30,38}. Accordingly, matrix-degrading enzymes, such as dextranase and mutanase, have been explored to destabilize glucan-rich biofilm architecture and improve antimicrobial access to embedded cells^{30,38–40}. While these approaches remain important for weakening established biofilms, the present findings suggest an additional and conceptually distinct role for the matrix. Under appropriate physicochemical conditions, an organized EPS

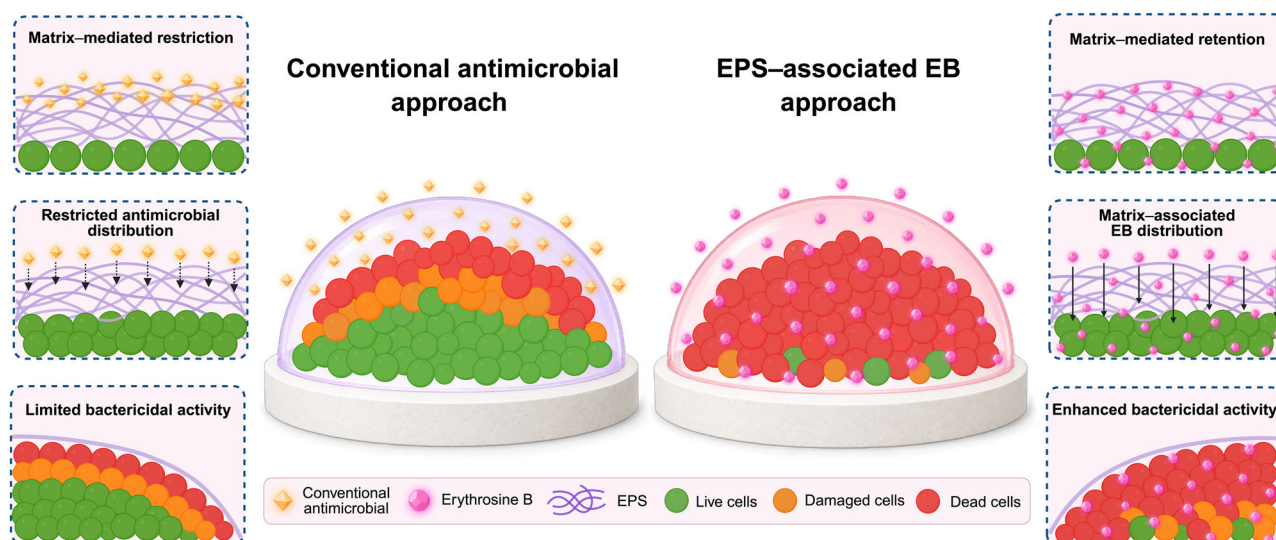


Fig. 9 | Conceptual model illustrating the dual role of the biofilm matrix in antimicrobial activity. The conventional barrier-dominated view depicts the EPS matrix as restricting antimicrobial distribution and limiting exposure of embedded bacteria. In contrast, the findings of the present study support an additional matrix-associated retention model for erythrosine B (EB), in which EB localizes within EPS-

rich regions and remains associated with the biofilm after washing. This post-wash retention is associated with greater bactericidal activity under the tested conditions. The schematic represents a working model and does not imply direct molecular binding between EB and an EPS component. Figure created in BioRender. Kim (2026) <https://BioRender.com/ww99ja>.

matrix may also promote the local retention, thereby enhancing localized activity. The matrix, therefore, acts not solely as a diffusion barrier, but also as a microenvironment that influences the spatial persistence and functional performance of exogenous compounds (Fig. 9). In this context, matrix–compound compatibility may represent a potentially important parameter in the development of short-contact antimicrobial strategies for EPS-rich biofilm communities.

The EB regimen used here should also be considered in the context of its clinical use as a plaque-disclosing dye. The concentration used in this study, 0.1 mg/mL or 0.01%, is relatively low compared with that used in many plaque-disclosing applications⁴¹, supporting further investigation of its translational potential. However, the 10-min exposure period is longer than the contact time typically associated with routine plaque disclosure. Therefore, the present condition should be viewed as a proof-of-concept topical exposure-and-rinse model designed to test EB matrix-associated localization, retention, and antimicrobial activity, rather than as a direct simulation of current plaque-disclosing practice.

The cytocompatibility findings strengthen the translational relevance of EB by demonstrating a favorable distinction between antibiofilm activity and oral cell compatibility over the same exposure duration. Consistent findings from the MTT and LIVE/DEAD assays support this differential response, although validation in physiologically representative models is required before broader safety conclusions can be drawn.

The present study has several limitations. First, the findings were obtained using an in vitro, single-species *S. mutans* biofilm model and therefore do not fully capture the ecological complexity of natural dental plaque, where interspecies interactions, host-derived factors, and environmental fluctuations contribute to biofilm behavior^{7,9}. Second, the static 10-min topical exposure used in this study does not reproduce the dynamic conditions of the oral cavity, including salivary dilution, clearance, and the shorter or repeated exposure periods likely to occur during clinical use. Therefore, the retention and antimicrobial activity of EB under clinically relevant application conditions remain to be established. Third, because a validated chemical neutralization step was not included, continued antimicrobial activity during the initial biofilm-recovery and dilution steps cannot be completely excluded. For CHX, washing and serial dilution reduce residual antimicrobial activity but may not completely eliminate it. For EB, which remained associated with the glucan-rich matrix after washing, retained EB may have continued to exert antimicrobial activity during subsequent sample processing. Therefore, CFU results should be interpreted as recoverable viable bacteria following treatment rather than as bacterial killing occurring exclusively during the nominal 10-min exposure period. Fourth, the proposed mechanism is inferred from retention, imaging, EPS perturbation, and functional killing assays rather than direct molecular interactions between EB and specific EPS components. Future studies incorporating multispecies biofilms, clinically derived communities, clinically relevant topical-exposure protocols, and direct physicochemical analyses of matrix–compound interactions will be important for defining the broader relevance and mechanistic basis of this phenomenon³⁵.

Despite these limitations, this study supports a model in which the glucan-rich EPS architecture influences EB localization, retention, and bactericidal activity within mature biofilms. More broadly, these findings suggest that antimicrobial performance in structured biofilm communities may depend not only on the ability of an agent to enter the matrix but also on its capacity to remain spatially associated with EPS-rich microenvironments following topical exposure. These findings highlight matrix–compound compatibility as a relevant factor in the design of short-contact antimicrobial strategies for biofilm-associated diseases.

Methods

Bacterial strains and culture conditions

Streptococcus mutans UA159 was used to generate single-species biofilms. For inoculum preparation, bacterial cultures were grown in ultrafiltered (10-kDa molecular-weight cut-off membrane; Millipore, MA, USA) tryptone–yeast extract broth (UFTYE; 2.5% (w/v) tryptone and 1.5% (w/v)

yeast extract; BD Biosciences, San Jose, CA, USA) supplemented with 1% (w/v) glucose at 37 °C under a 5% CO₂ atmosphere until reaching the mid-exponential phase (optical density at 600 nm (OD₆₀₀) ≈ 1.0)⁴².

Preparation of the treatment agents

Erythrosine B (EB; C.I. 45430; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was prepared in 5 mM phosphate buffer containing 20% (v/v) ethanol, and the pH was adjusted to 5.8. The solution was serially diluted to the required working concentrations using the same vehicle, sterilized by filtration through 0.22 µm membrane filters (Millex-GP; Merck KGaA, Darmstadt, Germany), and stored at 4 °C protected from light until use. Chlorhexidine digluconate (CHX; 20% in H₂O; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was likewise diluted to the required working concentration in 5 mM phosphate buffer containing 20% (v/v) ethanol and used as a positive control. The corresponding vehicle control consisted of the same phosphate buffer containing 20% (v/v) ethanol without EB or CHX, ensuring equivalent ethanol exposure across all treatment groups.

Planktonic growth kinetics

S. mutans UA159 (1 × 10⁵ colony-forming units (CFU)/mL) was inoculated into 96-well plates containing UFTYE medium supplemented with 1% (w/v) glucose and EB (0.00125–1 mg/mL), with CHX (0.012% and 0.12%) used as positive controls. Plates were incubated at 37 °C for 24 h in a microplate reader (Hidex Oy, Turku, Finland), and optical density at 600 nm (OD₆₀₀) was recorded every 30 min to monitor planktonic growth kinetics⁴³.

Crystal violet biofilm biomass assay

S. mutans UA159 (1 × 10⁵ CFU/mL) was inoculated into 96-well plates containing UFTYE medium supplemented with 1% (w/v) sucrose and EB (0.00125–1 mg/mL), with CHX (0.012% and 0.12%) used as positive controls. After incubation at 37 °C under 5% CO₂ for 24 h, non-adherent cells were removed, and biofilms were gently washed with water and blotted dry. The remaining biofilms were stained with 0.01% (w/v) crystal violet for 10 min, washed to remove excess dye, blotted dry, and solubilized with 30% (v/v) acetic acid. Absorbance was measured at 570 nm using a microplate reader⁴⁴.

A brief topical erythrosine B treatment of preformed *S. mutans* biofilms

To mimic the pellicle-coated tooth surface, *S. mutans* biofilms were established on saliva-coated hydroxyapatite disks (sHA; surface area 2.7 ± 0.2 cm²; Clarkson Chromatography Products, South Williamsport, PA, USA). Disks were coated with filter-sterilized human saliva for 1 h at 37 °C to allow salivary pellicle formation. For biofilm formation, sHA disks were held vertically in a Teflon holder and positioned within 12-well plates using a custom-made orthodontic wire apparatus⁴⁵. Each well contained 5 mL UFTYE medium (pH 7.0) supplemented with 1% (w/v) sucrose. The medium was inoculated with *S. mutans* at a final concentration of 10⁵ CFU/mL, and biofilms were cultivated at 37 °C under 5% CO₂ for 24 h. To maintain nutrient availability during biofilm development, the culture medium was replaced after 19 h with fresh UFTYE medium containing 1% sucrose. After 24 h of growth, preformed biofilms were subjected to a single 10-min topical treatment. Biofilms were exposed in situ to EB (0.1 mg/mL; 0.01% (w/v)) for 10 min, with CHX (0.12% (w/v)) included as a positive antimicrobial control. Following treatment, the disks were dip-washed in 0.89% NaCl to remove residual compounds. No specific chemical neutralizer was used for CHX; therefore, washing and dilution were used to reduce residual antimicrobial carryover prior to viable-cell enumeration. Viable bacterial counts were determined by CFU enumeration on blood agar plates. The overall workflow for single topical treatment of preformed biofilms is summarized in Fig. 1a. Biofilm structure and metabolic activity were further analyzed using confocal laser scanning microscopy and glycolytic pH-drop assays, as described below.

Biofilm viable-cell counts

After the topical treatment and washing steps described in the “A brief topical erythrosine B treatment of preformed *S. mutans* biofilms” section, biofilms were processed for viable-cell enumeration by CFU analysis. Briefly treated biofilms were washed three times with 0.89% NaCl to remove residual agents and separated from the Teflon holder. Each disc was transferred to 2 mL of 0.89% NaCl and sonicated in an ultrasonic bath (Power Sonic 410; Hwashin Technology Co., Seoul, Korea) for 10 min to disperse the biofilm. After adding 3 mL of 0.89% NaCl, the suspension was further homogenized by probe sonication (VCX 130PB; Sonics and Materials Inc., Newtown, CT, USA) at 7 W for 30 s. The homogenized suspension was serially diluted, plated on blood agar plates, and incubated at 37 °C for 48 h. No specific chemical neutralizer was used for CHX; therefore, washing and dilution were used to reduce residual antimicrobial carryover prior to viable-cell enumeration. Results were expressed as CFU per biofilm⁴⁶.

Glycolytic pH-drop assay of preformed biofilms

Preformed 24-h *S. mutans* biofilms on sHA disks were washed with salt solution (50 mM KCl, 1 mM MgCl₂, pH 7.0) and treated with EB (0.1, 1, or 10 mg/mL; 0.01%, 0.1%, or 1% (w/v)), CHX (0.12% (w/v)), or NaF (0.055% (w/v); 250 ppm F⁻) for 10 min. After treatment, the disks were washed and transferred to 20 mM potassium phosphate buffer (1 mM MgCl₂, 50 mM KCl, pH 7.2) for 1 h to deplete endogenous carbohydrate reserves. Biofilms were then transferred to 6-well plates containing salt solution adjusted to pH 7.0 with 0.2 M KOH. Glycolysis was initiated by adding glucose to a final concentration of 1% (w/v), and pH was recorded at 10-min intervals for 120 min using a calibrated glass electrode (Orion 3-Star, Thermo Scientific, Waltham, MA, USA)⁴⁷. To exclude the possible effect of residual EB on pH measurements, biofilm-free sHA disks were subjected to the same EB treatment and glucose-challenge procedure as explained above and monitored in parallel. The experimental workflow for glycolytic pH-drop analysis is summarized in Fig. 2a.

Spectral characterization and calibration of Erythrosine B

The absorption spectrum of EB (0.1 mg/mL) was recorded from 400 to 700 nm using a microplate reader (Hidex Oy) to determine the wavelength of maximum absorbance. A representative spectrum showed a maximum absorbance at 529 nm ($\lambda_{\text{max}} = 529 \text{ nm}$) (Supplementary Fig. 10a), which was subsequently used for absorbance-based quantification. EB standards were prepared by serial dilution at concentrations of 0–40 µg/mL in 5 mM phosphate buffer (pH 5.8) containing 20% (v/v) ethanol. The same buffer was used as the blank. Blank-corrected absorbance at 529 nm was measured in triplicate for each standard concentration, and the mean absorbance was fitted against EB concentration by linear regression to generate the calibration curve (Supplementary Fig. 10b). EB concentrations in experimental samples were determined from the resulting standard curve and expressed as µg/mL.

Erythrosine B retention on sHA surfaces with/without biofilms

EB retention was quantified on HA disks under four surface conditions: plain HA disks, saliva-coated HA disks, 24-h preformed *S. mutans* biofilms, and EPS-disrupted preformed biofilms. Saliva-coated HA disks were prepared by incubating HA disks with filter-sterilized human saliva for 1 h at 37 °C. Preformed biofilms were generated by inoculating saliva-coated HA disks with *S. mutans* UA159 (1×10^5 CFU/mL) in UFTYE medium supplemented with 1% (w/v) sucrose and culturing for 24 h at 37 °C under 5% CO₂. For glucan-matrix disruption, 24-h preformed biofilms were treated with dextranase (10 U; Sigma) and mutanase (10 U; HLB Genex, Daejeon, Korea) in 1 mL of 0.1 mM sodium acetate (NaOAc) buffer (pH 5.5) at 37 °C for 45 min⁴⁸. All specimens were topically treated with EB (0.1 mg/mL) for 10 min. Disks were then washed three times by immersion in 0.89% NaCl to remove unbound dye. Retained EB was extracted by transferring each disc to a glass tube containing 1 mL of 70% (v/v) ethanol and gently agitating for 5 min. The extracts were centrifuged at $16,000 \times g$ for 5 min, and the

supernatants were transferred to a 96-well plate. Absorbance was measured at 529 nm, and EB concentrations were calculated as described above. The workflow for EB retention analysis after washing is summarized in Fig. 3a.

Biofilm labeling and quantitative image analysis

Following single topical treatment with EB or a control agent, biofilm-covered sHA disks were gently dip-washed with 0.89% NaCl to remove loosely adherent cells and unbound compounds while preserving native biofilm architecture. Bacterial biomass was stained with DAPI, a blue-fluorescent nucleic acid stain (Molecular Probes Inc., Eugene, OR, USA; 2.5 µM final concentration; Ex 408/ Em 461 nm), for 20 min immediately before confocal imaging. DAPI was excited at 405 nm and detected in the blue emission range; the DAPI channel was pseudo colored green for visualization. For EPS labeling, Alexa Fluor 647-dextran conjugate (Molecular Probes Inc., Eugene, OR, USA; 1 µM, 10 kDa molecular weight; 647/668 nm) was added to UFTYE supplemented with 1% sucrose throughout the 24-h biofilm formation period. Biofilm structural analysis for both treatment models was performed using a Nikon C2 Plus confocal microscope (Nikon, Tokyo, Japan) equipped with a 20× objective (0.75 numerical aperture). Images were acquired at 512×512 pixels with a calibrated pixel size of 1.23 µm/pixel, corresponding to a field of view of approximately $630 \times 630 \mu\text{m}$. Z-stack images spanning full biofilm depth were collected at 2 µm intervals and acquired by sequential diode laser scanning with multi-alkali photomultiplier tube (PMT) detection using identical acquisition parameters across experimental groups⁴⁹. Three-dimensional (3D) reconstructions were generated using Amira 5.4.1 (Visage Imaging GmbH, Berlin, Germany), ImageJ (NIH), and NIS-Elements 5.21 (Nikon). Images represent maximum intensity projections of the acquired z-stacks and therefore visualize the three-dimensional biofilm architecture rather than a single optical plane. Bacterial and EPS biovolumes were quantified from complete Z-stack series using COMSTAT implemented as a script in MATLAB software (version R2023b; MathWorks)^{15,50}.

Live/Dead biofilm viability assay

Preformed 24-h *S. mutans* biofilms on sHA disks underwent single topical treatment with EB (0.1 mg/mL; 0.01%) or CHX (0.12%) for 10 min, followed by gentle dip-washing with 0.89% NaCl prior to staining at room temperature in the dark for 30 min with FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Invitrogen™, Molecular Probes Inc., Eugene, OR, USA). Final concentrations of SYTO9 (6.0 µM, live cells; Ex 483/ Em 503 nm) and propidium iodide (PI; 30 µM, dead cells; (493 (free state) or 535 nm (DNA-bound state)/ 636 (free state) or 617 nm (DNA-bound state)) were applied simultaneously⁵¹. Confocal imaging and quantitative live/dead cell biovolume analysis were performed using COMSTAT as described above. Confocal z-stacks were acquired throughout the biofilm depth and reconstructed as projected images to visualize the three-dimensional distribution of SYTO9 and PI-associated fluorescence. Because projection integrates fluorescence originating from different z-positions, spatial overlap of green and red signals in the projected images was not interpreted as dual staining of individual bacterial cells.

To assess potential spectral interference between EB and PI, additional fluorescence controls were performed using unstained, EB-only, PI-only, and SYTO-only biofilms. Each control was imaged using the same excitation wavelengths, emission windows, laser power, detector gains, and scanning conditions used for the experimental LIVE/DEAD images, and individual control samples were examined across the relevant detection channels. Under the acquisition settings optimized for PI detection, EB-only biofilms showed fluorescence comparable to the unstained background, indicating negligible detection contribution of the residual EB fluorescence to the PI channels. These controls confirmed that the red fluorescence detected in the Live/Dead images predominantly represented PI-positive, membrane-compromised cells rather than residual EB fluorescence. Representative single stain and spectral bleed-through controls used to validate the LIVE/DEAD imaging are shown in Supplementary Fig. 11.

EB localization, retention and matrix-dependency analysis

Pre-formed 24-h *S. mutans* biofilms on sHA disks and EPS-labeled with Alexa Fluor 647 during biofilm development as described above. After biofilm formation, disks were gently washed with 0.89% NaCl and treated topically with EB at 0.1 mg/mL for 10 min. After treatment, biofilms were washed again with 0.89% NaCl to remove unbound EB and counterstained with DAPI (Sigma-Aldrich; final 10 µg/mL, 20 min, dark) to visualize bacterial cells.

For the time-reserved confocal analysis, mature 24-h biofilms were first stained with DAPI for 20 min immediately before EB treatment. This prestaining step was used to ensure the defined exposure periods represented the actual duration of EB contact with the biofilm. The DAPI-stained biofilms were subsequently exposed to EB at 0.1 mg/mL for 1, 5, or 10 min, followed immediately by standardized washing and confocal imaging. For viable count analysis, biofilms were exposed to EB for 1, 3, 5, 10 min, followed by standardized washing and CFU enumeration as described above.

To assess the contribution of the biofilm matrix EB retention, parallel biofilms were enzymatically disrupted before EB exposure. Biofilms were treated with dextranase and mutanase (10 U each) in 1 mL of 0.1 mM NaOAc buffer (pH 5.5) at 37 °C for 45 min. Followed by washing, EB treatment, and DAPI staining as described above.

Quantitative EPS/EB fluorescence analysis was performed using Fiji (ImageJ) to measure EPS (Ex 640 nm, Em 663–720 nm) and EB (Ex 566.3 nm, Em 570–620 nm) intensities⁵². Confocal z-stack images underwent sequential processing: (1) rolling ball background subtraction (radius = 80 pixels); (2) median filtering (radius = 1 pixel); (3) Otsu automatic thresholding for EPS/EB binary masks; (4) The total positive area (Sum Area) was calculated from the binary masks, which were then applied to the corresponding original fluorescence image stacks using the Multi Measure tool in the ROI (region of interest) Manager to obtain the integrated fluorescence intensity (Sum IntDen) across all z-slices. Average fluorescence intensity per voxel was calculated as:

$$I_{\text{voxel}} = \frac{\text{Sum IntDen (original fluorescence image)}}{\text{Sum Area (binary mask)}} \quad (1)$$

For EB-EPS spatial association analysis, higher-magnification (5× zoom) confocal z-stacks were analyzed using Coloc 2 in Fiji. Whole-biofilm ROIs were applied consistently to the EB and EPS channels, and Pearson's correlation coefficient and Costes-thresholded Manders' coefficients were calculated. EB was assigned as channel 1 and EPS as channel 2; therefore, tM1 represents the thresholded fraction of EB fluorescence overlapping EPS-positive regions, whereas tM2 represents the thresholded fraction of EPS fluorescence overlapping EB-positive regions. These analyses were used to assess EB-EPS spatial association rather than direct molecular binding.

Selective enzymatic matrix-disruption analysis of EB retention

To further assess which matrix components contribute to EB retention, 24-h *S. mutans* biofilms formed on sHA disks were subjected to selective enzymatic matrix disruption before EB exposure. Biofilms were treated with proteinase K, dextranase, mutanase, or combined dextranase-mutanase treatment. Dextranase and mutanase treatments were performed in the same buffer and under the incubation conditions described above. Proteinase K was used to assess the contribution of protein-containing matrix components and was applied at 0.1 mg/mL in PBS (pH 7.5) at 37 °C for 45 min, with PBS serving as its corresponding buffer control. Following enzymatic treatment, biofilms were washed with 0.89% NaCl, exposed to EB at 0.1 mg/mL for 10 min, washed again to remove unbound EB, and counterstained with DAPI to visualize bacterial biomass. As an enzymatic control, the combined dextranase-mutanase preparation was heat-inactivated at 95 °C for 10 min, cooled to room temperature, and applied to biofilms at the same nominal enzyme concentrations and under the same incubation conditions as the active enzyme mixture^{53,54}. Parallel biofilms were incubated with the corresponding enzyme buffers alone (NaOAc

buffer for glucanohydrolases treatment and PBS for proteinase K treatment) under identical conditions. These buffer-only controls were processed for confocal imaging to assess any effects of the incubation buffers on biofilm integrity. EB retention was quantified from confocal images using Fiji/ImageJ. Mean EB fluorescence intensity was measured within whole-biofilm ROIs and normalized to the corresponding mean DAPI fluorescence intensity from the same ROI. DAPI-normalized EB fluorescence was then expressed relative to the buffer-treated EB control. This analysis was used to assess EB retention relative to the remaining bacterial biomass after enzymatic matrix disruption.

Influence of glucan matrix on EB bactericidal activity

Preformed 24-h *S. mutans* biofilms were developed on sHA disks in UFTYE supplemented with 1% sucrose. After washing with 0.89% NaCl, the disks were assigned to control, intact-EPS, and EPS-disrupted groups. Biofilms in the EPS-disrupted group were incubated with mutanase and dextranase (10 U each) in 1 mL of 0.1 mM NaOAc buffer (pH 5.5) at 37 °C for 45 min, whereas those in the control and intact-EPS groups were incubated with the same buffer alone under identical conditions. The intact-EPS and EPS-disrupted groups were then treated with EB (0.1 mg/mL) for 10 min, followed by live/dead staining and confocal imaging. Full-thickness confocal z-stacks were analyzed to quantify biofilm structural parameters following matrix disruption. Bacterial biovolume was determined from SYTO and PI channels, while EPS biovolume was quantified from the Alexa Fluor 647-dextran channel. Total biofilm biovolume, mean bacterial thickness, mean EPS thickness, and mean overall biofilm thickness were calculated from the corresponding z-stacks using identical image processing and threshold settings across all groups. The EPS-to-bacterial biovolume ratio was calculated by normalizing EPS biovolume to the corresponding bacterial biovolume for each biological replicate. To examine the effect of matrix disruption on CHX-mediated killing, mature 24-h *S. mutans* biofilms with an intact or dextranase-mutanase disrupted matrix were exposed to CHX (0.12%) for 10 min and washed under standardized conditions. An enzyme-only control was included to assess whether matrix-disrupting enzyme treatment itself affected bacterial viability. Bacterial viability was assessed using the LIVE/DEAD assay as described above. The workflow for live/dead imaging analysis under intact and enzymatically disrupted glucan-rich matrix conditions is summarized in Fig. 7a.

Glucan-rich matrix-dependent bactericidal activity of EB

S. mutans biofilms were cultured for 18 h in sterile Eppendorf tubes using an initial inoculum of 10⁵ CFU/mL. Because the intact, enzymatically disrupted, and glucan-deficient conditions can differ substantially in biofilm growth and surface adhesion, a tube-based format was used to minimize differences in cell recovery associated with surface attachment and enable direct comparison across matrix conditions. This format also allowed uniform enzymatic treatment, EB exposure, washing, and complete recovery of biofilm cells for CFU enumeration. Three matrix conditions were examined: (1) Sucrose-grown biofilms with intact glucan-rich matrix, cultured in UFTYE supplemented with 1% sucrose; (2) Sucrose-grown biofilms with enzymatically disrupted glucan-rich matrix, generated by treating sucrose-cultured biofilms with dextranase and mutanase (10 U each) in 0.1 mM sodium acetate buffer (pH 5.5) at 37 °C for 45 min; and (3) glucose/fructose-grown non-sucrose biofilms, cultured in UFTYE supplemented with 0.5% glucose and 0.5% fructose to limit sucrose-dependent glucan-rich matrix development. After matrix conditioning, biofilms were exposed to EB treatment (0.1 mg/mL) for 10 min. Biofilms were then washed twice with 0.1 mM NaOAc buffer (pH 5.5) and once with 0.89% NaCl. Vehicle treated (20% ethanol in PBS) for 10 min served as control. Biofilm cells were harvested, serially diluted in 0.89% NaCl, plated on blood agar, and colony-forming units were enumerated. To determine whether the influence of biofilm matrix condition was specific to EB, a parallel experiment was performed using CHX. *S. mutans* biofilms were prepared under the same three conditions used for the EB experiment as described above. Biofilms were then exposed to CHX (0.12%) for 10 min, followed by standardized

washing and CFU enumeration. The experimental workflow for assessing glucan-rich matrix-dependent EB bactericidal activity is summarized in Fig. 6a and Supplementary Fig. 6a.

Human oral cell culture

Human gingival fibroblasts (HGF; ATCC PCS-201-018) and human oral keratinocytes (HOK-16B, kindly provided by Dr. No-Hee Park, UCLA School of Dentistry) were cultured at 37 °C, 5% CO₂, with medium replacement every 3 days. HGF was maintained in DMEM (Gibco) supplemented with 10% FBS, 300 mg/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). HOK-16B were grown in Keratinocyte Basal Medium 2 (PromoCell C-20211) with Growth Supplement Mix (C-39016), 150 µM CaCl₂ (C-34006), and 30 µg/mL gentamicin (Gibco). At 80–90% confluence, cells were subcultured 1:4 from 100 mm dishes using TrypLE™ Express (HOK-16B) or 1× Trypsin-EDTA (HGF). Experiments utilized passages 3–8.

Cell viability assay

Cytocompatibility was evaluated using human gingival fibroblasts (HGF) (5×10^5 cells/well) and human oral keratinocytes (HOK-16B) (2×10^5 cells/well). For the continuous-exposure assessment, cells were exposed to EB (0.1 mg/mL) or CHX (0.012% and 0.12%) in 24-well plates (SPL Life Sciences) for 24 or 48 h, after which cellular metabolic activity was assessed using the MTT assay. Cells were washed twice with 1× PBS, then incubated with MTT (5 mg/mL stock diluted 1:50 in culture medium) at 37 °C for 3 h. Formazan crystals were solubilized in DMSO (gentle shaking, 10 min), and absorbance was measured at 570 nm using a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT, USA)⁵⁵. These prolonged exposure conditions were included as a conservative assessment of cytocompatibility. To evaluate cytocompatibility under conditions that more closely reflect the single-contact topical treatment protocol, an additional single-contact assay was performed. Cells were exposed to EB (0.1 mg/mL) or CHX (0.012% and 0.12%) for 10 min, after which the treatment solutions were washed, and fresh culture medium was added. Cellular metabolic activity was assessed using the MTT assay at 0, 24, and 48 h post-exposure recovery as described above. To account for possible interference from treatment-associated absorbance, treatment-matched background controls were included in parallel. Background absorbance for each treatment condition was measured and subtracted from the corresponding sample absorbance before calculating cell viability. Cell viability was expressed relative to the corresponding vehicle control. Additionally, as an orthogonal assessment of cytocompatibility, cells subjected to the 10-min protocol were evaluated using LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells according to the manufacturer's instructions. The assay uses calcein-AM, which is converted by intracellular esterases in viable cells to generate green fluorescence, and ethidium homodimer-1 (EthD-1), which enters the cells with compromised plasma membranes and produces red fluorescence upon binding to nucleic acid. Cells were stained immediately after 10-min exposure or 24 or 48 h of post-exposure recovery, with 2 µM calcein-AM and 4 µM EthD-1 for 30 min at room temperature. The cells were then washed twice to remove the residual staining solution. Calcein-AM and EthD-1 were excited at 488 and 566 nm, and fluorescence was collected using the corresponding green (500–550 nm) and red (570–620 nm) fluorescence detection channels imaged by a Nikon C2 Plus confocal microscope (Nikon, Tokyo, Japan) equipped with a 10× objective.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical tests were selected according to the experimental design and the nature of the planned comparison. Comparisons involving multiple groups against a single control were analyzed using one-way ANOVA followed by Dunnett's multiple-comparisons test. For selected preplanned comparisons among specific groups, one-way ANOVA followed by Sidák's multiple-comparisons test was used. Comparisons among multiple groups were analyzed using one-way ANOVA with Tukey's HSD test or, for non-

parametric data, the Kruskal–Wallis test followed by Dunn's post hoc test. Pairwise comparisons were analyzed using the unpaired two-tailed Student's *t*-test. For experiments involving treatment and time as independent factors, two-way ANOVA followed by the appropriate multiple-comparisons test was used. CFU values were log₁₀-transformed before statistical analysis. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using GraphPad Prism (version 10.4.0; GraphPad Software, San Diego, CA, USA). Unless otherwise stated, all experiments were performed using three independent biological replicates ($n = 3$), with each replicate derived from an independently prepared biofilm.

Ethics statement

The studies involving human participants were approved by the Institutional Review Board of Jeonbuk National University (IRB No. JBNU 2021-12-008-003). Written informed consent was obtained from all participants prior to saliva collection.

Data availability

No datasets were generated or analyzed during the current study.

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Author contributions

X.B.H., K.R., and D.K. designed experiments; X.B.H. and K.R. performed the experiments; M.H.D., J.G.J., and D.K. contributed reagents/materials/analysis tools; X.B.H. and D.K. analyzed the data; X.B.H. and K.R. drafted the manuscript. X.B.H., K.R., and D.K. critically revised the manuscript. All authors reviewed and approved the final manuscript.

Competing interests

D.K. and X.B.H. are co-inventors on a pending Patent Cooperation Treaty (PCT) application (application no. PCT/KR2026/009809) filed by Jeonbuk National University Industrial Cooperation Foundation related to a composition for the simultaneous labeling and inhibition of virulence factors in oral biofilms. The other authors have no competing interests.

Additional information

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