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# Ursolic acid disrupts cariogenic biofilms with selective antibacterial activity against *Streptococcus mutans* and *Streptococcus sobrinus*

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## ABSTRACT

Prevention of dental caries requires effective control of biofilm-forming *Streptococcus* spp. The present study evaluated the antimicrobial and antibiofilm properties of ursolic acid (UA) and beta-sitosterol against cariogenic bacteria. UA demonstrated selective antimicrobial activity, with MIC/MBC values of 8 and 16 μM against *Streptococcus mutans* and *Streptococcus sobrinus*, respectively. At 16 μM, UA reduced biofilm adherence by 95% and 90% in *S. mutans* and *S. sobrinus*, respectively. Confocal microscopy revealed a dose-dependent reduction in biofilm thickness, decreasing from 35.2 ± 2.3 μm to 17.3 ± 1.5 μm for *S. mutans* and from 64.3 ± 1.8 μm to 42.1 ± 1.2 μm for *S. sobrinus* following treatment with 64 μM UA. At this concentration, bacterial viability decreased to 40% and 62% for *S. mutans* and *S. sobrinus*, respectively. In comparison, 0.12% chlorhexidine (positive control) reduced biofilm thickness to 15.1 ± 2.5 μm and 32.5 ± 1.9 μm, with viability decreasing to 36% and 52% for *S. mutans* and *S. sobrinus*, respectively. Unlike UA, beta-sitosterol showed negligible antibacterial activity. These findings demonstrate the potential of UA as a promising natural therapeutic agent against cariogenic biofilms, although its efficacy remains lower than that of chlorhexidine.

**Keywords:** dental caries; ursolic acid; beta-sitosterol; biofilm; *Streptococcus mutans*; *Streptococcus sobrinus*; ethnopharmacology

## 1. INTRODUCTION

Dental caries remains a significant global health concern, affecting millions of people worldwide (Pitts et al., 2021). The disease is characterized by the demineralization of tooth enamel, which is driven by the interplay of biological and environmental factors. Among the primary causative agents, *Streptococcus mutans* and *Streptococcus sobrinus* are notable for their acid production (acidogenicity) and their ability to survive in acidic environments (aciduricity), both of which contribute significantly to biofilm formation on tooth surfaces (Balakrishnan et al., 2000; Krzyściak et al., 2014). These biofilms metabolize dietary sucrose to produce water-insoluble glucans,

facilitating bacterial adherence and generating lactic acid, which demineralizes tooth enamel (Bowen & Koo, 2011; Forssten et al., 2010; Kolenbrander et al., 2002). The progression of dental caries is influenced by a complex interplay of four factors: cariogenic microorganisms, fermentable carbohydrates, susceptible host factors, and time (Takahashi & Nyvad, 2010). Understanding the pivotal role of *Streptococcus* spp. in caries development highlights the need for targeted prevention and management strategies (Lin et al., 2021).

Chlorhexidine (CHX) is widely employed as an antimicrobial agent in dental research (Lamarque et al., 2019). Clinically, mouthwash formulations containing CHX (0.12% to 0.2%) have demonstrated superior effectiveness in inhibiting dental plaque formation (Poppolo Deus & Ouanounou, 2022). CHX exhibits broad-spectrum antibacterial activity and potent efficacy against oral biofilms (Lamarque et al., 2019). The antimicrobial mechanism of CHX involves electrostatic binding to bacterial cell surfaces, disrupting membrane integrity and causing cytoplasmic leakage and coagulation (Chatzigiannidou et al., 2020; Shen et al., 2011). However, adverse effects associated with CHX include tooth staining, taste alteration, oral numbness or pain, xerostomia, and calculus formation (Poppolo Deus & Ouanounou, 2022). Therefore, natural products derived from plants and other organisms have gained attention as potential agents for disrupting biofilm formation and bacterial growth with reduced toxicity (Amanpour et al., 2023; Gouda et al., 2023; Manosroi et al., 2006). These compounds often exhibit multiple mechanisms of action, including the inhibition of bacterial growth and the prevention of biofilm formation, which are crucial for managing oral diseases such as dental caries and periodontitis.

*Paederia foetida* (Linn.), a member of the Rubiaceae family, is widely distributed throughout tropical Asia and has a long history of use in traditional medicine systems, including Ayurveda and traditional Chinese medicine. The plant's extract has been shown to possess anti-inflammatory, analgesic, and antipyretic properties (Dutta et al., 2023; Wang et al., 2014). Although its antibacterial and antifungal activities have been previously reported, the activity of *P. foetida* against oral pathogens responsible for dental caries, such as *S. mutans* and *S. sobrinus*, remains poorly documented. Prior studies have identified beta-sitosterol and ursolic acid (UA) as bioactive compounds found in *P. foetida* (Pouyfung et al., 2023); however, their antimicrobial activity against these specific pathogens has not been thoroughly studied. The present study aimed to investigate the antibacterial activity of beta-sitosterol and UA through assays of their minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs), as well as their ability to disrupt biofilm formation and bacterial adhesion using *in-vitro* biofilm assays and confocal microscopy. In the present study, 0.12% CHX was selected as the positive control for assessments of bacterial growth and biofilm formation due to its near-complete biofilm eradication (Chatzigiannidou et al., 2020; Shen et al., 2011). The findings of this study could contribute to the development of nature-derived approaches to preventing dental caries and other oral diseases.

## 2. MATERIALS AND METHODS

### 2.1 Bacterial strains and culture conditions

*S. mutans* (ATCC 25175), *S. sobrinus* (ATCC 33478), and *Aggregatibacter actinomycetemcomitans* (ATCC 29523) were cultured in brain heart infusion (BHI) broth at 37°C in a 5% $CO_2$ atmosphere for 48 h.

### 2.2 Effects of UA and beta-sitosterol on bacterial growth: determination of MICs and MBCs

The MICs and MBCs were determined according to previously published protocols (Klaophimai et al., 2023). Briefly, overnight cultures of *S. mutans*, *S. sobrinus*, and *A. actinomycetemcomitans* were grown at 37°C in a 5% $CO_2$ atmosphere and adjusted to a concentration of $10^6$ CFU/mL. UA (CAS 77-52-1, Sigma-Aldrich, St. Louis, MO, USA) and beta-sitosterol (CAS 83-46-5, Sigma-Aldrich) were serially diluted in 96-well plates over a concentration range of 0 to 512 µM. Subsequently, 100 µL of the bacterial suspension ($1 \times 10^6$ CFU/mL) was added to each well, resulting in a final volume of 200 µL per well with a final bacterial concentration of $5 \times 10^5$ CFU/mL in each well (Smith & Kirby, 2018). The plates were incubated at 37°C for 24 h, and bacterial growth was monitored spectrophotometrically at 600 nm ($OD_{600}$).

### 2.3 Effect of UA on biofilm formation

Biofilm formation was quantitatively evaluated using crystal violet staining in 96-well polystyrene microtiter plates. To closely simulate the natural oral environment and support the development of clinically relevant biofilms, each well was pre-coated with 10% saliva collected from a healthy volunteer for 24 h, in accordance with ethical guidelines (Choi et al., 2024). Subsequently, *S. mutans* and *S. sobrinus* were cultured in BHI broth at 37°C with 5% $CO_2$ for 18 to 24 h and then diluted to $5 \times 10^5$ CFU/mL in BHI broth supplemented with 1% sucrose. The addition of sucrose provides a substrate for bacterial glucosyltransferases to synthesize extracellular polysaccharides (EPSs), which are essential for robust biofilm matrix formation and bacterial adherence (Waldman et al., 2023). This suspension was combined with various concentrations of UA (0, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µM). The plates were then incubated under aerobic conditions at 37°C. After a 24-h incubation, the biofilms were fixed with anhydrous ethanol for 15 min. The fixed biofilms were then stained with 0.1% (w/v) crystal violet for 5 min, followed by thorough rinsing to remove the excess stain. The bound crystal violet was subsequently solubilized with 150 µL of 90% ethanol per well, and the absorbance was measured at 590 nm using a microplate reader.

### 2.4 Effect of UA on biofilm biomass and viability

Cultures of *S. mutans* and *S. sobrinus* were prepared as described by Klaophimai et al. (2023). These cultures were adjusted to the 0.5 McFarland standard in BHI broth containing 1% sucrose. The adjusted suspension was then applied to 8-well chamber slides, and the setup was incubated with human saliva at 37°C and 5% $CO_2$ for 24 h to facilitate biofilm formation. Next, biofilms were treated with varying concentrations of UA (16, 32, and 64 µM) and incubated for an additional 24 h. For visualization, the biofilms were stained using SYTO 9 and propidium

iodide and examined by confocal laser scanning microscopy (CLSM). Image data were analyzed using COMSTAT software to measure the biomass and calculate the percentage of live versus dead bacteria (Heydorn et al., 2000; Vorregaard, 2008).

### 2.5 Statistical analysis

All experiments were performed in triplicate and independently repeated on at least three separate occasions. Data were expressed as the mean ± standard deviation (SD). Statistical comparisons were performed using one-way or two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. For comparisons between two groups, the Student's t-test (paired or unpaired) was employed. Differences were considered statistically significant at a *p*-value of less than .05. Statistical analyses were performed using GraphPad Prism (version 10).

## 3. RESULTS

### 3.1 MIC and MBC

The antimicrobial effect of UA, a major compound found in *Paederia foetida* (L.), was evaluated against two Gram-positive bacteria (*S. mutans* and *S. sobrinus*) and a Gram-negative bacterium (*A. actinomycetemcomitans*) using MIC and MBC assays. UA exhibited antimicrobial activity against the Gram-positive species.

The MIC of UA was 8 μM for *S. mutans* and 16 μM for *S. sobrinus* (Table 1). In contrast, the MIC exceeded 128 μM against the Gram-negative *A. actinomycetemcomitans*. Notably, the MBC values for *S. mutans* and *S. sobrinus* were consistent with their corresponding MIC values. The results indicated that UA possesses both bacteriostatic (growth-inhibitory) and bactericidal (bacteria-killing) activities against *S. mutans* and *S. sobrinus* growth kinetics (Figure 1) confirmed these findings; overnight cultures of *S. mutans* (Figure 1A) and *S. sobrinus* (Figure 1B) exposed to UA (0 to 16 μM) showed a dose-dependent suppression of bacterial growth (shown by the reduction in optical density) over 24 h.

### 3.2 Inhibitory effect of UA on *S. mutans* and *S. sobrinus* biofilm formation

The inhibitory effects of UA on biofilm formation by *S. mutans* (Figure 2A) and *S. sobrinus* (Figure 2B) were evaluated following a 24-h incubation in the presence of human saliva. Crystal violet staining revealed concentration-dependent anti-biofilm activity. For *S. mutans*, UA at 8, 16, and 32 μM reduced biofilm formation to 14.2 ± 3.5%, 4.8 ± 1.2%, and 3.5 ± 0.8% of the control, respectively. *S. sobrinus* demonstrated greater resistance, with the same concentrations yielding a biofilm formation of 54.5 ± 8.2%, 9.8 ± 2.3%, or 4.1 ± 0.9% relative to the untreated control. The positive control (0.12% CHX) exhibited nearly total inhibition of biofilm formation (~ 98% to 99%) for both bacterial species. Higher UA concentrations (64 μM and 128 μM) maintained inhibitory levels comparable to those of 32 μM, with minimal biofilm formation observed (2.5% to 3.0% for *S. mutans* and 2.8% to 3.2% for *S. sobrinus*). Visual assessment via crystal violet staining (Figures 2C and 2D) confirmed these quantitative findings, demonstrating the potent dose-dependent activity of UA against cariogenic streptococci.

**Table 1.** Antimicrobial activity (MIC and MBC) of UA and beta-sitosterol against *S. mutans*, *S. sobrinus,* and *A. actinomycetemcomitans*

| | **Gram-positive** | | | | **Gram-negative** | |
|---|---|---|---|---|---|---|
| | ***S. mutans*** | | ***S. sobrinus*** | | ***A. actinomycetemcomitans*** | |
| | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** |
| UA | 8 | 8 | 16 | 16 | >128 | >128 |
| Beta-sitosterol | >128 | >128 | >128 | >128 | >128 | >128 |

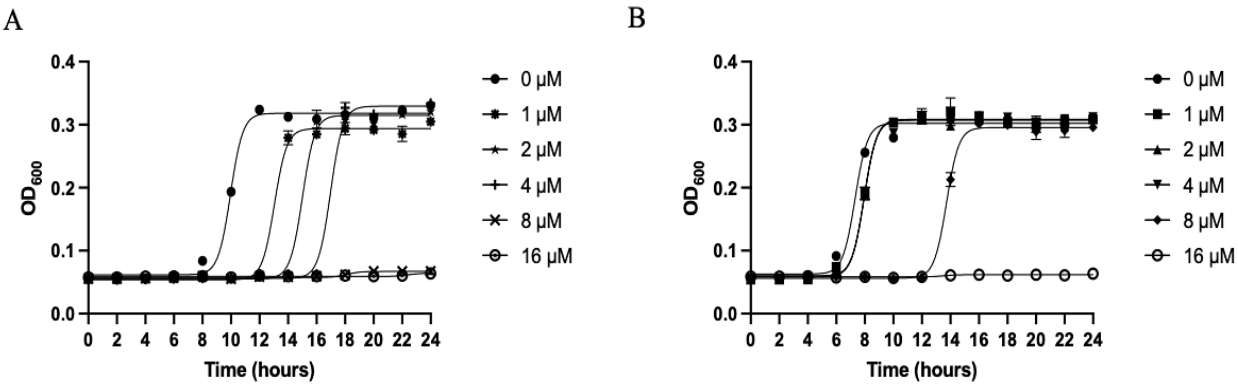



**Figure 1.** Growth inhibitory effects of UA against (A) *S. mutans* and (B) *S. sobrinus*

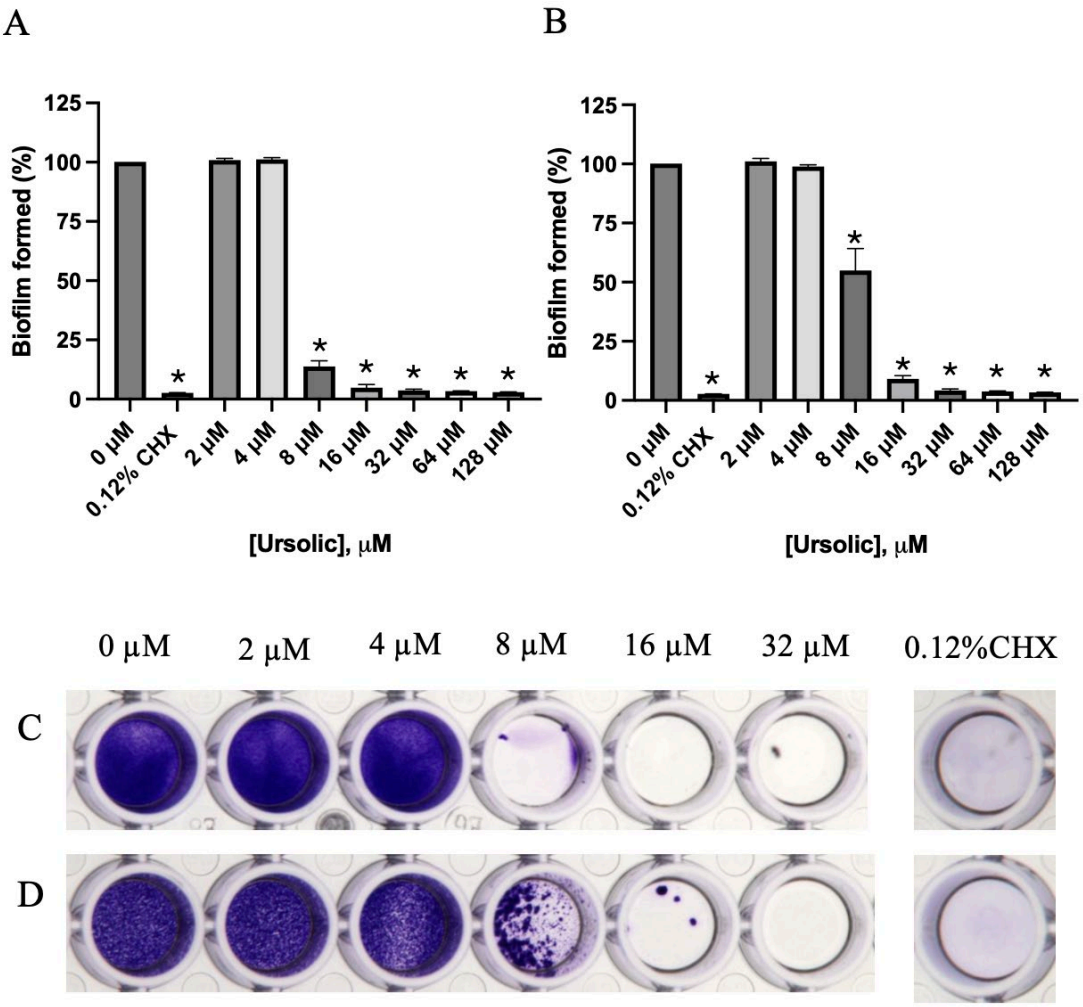



**Figure 2.** Inhibitory effects of UA on biofilm formation of *S. mutans* and *S. sobrinus*; quantitative analysis of biofilm formation for (A) *S. mutans* and (B) *S. sobrinus* following incubation with increasing concentrations of UA (0 – 64 μM) or 0.12% CHX in the presence of human saliva, representative images of stained biofilms are shown for (C) *S. mutans* and (D) *S. sobrinus*.

*Note:* Data represent the mean ± SD of three independent experiments. Statistical significance was determined using Student's t-test; asterisks denote significant differences compared to the control group ($p < .05$).

### 3.3 Efficacy of UA on streptococcal biofilm viability and thickness

*S. mutans* and *S. sobrinus* are key contributors to the pathogenesis of dental caries due to their robust biofilm on tooth surfaces. Based on preliminary screening (Figure 2), concentrations of 16, 32, and 64 μM were selected to assess the impact on established biofilm biomass and bacterial viability. For comparative purposes, 0.12% CHX was included as a positive control. For *S. mutans* biofilms (Figures 3A–C), UA treatment resulted in concentration-dependent reductions in cell viability, with 69%, 53%, and 40% viable cells remaining at 16, 32, and 64 μM, respectively, compared to 100% viability in the untreated control. The positive control (0.12% CHX) demonstrated 36% cell viability (Figure 3B). Biofilm thickness measurements revealed significant structural disruption, with mean thicknesses decreasing from 35.2 ± 2.3 μM (control) to 23.5 ± 0.8, 20.2 ± 2.1, and 17.3 ± 1.5 μM following treatment with 16, 32, and 64 μM UA, respectively. It was noted that the CHX treatment resulted in the most substantial reduction in biofilm thickness (15.1 ± 2.5 μM). *S. sobrinus* biofilms (Figures 3D–F) demonstrated greater resistance to UA, cell viability analysis showed 98%, 78%, and 62% viable cells at 16, 32, and 64 μM UA, respectively, while CHX reduced viability to 52% (Figure 3E). Biofilm thickness measurements corroborated these findings, with mean values of 63.8 ± 1.5, 46.7 ± 1.3, and 42.1 ± 1.2 μm at increasing UA concentrations (16, 32, and 64 μM) compared with 64.3 ± 1.8 μm in the control and 32.5 ± 1.9 μm with CHX (Figure 3F). These results demonstrate that UA possesses significant concentration-dependent antimicrobial and antibiofilm properties against cariogenic streptococci, though its efficacy remains slightly lower than the clinical standard, CHX.

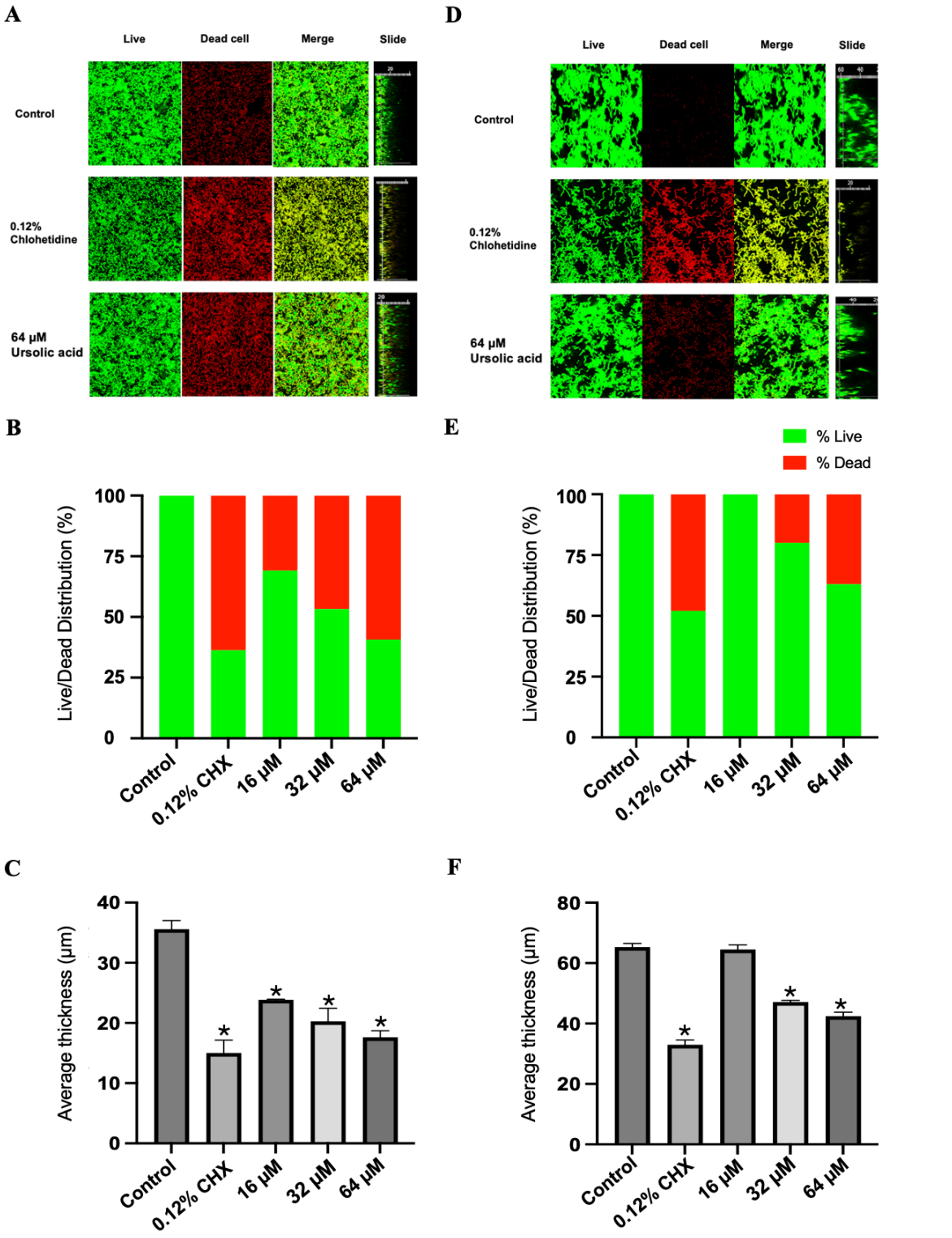



**Figure 3.** Effects of UA on the viability and structural integrity of established *S. mutans* and *S. sobrinus* biofilms; preformed biofilms of *S. mutans* (A–C) and *S. sobrinus* (D–F) were grown on chamber slides for 24 h and subsequently treated with 0 μM UA (control), 0.12% CHX, or 16, 32, or 64 μM UA for an additional of 24 h.

*Note:* Biofilms were stained with LIVE/DEAD BacLight fluorescent dye (SYTO 9/propidium iodide), and visualized using confocal laser scanning microscopy (CLSM). Representative CLSM images where green fluorescence indicates viable cells and red fluorescence indicates dead or membrane-compromised cells (A, D), Quantitative analysis of bacterial viability (%) based on fluorescence intensity. (C, F) Measurement of average biofilm thickness (μm) (B, E). Data are presented as the mean ± SD of three independent experiments. Statistical significance compared to the control group was determined using Student's t-test (*p < .05).

## 4. DISCUSSION

Dental caries is a complex polymicrobial disease significantly influenced by biofilm formation on tooth surfaces. *S. mutans* and *S. sobrinus* play pivotal roles in this process due to their abilities to form robust biofilms and produce organic acids (Loesche, 1986; Veenman et al., 2024). This study explored the selective antimicrobial activity of UA, a phytochemical, against cariogenic bacteria. UA displayed substantial antimicrobial efficacy, demonstrating bactericidal effects with MIC/MBC values of 8 μM and 16 μM for *S. mutans* and *S. sobrinus*, respectively. In contrast, the Gram-negative bacterium *A. actinomycetemcomitans* exhibited reduced susceptibility, underscoring the selectivity of this compound (Table 1, Figure 1). These results are consistent with Kim et al. (2011), who reported MIC values for UA of 2 μg/mL (~4.38 μM) and 4 μg/mL (~8.76 μM) against *S. mutans* and *S. sobrinus,* respectively. The differential sensitivity of these bacteria to UA may be attributed to structural variations in their cell walls. The relatively simple cell wall structure of Gram-positive bacterial likely increases their susceptibility to UA compared to Gram-negative species, which possess an outer membrane that acts as a permeability barrier (Trombetta et al., 2005). Furthermore, the distinct structural differences between beta-sitosterol and UA explain their contrasting efficacies. Beta-sitosterol, a phytosterol with a cholesterol-like ring, lacked significant antibacterial activity (MIC > 128 μM). In contrast, UA, a pentacyclic triterpenoid with polar hydroxyl groups, demonstrated potent activity. Studies suggest that UA may damage bacterial cell walls and inhibit biofilm formation by interfering with membrane integrity (Kim et al., 2011; Lyu et al., 2021), whereas the high hydrophobicity of beta-sitosterol may hinder its interactions with the bacterial cell envelope. The inhibitory effects of UA on *S. mutans* and *S. sobrinus* biofilm formation align with existing literature (Liu et al., 2024; Liu et al., 2021). Our results demonstrated that UA at 8, 16, and 32 μM notably reduced bacterial adherence by approximately 14.2 ± 3.5%, 4.8 ± 1.2%, and 3.5 ± 0.8% for *S. mutans* and by 54.5 ± 8.2%, 9.8 ± 2.3%, and 4.1 ± 0.9% for *S. sobrinus* relative to an untreated control (see Figure 2). The potential mechanisms by which UA impedes biofilm formation may include the down-regulation of *gtf* genes, which encode glucosyltransferases (GTFs) responsible for synthesizing the water-insoluble glucans essential for biofilm stability (Hasan et al., 2012; Subramenium et al., 2015). Moreover, the *spaP* gene, encoding the antigen I/II surface protein, is pivotal for initial adherence to salivary agglutinins on tooth enamel (Hasan et al., 2012). The complete inhibition of biofilm formation by UA at elevated concentrations suggests that UA can effectively penetrate and dismantle the established biofilm matrix.

This study demonstrated the effectiveness of UA in disrupting biofilms formed by *S. mutans* and *S. sobrinus,* which are key for the prevention of dental caries. The observed concentration-dependent reductions in biofilm viability and thickness were consistent with those in a previous study by Klaophimai et al. (2023) on the reduction in biofilm viability and thickness by another natural compound called rhinacanthin-C, which is isolated from *Rhinacanthus nasutus.* Moreover, curcumin, another natural product, exhibits similar antibiofilm activity against *S. mutans* (Li et al., 2018; Li et al., 2019). In comparison with 0.12% CHX (Brookes et al., 2020), the established antibiofilm agent, UA demonstrated a clear dose-dependent effect but was generally less potent. This suggests that UA may require further optimization or formulation strategies to achieve efficacy comparable to that of CHX (Lu et al., 2019). Interestingly, while CHX completely inhibited initial biofilm formation (adherence assays, Figures 2A and B), it did not completely eliminate preformed mature biofilms (viability/thickness assays, Figures 3C and F). This difference is likely due to the protective nature of the EPS matrix in mature biofilms, which restricts the diffusion of antimicrobial agents (Hope & Wilson, 2004; Maruo et al., 2008). While CHX exerts potent bactericidal activity through electrostatic binding to negatively charged bacterial membranes, resulting in membrane disruption, cytoplasmic leakage, and coagulation (Chatzigiannidou et al., 2020; Lamarque et al., 2019; Poppolo Deus & Ouanounou, 2022; Shen et al., 2011), when applied during the early stages of colonization, CHX effectively kills planktonic cells before adherence, thereby preventing biofilm initiation. In contrast, CLSM analysis (Figure 3) was used to assess the effect of CHX on mature biofilms that had already developed structural integrity and EPS networks. Mature biofilms provide substantial protection for the bacterial communities, hindering the penetration of antimicrobial agents and restricting the access of CHX to all viable cells within the biofilm. Therefore, while CHX substantially reduces viability and thickness, some residual biofilm mass remains owing to the mechanical robustness and resistance of the EPS matrix. Further investigation is needed to elucidate the specific mechanisms by which UA disrupts biofilms in *S. mutans* and *S. sobrinus* (Chen et al., 2022; Zhang et al., 2021). Additionally, studies exploring formulations or combinations with other agents that could enhance the efficacy of UA would be valuable for determining the potential clinical applications of this compound (Burne et al., 2009; Ham & Kim, 2023).

## 5. CONCLUSION

In summary, UA exhibits selective antibacterial and antibiofilm effects against *S. mutans* and *S. sobrinus*. These results highlight the potential of UA as a natural therapeutic agent in dental care. Future research should focus on the specific molecular mechanisms of biofilm disruption and the development of optimized delivery systems to enhance its clinical anticariogenic potential.

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## AUTHOR CONTRIBUTIONS

A.K., P.P., K.C., C.P. responsible for the initial study concept and design, shaping the scientific inquiry and methodology. A.K. and S.K. conducted the experiments, including Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays, as well as biofilm formation evaluations. A.K., P.P., S.K. analyzed the experimental data, focusing on confocal

microscopy images and statistical analysis. A.K., P.P., and S.K. drafted the manuscript, incorporating critical revisions concerning the research content; all co-authors reviewed and critically revised the manuscript for important intellectual content; approved the final version to be published. A.K. secured funding for the research project and provided necessary resources. K.C. supervised the overall project, provided mentorship to co-authors, and oversaw research progress.

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