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Antibacterial activity of chrysophsin-3 against oral pathogens and *Streptococcus mutans* biofilms

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ABSTRACT

Dental caries and pulpal diseases are common oral bacterial infectious diseases, the prevention and treatment of these diseases require the control of the causative pathogens, such as S*treptococcus mutans* (*S. mutans*) and *Enterococcus faecalis*. As a cationic antimicrobial peptide, Chrysophsin-3 has broad-spectrum bactericidal activity against both Gram-positive and Gram-negative bacteria which may cause a variety of oral infectious diseases. The present study evaluated the potential of chrysophsin-3 against several oral pathogens and *S.mutans* biofilms. The cytotoxic activity of chrysophsin-3 against human gingival fibroblasts (HGFs) was investigated for potential oral application. We use minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and time-kill assay to evaluate the killing effect of chrysophsin-3. Then scanning electron microscopy (SEM) and transmission electron microscope (TEM) were used to analyze the change of morphology and membrane of the pathogens, Live/Dead staining and confocal scanning laser microscopy (CSLM) was used to observe *S. mutans* biofilms. The results indicate that chrysophsin-3 has varying antimicrobial activities against different oral bacteria. Chrysophsin-3 did not cause obvious cytotoxicity in HGFs at concentrations of 32-128 μg/ml for 5 min or 8 μg/ml for 60 min. SEM revealed membranous blebs and pore formation on the bacterial cell surface, and TEM showed loss of the nucleoid and dissolution of the cytoplasmic space. Furthermore, the CSLM images indicate that chrysophsin-3 can reduce the viability of the cells within the biofilms significantly and had a comparatively lethal effect against *S. mutans* biofilms. Taken together, our finding suggests that chrysophsin-3 has potential clinical application in oral infectious disease, especially in preventing and treating dental caries.

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Introduction

It is well known that numerous microorganisms are harbored in oral cavity, in which many of them have been implicated in a variety of oral infectious diseases, such as dental caries, pulpal and periapical disease. As one of the most prevalent infectious oral diseases, dental caries has led to a heavy burden on both the economy and public health worldwide(1, 2). Regarding pathogenesis, many species of cariogenic bacteria play an important role during the development of caries, such as *Streptococcus spp., Lactobacillus* spp., *Actinomyces* spp.(3). *Streptococcus* spp. and *Lactobacillus* spp. can produce acidic by-products, disrupting demineralization and remineralization of the hard tissue(4). *Actinomyces* spp. is involved in early plaque development on tooth surfaces and contributes to root caries(5). With the spread of the infection, pulpal inflammation and apical periodontitis may arise(6). As an opportunistic pathogen, *Enterococcus faecalis* is commonly detected in asymptomatic, persistent endodontic infections, especially from the teeth with root canal treatment failure, for its ability to compete with other microorganisms, invade dentinal tubules, and resist nutritional deprivation(7). It is well accepted that most microorganisms exist as biofilm communities that process special physical and biochemical properties, helping enhance adhesion strength, defend against external aggression and maintain the acidic microenvironment, thus increasing the survival capacity of the bacteria(8). *Streptococcus mutans* (*S. mutans*) has been demonstrated to be a primary etiologic agent of dental caries(3), it can express glucosyltransferases for the synthesis of extracellular glucans, promoting bacterial adhesion and biofilm formation(9). Moreover, *Candida albicans* can increase the cariogenicity of oral biofilm by altering its microbial ecology, leading to a polymicrobial biofilm with enhanced acidogenicity(10). Thus, effective control of these pathogenic bacteria and biofilms is key to the prevention and treatment of these oral infectious diseases. So far, many kinds of antimicrobial agents such as chlorhexidine, antibiotics and phenolic compounds were used to inhibit the development of biofilm, however, they can lead to some undesirable side effects, such as antimi-

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crobial resistance, and tooth staining(11, 12). Thus, it is of great importance to developing new antibiotics or substitutes(13, 14).

Antimicrobial peptides (AMPs) are a wide-ranging class of small-molecule peptides existing widely in nature(15). As amphiphilic cations, AMPs have a variety of biological functions, such as antimicrobial, anticancer, immunomodulatory, and wound-healing activities(16-18). Thus, they are important effectors in the innate immune system and the first line of defense protecting against pathogen infection(19). Furthermore, most AMPs have shown the advantages of low toxicity, strong thermal stability, and lack of resistance(20). In light of recent studies, AMPs have potent antimicrobial activities against a variety of bacteria, fungi, and viruses(21). They can enter the cell and target different points, causing multiple activities, such as inhibiting the synthesis of nucleic acids and proteins and affecting the cell cycle(22, 23). Therefore, AMPs have been recognized as one of the important candidates to conventional antibiotic treatment for oral diseases caused by oral pathogenic bacteria(24), especially the prevention and treatment of dental caries(25).

At present, there are more than 3100 natural AMPs have been found, in which, chrysophsin-3 derived from the gills of the red sea bream, chrysophsin major, drew our interest. Chrysophsin-3 is an amphipathic cationic 20-amino acid peptide that is rich in histidine residues(26). It has been recently demonstrated that chrysophsin-3 can kill the sporulated, germinated and vegetative stages of *Bacillus anthracis*, and penetrate and kill the spores without full germination (27), indicating the significant bactericidal activity of chrysophsin-3. Chrysophsin-3 has an unusual C-terminal domain (RRRH sequence) that makes it the possibility for lipid bilayer insertion as a single molecule or as an aggregate, which is similar to chrysophsin-1(26, 28). We have previously demonstrated that chrysophsin-1 has a promising effect on a variety of oral pathogens and *S. mutans* biofilms(29). Accordingly, it can be speculated that Chrysophsin-3 may have potential antimicrobial activities, providing an alternative antimicrobial therapy for the treatment and prevention of oral infectious disease, especially dental caries.

In this study, we investigated the antimicrobial activity and mechanism of chrysophsin-3 against several major oral pathogens and *S. mutans* biofilms in vitro. Furthermore, given the therapeutic potential of chrysophsin-3 in the oral cavity, the toxicity of the peptide against human gingival fibroblasts (HGFs) was evaluated, hoping to set a foundation for the further practical application of chrysophsin-3 in the treatment and prevention of oral infectious diseases, especially dental caries..

Materials and Methods

Bacterial strains and growth conditions

Streptococcus mutans UA159, *Streptococcus gordonii* ATCC 10558, *Streptococcus sobrinus* ATCC 6715, *Streptococcus sanguinis* ATCC 10556, Actinomyces naeslundii ATCC 12104, *Actinomyces viscosus* ATCC 15987, *Enterococcus faecalis* ATCC 29212 and *Candida albicans* ATCC 90028 (Table 1) were grown in Brain Heart Infusion (BHI) broth (BD-Difco, Detroit, MI, USA). *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus casei* ATCC 393 and *Lactobacillus fermenti* ATCC 9338 were grown on de Man, Rogosa and Sharpe (MRS) medium (BD-Difco). *Candida albicans* was grown aerobically, whereas the other strains were cultured anaerobically (80% N_2, 10% H2 and 10% CO_2) for 24 or 48 h (37°C) prior to use.

Peptide synthesis and purification

Chrysophsin-3 (FIGLLISAGKAIHDLIRRRH) was synthesized using standard solid-phase (Fmoc) methods (CL Bio-scientific company (Xi'an, China)). Purification and quality were confirmed by high-performance liquid chromatography (HPLC). The peptide was dissolved in sterile deionized water (5120 μg/ml) and stored at –20 °C.

MIC and MBC assays

Peptide MICs were determined using a broth microdilution assay(30). Briefly, bacterial cells were grown overnight in BHI or MRS and diluted to 10^5CFU/ml in a culture medium prior to use. Two-fold serial dilutions of peptides were prepared in the medium at a volume of 200 μl/well in 96-well plates. The final concentration of peptides ranged from 0.125 to 256 μg/ml. Water was added to a separate well to serve as a control. The plates were then incubated at 37°C under anaerobic conditions for 24 or 48 h. The MIC was defined as the lowest peptide concentration present in the clear well by visual inspection(31). For the MBC, 100 μl of the well contents were spread on agar and grown at 37°C for 24 or 48 h. The MBC was defined as the lowest peptide concentration resulting in no bacterial growth in the medium(32). Each assay was performed at least three times for all bacteria.

Time-killing assays

The killing kinetics of chrysophsin-3 against *S. mutans* were analyzed using a time-kill assay as previously described(33). *S. mutans* was grown to an exponential phase and diluted to 10^5 CFU/ml in a growth medium. Chrysophsin-3 was added to the *S. mutans* suspension at concentrations of 2- and 4-times the MICs using the time-kill methodology (CLSI M26-A). At 0, 1, 2, 5, 15, 30, 60, 120 and 240 min, 10 μl of cell suspension was collected, diluted in medium (1:50) and immediately put on ice to halt growth. Aliquots (50 to 200 μl) were then spread on agar plates, and colonies were counted after 24 h under anaerobic conditions(37°C). The assays were performed at least three times.

Cytotoxicity assay

HGFs were thawed and cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, USA) supplemented with 10% newborn calf serum (GIBCO, USA) and 1% penicillin-streptomycin (GIBCO BRL, USA) as previously described(29). The sixth passage of these cell cultures was used for the experimental studies. HGFs were seeded at 2×10^3 cells per well in 96-well plates and at 10^5 cells per well in 12-well plates. After the cells were cultured for 48 h, they were exposed to chrysophsin-3 at concentrations of 8, 32, or 128 μg/ml for 5, 60, or 240 min. Cells that were exposed to the medium alone served as controls. The cell viability and levels of extracellular lactate dehydrogenase (LDH) were evaluated as previously performed(29).

SEM and TEM observations of oral pathogens

Exponential-phase *S. mutans* and *L. fermenti* cells were

treated with chrysophsin-3 (at 10 or 60 times their MICs) for 4 h at room temperature. After treatment, the bacterial suspensions were collected by centrifugation for 5 min at 2500 rpm, and the supernatants were removed. The deposits were fixed with 2.5% buffered glutaraldehyde for 24 h and processed for SEM (S-4800, Hitachi, Japan) and TEM (JEM-2000EX, JEOL Ltd, Japan) observations. Bacteria that were not treated with Bac8c were processed in an identical fashion to the experimental samples and served as controls.

Biofilm susceptibility assay

S. mutans biofilms for CLSM (FluoView 1000, OLYMPUS, Japan) were generated by placing 100 µl aliquots of an overnight culture (diluted to 10^7 CFU/ml) on each of the chambered cover glasses (Nagle Nunc International, Rochester, NY) and allowing the attachment of the bacteria for 30 min at room temperature. BHI (2 ml) supplemented with 1% sucrose was added to each well, and the chambers were incubated for 24 h at 37°C anaerobically. The biofilms were then washed twice with sterile deionized water and treated with chrysophsin-3 at concentrations of 64 µg/ml and 128 µg/ml. Biofilms treated with 0.12% chlorhexidine (CHX) for 1 h served as a positive control, and biofilms left untreated served as a negative control. To examine the effects of chrysophsin-3 against *S. mutans* biofilms, biofilms were stained in the dark for 15 min using a LIVE/DEAD® BacLightTM Bacterial Viability kit (L13152, Invitrogen Inc. USA). The stained biofilms were observed under a confocal laser scanning microscope (CLSM) (FluoView 1000, Olympus, Japan), and stacks of images were obtained at 60× magnification. The "Image to Stack" and "Reslice" commands of Image J were used to generate Z-axis images. The fluorescence intensity (FI) of the images was analyzed using Image Pro-Plus software 6.0(34).

Statistical analysis

Cell viability, levels of extracellular LDH and *S. mutans* biofilm viability were evaluated using one-way ANOVA and Tukey's HSD tests. Statistical analyses were performed by SPSS 18.0 software at a significance level of $P < 0.05$.

Results

Antimicrobial activity

Chrysophsin-3 displayed different antimicrobial activities on different oral bacteria (Table 1). The MIC values ranged from 8 to 128 µg/ml, and the MBC values ranged from 16 to 256 µg/ml. *S. sanguinis* and *L. fermenti* were more susceptible to chrysophsin-3, whereas *C.albicans* and *S. sobrinus* were relatively resistant to chrysophsin-3. In general, the MBC values of the oral bacteria were two to four times higher than their MIC values. The killing kinetics of chrysophsin-3 against *S. mutans* were analyzed by time-kill assays (Fig.1). The killing of *S. mutans* by chrysophsin-3 was time-dependent within 240 min of incubation. At high concentrations, chrysophsin-3 had increased killing kinetics compared to chrysophsin-3 at low concentrations, i.e., at 2 times the MIC, *S. mutans* was killed within 240 min, whereas at 4 times the MIC, *S. mutans* was killed within 30 min.

Cytotoxicity

Chrysophsin-3 did not cause obvious cytotoxicity in HGFs at 8-128 µg/ml for 5 min or at 8 µg/ml for 60 min (Fig. 2A). There were no significant differences between the chrysophsin-3-treated groups under the above conditions and the control groups ($P>0.05$). However, chrysophsin-3 significantly inhibited HGF proliferation at 32-128 µg/ml for 60-240 min. In addition, chrysophsin-3 did not compromise the membrane integrity of HGFs at 8-128 µg/ml for 5 min or at 8-32 µg/ml for 60 min (Fig. 2B). Similar levels of extracellular LDH were observed between

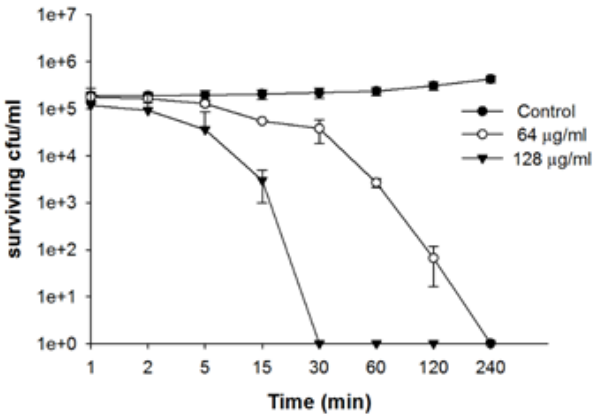

Figure 1. Time–kill curves of *S. mutans* treated with different concentrations of chrysophsin-3. The surviving bacteria were plated at various time points (1 to 240 min). All data points represent the mean ± SD from at least three independent experiments.

Table 1. In vitro susceptibility of oral microbes to chrysophsin-3.

Microbe	Source	MIC range (µg/ml)	MBC range (µg/ml)
Streptococcus mutans	UA 159	32	64
Streptococcus sanguinis	ATCC 10556	8-16	16-32
Streptococcus sobrinus	ATCC 6715	32-64	128
Streptococcus gordonii	ATCC 10558	16	32-64
Actinomyces viscosus	ATCC 15987	32	64
Actinomyces naeslundii	ATCC 12104	16-32	32-64
Lactobacillus acidophilus	ATCC 4356	64	128
Lactobacillus casei	ATCC 393	16-32	32-64
Lactobacillus fermenti	ATCC 9338	2-8	8-64
Enterococcus faecalis	ATCC 29212	16	32
Candida albicans	ATCC 90028	128	256

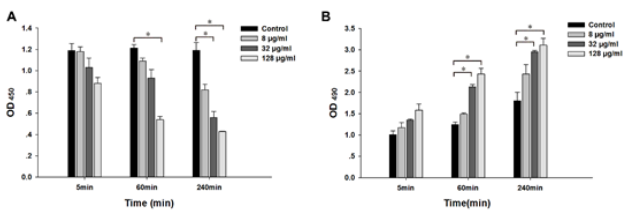

Figure 2. Cytotoxicity of chrysophsin-3 treatment on human gingival fibroblasts. (A) Inhibition of proliferation, determined by CCK-8 assay. (B) Membrane integrity, determined by LDH assay. Columns and bars represent the mean ± SD from at least three independent experiments. * P< 0.05, chrysophsin-3-treated group versus the corresponding control group.

the chrysophsin-3-treated cells and untreated cells. There were no significant differences between the chrysophsin-3-treated groups under the above conditions and the control groups (P>0.05).

Morphological observation by SEM and TEM

According to SEM and TEM (Fig. 3), chrysophsin-3 can induce damage of bacterial cells just like other cationic antimicrobial peptides. The SEM images revealed some cellular debris around the *S. mutans* and membranous blebs on the surface of the *L. fermenti* after exposure to chrysophsin-3 at 10 times their MICs. Membrane wrinkling was apparent both in *S. mutans* and *L. fermenti* after exposure to chrysophsin-3 at 60 times their MICs. Specifically, there appears to be cellular debris in *S. mutans* and obvious pore formation in *L. fermenti* (Fig. 3A). In the control group, bacteria had intact and smooth surfaces without any cell lysis or debris apparent. To further elucidate the possible mechanisms of chrysophsin-3 on bacteria, TEM was performed to observe any ultrastructural damage (Fig. 3B). Chrysophsin-3 at 10 times its MIC did not cause any obvious ultrastructural changes in *S. mutans* but led to the loss of nucleoid in some *S. mutans* cells at 60 times its MIC. The translocation of *L. fermenti* nucleoid at the division septa was found after exposure to chrysophsin-3 at 10 times its MIC. More severe effects occurred in *L. fermenti* after exposure to chrysophsin-3 at 60 times its MIC, including cell wall breaks and dissolution of the cytoplasmic space.

Biofilm susceptibility assay

Images of *S. mutans* biofilms treated with chrysophsin-3 and CHX for 1 h are shown in Fig.4, and the fluorescence intensity (FI) of the images was quantified in Fig.5, which served as the viability of S. mutans biofilms. There were many water channels in the 24h biofilms, which may be the sites of nutrient and metabolite exchange. Treatment of biofilms with chrysophsin-3 did not result in the dose-dependent killing of the bacteria because there was no significant difference between the 128 μg/ml and 64 μg/ml treatments. The cells around the channels were stained with the red dye PI (dead, Fig. 4A, arrow), whereas those cells away from channels were stained with the green dye

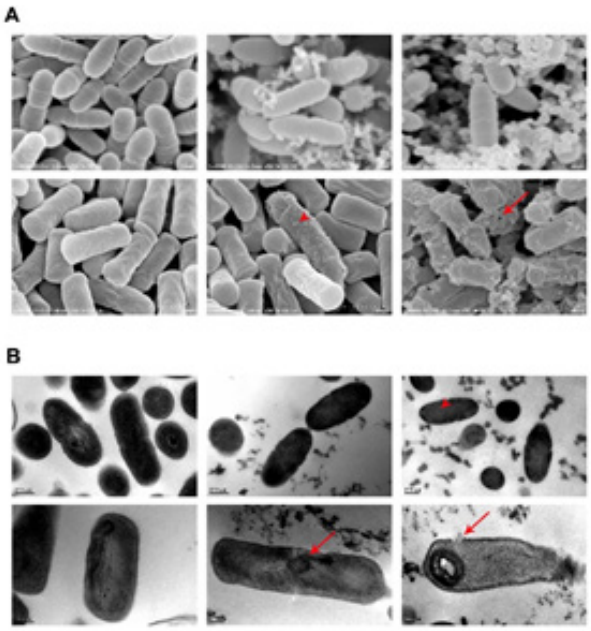

Figure 3. Structural observations of *S. mutans* and *L. fermenticells* subjected to chrysophsin-3 treatment (at 10 times and 60 times their MICs for 4 h at room temperature). Scanning electron microscopy (A) and transmission electron microscopy (B): Untreated *S. mutans* (upper left panel), *S. mutans* treated with chrysophsin-3 at 10 times its MIC (upper middle panel), *S. mutans* treated with chrysophsin-3 at 60 times its MIC (upper right panel); untreated *L. fermenti* (bottom left panel), *L. fermenti* treated with chrysophsin-3 at 10 times its MIC (bottom middle panel), *L. fermenti* treated with chrysophsin-3 at 60 times its MIC (bottom right panel). A: Red arrowhead indicates membranous blebs; Red arrow indicates pore formation. B: Red arrowhead indicates nucleoid loss; Red arrow indicates translocation of nucleoid and cell wall breaks, respectively.

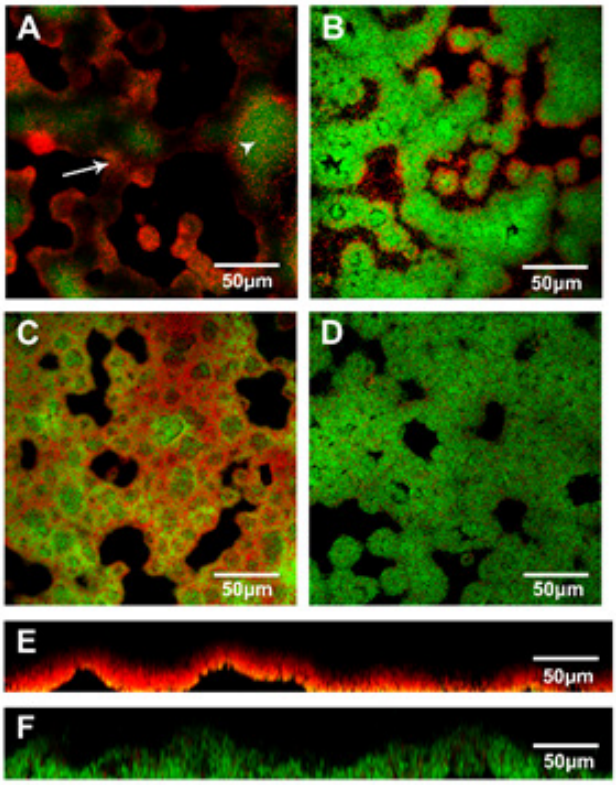

Figure 4. CLSM images of *S. mutans* biofilms treated with chrysophsin-3 and CHX. Dead cells were stained red, whereas live cells were stained green using the LIVE/DEAD BacLight viability stain. The biofilms for the 24 h incubations were treated with 128 μg/ml chrysophsin-3 (A), 64 μg/ml chrysophsin-3 (B), 0.12% CHX (C) and the control (D). The Z-axis images were reconstructed using ImageJ software for biofilms treated with chrysophsin-3 at 128 μg/ml (E) and the negative control (F).

STYO-9 (living, Fig. 4A, arrowhead). The cell viability in the biofilms treated with chrysophsin-3 was 67% at 64 μg/ml and 36% at 128 μg/ml. Meanwhile, the cell viability was 33.54% within biofilms treated with 0.12% CHX and 88.23% in the negative control. There were significant differences between the groups treated with chrysophsin-3 at 128 μg/ml and the negative control ($P < 0.05$), as well as between the groups treated with 0.12% CHX and the negative control. The reconstructed Z-axis image indicates that chrysophsin-3 at 128 μg/ml could kill most of the bacteria in the entire biofilms; however, there were still some viable bacteria at the bottom of the biofilms (Fig 5).

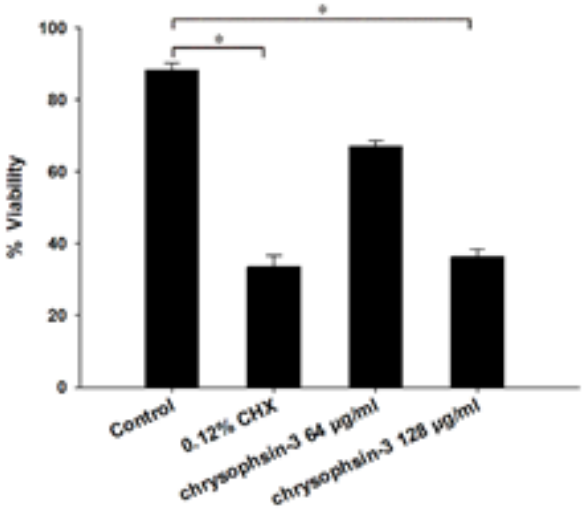

Figure 5. The viability of *S. mutans* biofilms treated with chrysophsin-3 and CHX.The fluorescence intensity (FI) of the images was quantified using Image ProPlus software 6.0. Each image taken in the green or red channel (FIg or FIr) is directly proportional to the number of bacteria with intact or compromised membranes, respectively. The FIg / (FIg +FIr) ratio served as an index of *S. mutans* biofilm viability (B). * $p < 0.05$, the treated group versus the negative control.

Discussion

So far, many kinds of AMPs have been recognized as promising alternatives to conventional antimicrobial strategies for their potent antimicrobial activities and pleiotropic bioactive functions(25). Few studies have explored the effects of chrysophsin-3. As the most prominent odontogenic infectious disease, dental caries can originate from lots of cariogenic bacteria. In this study, we investigated the antimicrobial activity of chrysophsin-3 against a panel of major oral pathogenic bacteria. Meanwhile, *S. mutans,* a causative agent of dental caries, was chosen as a model bacterium to explore the effect of chrysophsin-3 on bacterial survival in planktonic culture, as well as on biofilm formation.

According to the results, chrysophsin-3 can inhibit the growth of pathogenic bacteria significantly, and different oral microbes show varying susceptibility to it. The data showed that the MBC values of chrysophsin-3 were two to four times higher than the MIC values, suggesting a high bactericidal activity of chrysophsin-3 against the tested oral microbes. While putting chrysophsin-3 into clinical use, we have to make sure about its safety. On the basis of the cytotoxicity assay, treating with chrysophsin-3 at concentrations from 8 to 128 μg/ml for 5 min did not cause obvious cytotoxicity in HGFs. Generally, the typical treatment duration for oral rinse formulations may last 30 s to 2 min(35). Thus, we can infer that short-term treatment of chrysophsin-3 in the oral cavity will not affect the biological activity of HGFs, indicating that chrysophsin-3 has the potential for use in the prevention and treatment of dental caries.

The ability of AMPs to avoid pathogen resistance is largely due to the mechanism by which they kill bacteria. Currently, the barrel-stave, toroidal, and carpet models are the three most widely recognized mechanisms(18, 36, 37). In the barrel-stave and toroidal theories, AMPs insert themselves into the bacterial cell membrane perpendicularly, forming a bundle with a cylindrical central lumen or causing a continuous bending of the lipid monolayer in a toroidal pore, respectively, finally resulting in the loss of cellular constituents. The carpet model supposes that AMPs align themselves parallel to the membrane surface and induce membrane disintegration by micelle formation(38-41). Studies have suggested that the nature of chrysophsin-3 interactions with a PC bilayer is concentration-dependent and consistent with the barrel-stave model. At low concentrations, chrysophsin-3 preferentially inserts into the membrane over adsorption onto the surface. As the concentration increases, the peptide continues to form pores and begins to adsorb onto the bilayer surface. At a certain critical concentration, peptide lipid aggregates begin to be removed from the membrane(42). It has been suggested that chrysophsin-3 may act on vegetative *B. subtilis* cells by forming pores that destabilize the cell membrane, causing vital cellular contents to be lost from the cell(27). Currently, the use of microscopy to visualize the effects of antimicrobial peptides on microbial cells has helped to identify general target sites. Here, we chose *S. mutans* and *L. fermenti* to perform the study by SEM and TEM. These results are consistent with previous studies, i.e., that the effect of chrysophsin-3 on *S. mutans* and *L. fermenti* is concentration-dependent. As the concentration increased, pore formation and more damage were observed. Pore formation and membrane rupture verify the mechanism of barrel-stave action of chrysophsin-3 to some extent. Meanwhile, membrane blebbing was found both in *S. mutans* and *L. fermenti* (data not shown for *S. mutans*). Lehrer et al. noted the appearance of membranous blebs on HNP-treated *E. coli* followed by the loss of bacterial viability(43), indicating that the membrane blebbing of *S. mutans* and *L. fermenti* is an existing condition of cellular damage. Membrane blebbing is deemed to be the indication that lipopolysaccharide, existing in the cell wall of Gram-negative bacteria predominantly, has been released from the cell surface(44). The underlying mechanism needs to be elucidated in the future.

As is well known that *S. mutans* is a causative bacterium of dental caries and is recognized as an indicator of cariogenic biofilms. They can propagate bacterial adhesion and biofilm formation by promoting the synthesis of extracellular glucans(45). It is reported that *S. mutans* has developed fluoride resistance, which is one of the first-line interventions to prevent caries development(46). Furthermore, some *S.mutans* strains which are more resistant to AMPs may have an ecological advantage to preferentially colonizing within dental plaque(47). Thus, the elimination of plaque biofilms is key to the prevention of dental car-

ies, especially *S. mutans* biofilm. However, as mentioned above, the biofilm is one of the important factors for antibiotic resistance because bacteria within a biofilm are always less susceptible to antimicrobial agents than their planktonic counterparts(48). Most antibiotics tend to kill planktonic bacteria effectively but not bacteria in biofilms, mainly due to the slow growth rate and low metabolic activity of bacteria in such communities (49). To date, many agents have been introduced as antibacterial agents (50-52). Although chlorhexidine is a potent anti-biofilm chemical agent, its clinical application is limited because of its bitter taste and tooth staining when frequently used. A previous study demonstrated that some AMPs have promising antiplaque capabilities (49). Within the biofilms treated with chrysophsin-3 (128 μg/ml), it was obvious that the green fluorescence of the live bacteria decreased significantly, which was enwrapped by the red fluorescence of the dead bacteria. Cell viability of 36% represents a preferable anti-biofilm effect. According to the reconstructed Z-axis image, chrysophsin-3 (128 μg/ml) not only killed bacteria on the surface of biofilms but also at the bottom of biofilms. When the concentration was decreased to 64 μg/ml, there were more live bacteria, demonstrating that chrysophsin-3 at 64 μg/ml was not able to penetrate the interior of the biofilms and kill most of the bacteria. The inhibition of pre-formed biofilms is a significant challenge in treating dental caries. Our present results precisely demonstrate that chrysophsin-3 effectively prevented the development of mature biofilms, suggesting that chrysophsin-3 also has the potential ability to act on slow-growing or even non-growing bacteria.

In conclusion, chrysophsin-3 has a desirable antibacterial activity against *S.mutans*, as well as the other oral pathogens that were tested. Its inhibitory effect on *S. mutans* biofilms suggests that chrysophsin-3 might be useful in preventing and treating dental caries. Furthermore, it appears feasible to employ antibacterial peptides during future therapies for oral infections as one approach to dealing with the increase in microbial resistance to antibiotics. Being readily accessible for local application, the oral cavity may be particularly suitable for peptide therapy.

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Interest conflict
None.

Author's contribution
Wei Wang contributed to the conception of the study; Kejing Wang and Lipeng Hou Performed the experiment and manuscript preparation; Zi-ang Sun contributed to data analysis.

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