In vitro anti-bacterial activity of diosgenin on *Porphyromonas gingivalis* and *Prevotella intermedia*

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Abstract. Diosgenin (Dios), a natural steroidal sapogenin, is a bioactive compound extracted from dietary fenugreek seeds. It has a wide range of applications, exhibiting anti-oxidant, anti-inflammatory and anti-cancer activities. However, whether the extracts have beneficial effects on periodontal pathogens has so far remained elusive. The aim of the present study was to investigate the anti-bacterial effects of Dios on *Porphyromonas gingivalis* (*P. gingivalis*) and *Prevotella intermedia* (*P. intermedia*) *in vitro*. The anti-microbial effect of Dios on *P. gingivalis* and *P. intermedia* was assessed by a direct contact test (DCT) and the Cell Counting Kit (CCK)-8 assay at 60, 90 and 120 min. In addition, counting of colony-forming units (CFU) and live/dead cell staining were used to evaluate the anti-bacterial effects. The results of the DCT and CCK-8 assays indicated that Dios had beneficial dose-dependent inhibitory effects on *P. gingivalis* and *P. intermedia*. The CFU counting results also indicated that Dios had dose-dependent anti-bacterial effects on *P. gingivalis* and *P. intermedia*. Of note, Dios had significant anti-bacterial effects on the biofilms of *P. gingivalis* and *P. intermedia in vitro* as visualized by the live/dead cell staining method. In conclusion, the present results demonstrated that Dios had a marked anti-bacterial activity against *P. gingivalis* and *P. intermedia in vitro*, both in suspension and on biofilms. The present study highlighted the potential applications of Dios as a novel natural agent to prevent and treat periodontitis through its anti-bacterial effects.

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Introduction

Periodontitis (PD) is a chronic inflammatory disease that leads to progressive destruction of the periodontal ligament and alveolar bone and even causes the teeth to become loose and fall out (1). Currently, the incidence of chronic periodontitis is >90% in China, posing a serious threat to human oral health (2). Soft-tissue and bone-tissue destruction in PD caused by prolonged inflammation is initiated by bacterial colonization and invasion around teeth near the bottom of the periodontal pocket. It is well known that dental bacterial biofilms are the initiating factor of PD (3). Among these pathogenic bacteria, *Porphyromonas gingivalis* (*P. gingivalis*) and *Prevotella intermedia* (*P. intermedia*) have been indicated to have a strong relationship with PD initiation and progression (4,5). *P. gingivalis* is a key pathogenic factor in PD that accounts for a majority of periodontal tissue damage (6,7), and *P. intermedia* is frequently isolated from dental plaques of patients with periodontal diseases. *P. intermedia* is also associated with other oral infections, including pregnancy gingivitis (8,9). Therefore, PD is associated with *P. intermedia* and *P. gingivalis*. These species are considered periodontal pathogens that invade periodontal pockets and are frequently associated with periodontal breakdown (10-12). Typical initial therapy for PD involves mechanical and nanotechnological methods and near-infrared photodynamic therapy to clean bacterial plaques (13,14). However, complete elimination of pathogenic bacteria by mechanical cleaning is impossible, as certain pathogens can may be embedded in soft tissue (15). Nanotechnological methods may cause damage to the body; however, this remains uncertain at present. Hence, drug application is an important adjuvant therapy for PD. There are multiple anti-microbial options, such as metronidazole, chlorhexidine, minocycline, doxycycline and tetracycline, but these may result in drug resistance and oral dysbacteriosis (16).

Numerous natural products from Traditional Chinese Medicine have been indicated to be suitable for the treatment of PD due to their anti-bacterial effects, including herbal compounds (17), *Morus alba* leaves (18), psoralen and angelicin (19). Diosgenin (Dios) is a naturally occurring steroidal sapogenin and is one of the major bioactive compounds in dietary fenugreek seeds (Fig. 1). Dioscin, as

a derivative of Dios, has anti-*Candida* efficiency (20). Dios has a unique structural similarity to estrogen. In addition to being a lactation aid, Dios has been indicated to have hypocholesterolemic (21), gastro- and hepatoprotective (22), anti-oxidative (23), anti-inflammatory, anti-diabetic and anti-tumorigenic effects (24). These notable biological properties of Dios (e.g., anti-oxidative, anti-inflammatory, anti-diabetic and anti-osteoclastogenic) make it suitable for the treatment of PD. However, whether Dios has efficacy against PD-associated pathogenic bacteria (e.g., *P. intermedia* and *P. gingivalis*) has remained to be examined. As a potential novel clinical therapeutic application for the prevention and treatment PD, it is worthwhile to study the anti-bacterial activity of Dios, e.g. by investigating its anti-bacterial effects on PD-associated bacteria.

In the present study, the anti-bacterial effects of Dios on *P. gingivalis* and *P. intermedia* were evaluated by a direct contact test (DCT), the Cell Counting Kit (CCK)-8 assay and counting of colony-forming units (CFU) *in vitro*. In addition, the anti-bacterial biofilm effects of Dios on *P. gingivalis* and *P. intermedia* were determined by live/dead cell staining *in vitro*.

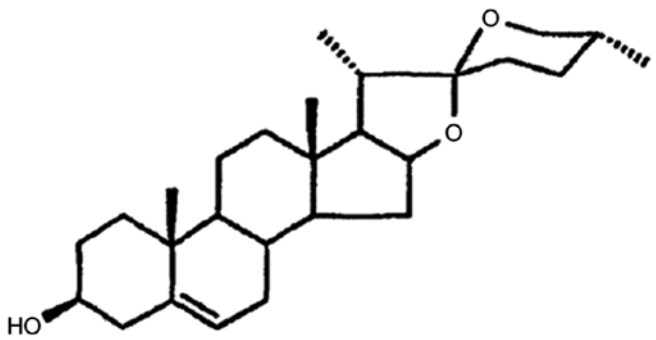

Figure 1. Molecular structure of diosgenin.

Materials and methods

Bacterial preparation. The anti-bacterial properties of Dios (cat. no. CSN12576; CSNpharm) were evaluated using *P. gingivalis* [no. American Type Culture Collection (ATCC)33227] and *P. intermedia* (no. ATTC 25671; both from ATCC) as model gram-negative bacteria. Glycerol stock solutions were used to inoculate defined overnight cultures in tryptic soy broth (TSB; Biti Medical Device Co., Ltd.) medium under anaerobic conditions (80% N_2, 10% H_2 and 10% CO_2) at 37˚C. One milliliter of each cell suspension was subcultured and harvested during the exponential growth phase. Subsequently, 100 μl of the *P. gingivalis* and *P. intermedia* solutions in a 96-well plate were monitored in a microplate spectrophotometer (Power Wave XS2; BioTek Instruments, Inc.) at 600 nm and samples with an optical density (OD) of ~0.12 were used in the following experiments. A 0.2% chlorhexidine (CHX) solution was used to establish a positive control group (25,26).

DCT. The test compounds were prepared at a concentration of 25 μM in anhydrous ethanol. A total of 90 μl TSB with different dilutions of Dios was added to a 96-well microplate and 10 μl bacterial suspension (prepared OD=0.12) was added. Subsequently, the plate with final concentrations of Dios of 1-100 μmol/l was measured in a microplate spectrophotometer. Wells containing media inoculated with bacteria but without compound were used as a control group. Each sample contained 0.1% (v/v) anhydrous ethanol. The 96-well microplates were incubated at 37˚C in an anaerobic incubator (80% N_2, 10% H_2 and 10% CO_2) and the absorbance of the 96-well microplate was read every 30 min in a microplate spectrophotometer to determine the absorbance value at 600 nm. Bacteria were treated with different concentrations of Dios for 2 h. Each group contained 5 replicate wells and the experiment was repeated three times.

CCK-8 assay. A CCK-8 assay was used to detect the viability of the bacteria in the present study. A total of 10 μl of *P. gingivalis* or *P. intermedia* bacteria (prepared OD=0.12) was cultured with 90 μl fresh TSB medium containing different concentrations of Dios. According to the bacterial dynamics, after culturing for 120 min in an anaerobic incubator at 37˚C, CCK-8 solution (10 μl/well) was added to the 96-well plate. After co-incubating for 30 min at a normal temperature in the dark, the 96-well plate was placed in a microplate spectrophotometer to determine the absorption value at 450 nm, which reflected the number of live cells in each well. Each group contained 5 replicate wells. Cell viability was expressed as the mean ± standard deviation (SD) of the absorbance for five wells for each group. The experiment was repeated three times.

CFU assay. The anti-bacterial activity of Dios against *P. gingivalis* and *P. intermedia* was determined by spread-plate CFU counting. The resulting colonies were counted to determine the CFUs and the growth inhibitory activity of the drug. The bacterial suspensions was incubated with Dios at different concentrations. Subsequently, 10 μl of 10-fold serial dilutions of the bacteria at different concentrations were plated onto brain heart infusion (BHI; Difco) agar plates and the plates were further incubated for 24 h at 37˚C under anaerobic conditions, with 3 replicate plates in each group. The colonies were counted after incubation at 37˚C for 24 h. Representative images of the BHI agar plates were acquired with an iPhone 7 Plus (Apple Inc.). Data from three replicate plates were acquired and the CFU count log reduction was calculated using GraphPad Prism 7 (GraphPad Software, Inc.). All experiments were performed under anaerobic conditions.

Biofilm viability. Cell slides were placed at the bottom of a 24-well plate. Subsequently, 300 μl of a *P. gingivalis* or *P. intermedia* suspension was cultured on each cell slide. After static growth for 1 h at 37˚C under anaerobic conditions, 2 ml fresh TSB culture solution was added to the 24-well plate and the cells were further cultured in an anaerobic incubator for 24 h to form bacterial biofilms. After washing with PBS 3 times, 2 ml of fresh medium containing 25 or 50 μM Dios was added for cocultivation for 1 h. Experiments with TSB and bacteria but no compound and with 0.2% CHX and an equal amount of bacterial suspension were also set up. After gently washing with PBS 3 times, 200 μl of working solution from the LIVE/DEAD BacLight Bacterial Viability Kit (Shanghai Yeasen Biotechnology Co., Ltd.) was added into the

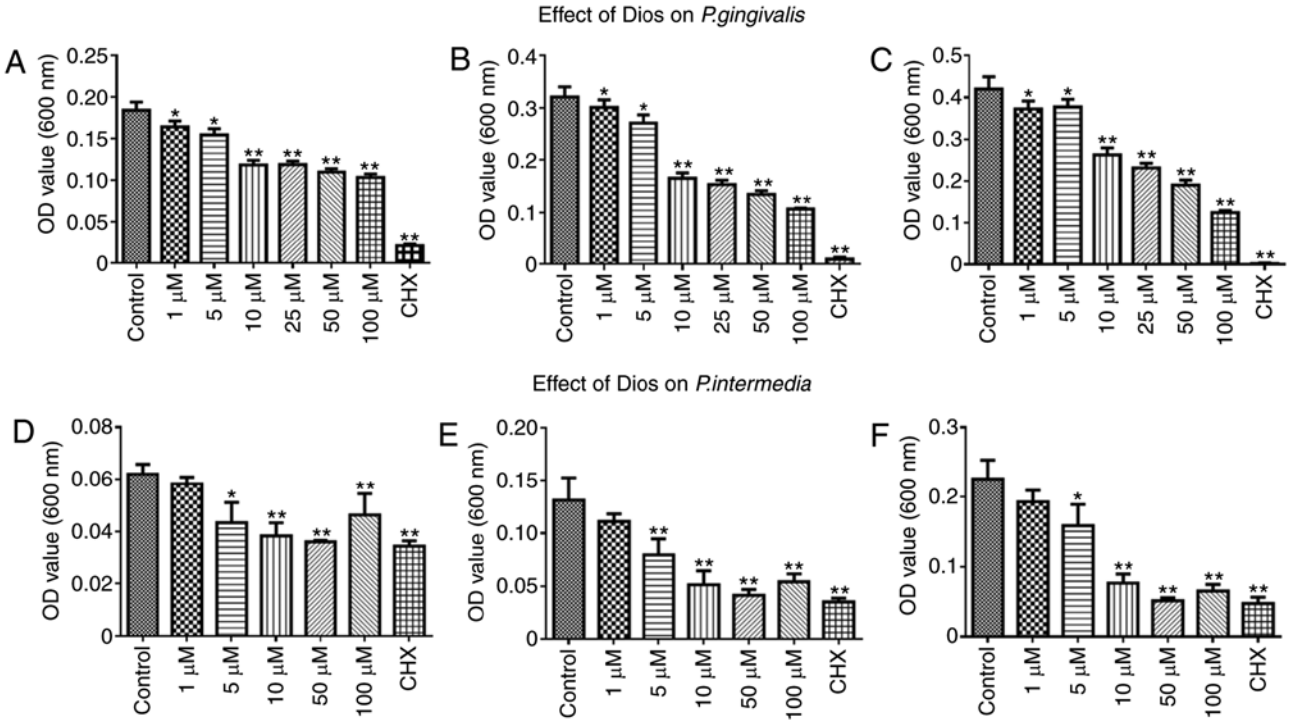

Figure 2. Effect of Dios on *P. gingivalis* and *P. intermedia* determined by a direct contact test. Effect of Dios against *P. gingivalis* over three periods: (A) 60 min, (B) 90 min, (C) 120 min; effect of Dios against *P. intermedia* over three periods: (D) 60 min, (E) 90 min, (F) 120 min. Bacterial suspensions in tryptic soy broth without Dios were used as a control. Data are presented as the mean ± SD (n=5). *P<0.05, **P<0.01 vs. control group. Dios, diosgenin; OD, optical density; CHX, chlorhexidine; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*.

24-well plate, followed by incubation in the dark at 37˚C for 15 min. After washing with PBS three times, the biofilm was imaged using confocal laser scanning microscopy (CLSM; Nikon AI Plus; Nikon Corp.) at excitation wavelengths of 488 nm (calcein-AM) and 561 nm (propidium iodide), with dead bacteria stained red and live bacteria stained green. The data were plotted to analyze the percent distribution of live and dead bacteria according to green and red fluorescence intensities. Images were obtained with a 20x objective and at least three images of randomly selected fields were collected for each sample.

Statistical analysis. All data were obtained from at least three independent experiments. Values are expressed as the mean ± SD. Analysis was performed using one-way ANOVA followed Dunnett's multiple-comparisons test with GraphPad Prism 7 (GraphPad Software, Inc.) and Microsoft Excel (Office 365; Microsoft Corp.). P<0.05 was considered to indicate statistical significance.

Results

Effects of Dios on planktonic P. gingivalis and P. intermedia. The anti-bacterial activity of Dios against *P. gingivalis* and *P. intermedia* was assessed by a DCT. As presented in Fig. 2, it was demonstrated that the growth of *P. gingivalis* or *P. intermedia* was inhibited after incubation with Dios for 60, 90 and 120 min. Furthermore, increasing concentrations of Dios led to increasingly obvious growth inhibition of *P. gingivalis* and *P. intermedia* (5-100 μM, P<0.05). However, there was no significant difference between the control group and the 1 μM group (P>0.05). In summary, the growth of *P. gingivalis* or *P. intermedia* was dose-dependently inhibited by Dios.

Effects of Dios on planktonic P. gingivalis and P. intermedia. The anti-bacterial activity of Dios was further confirmed by the CCK-8 assay. As presented in Fig. 3, the bacterial activity decreased after treatment with Dios and was negatively correlated with the dose of Dios. Consistent with the previous results, 1 μM Dios did not have any marked effects on bacterial activity; furthermore, the growth of *P. gingivalis* or *P. intermedia* was dose-dependently inhibited by Dios.

Effects of Dios on planktonic P. gingivalis and P. intermedia. To investigate the anti-bacterial activity of Dios against *P. gingivalis* or *P. intermedia*, the CFU counting method was used (Fig. 4). After coculture with Dios for 24 h, the number of bacterial CFU was markedly decreased. As the concentration of Dios increased to 5, 10, 50 and 100 μM, the number of bacterial CFU decreased correspondingly (Fig. 4A and C). The log statistics of the clone count indicated a significant anti-bacterial effect (Fig. 4B and D). The levels of both *P. gingivalis* and *P. intermedia* in the 5, 10, 50, 25 and 100 μM groups were lower than those in the control group by one order of magnitude (P<0.05). The results obtained confirmed the dose-dependent anti-microbial activity of Dios.

Anti-biofilm effects of Dios. The viability of mature biofilms was investigated by CLSM. The biofilm viability in the 25 and 50 μM treatment groups was markedly lower than that in the control group (P<0.05). The CLSM images displayed the distributions of live (green) and dead (red) bacteria within

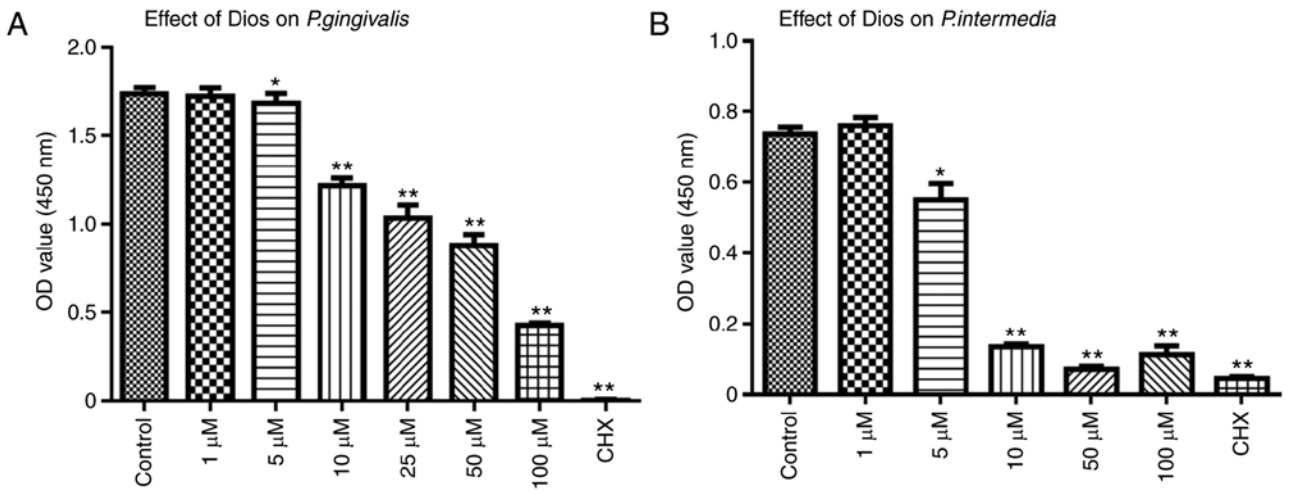

Figure 3. Inhibitory effects of Dios on (A) *P. gingivalis* and (B) *P. intermedia*. The viability of *P. gingivalis* and *P. intermedia* was tested by a Cell-Counting-Kit 8 assay. Bacterial suspensions in tryptic soy broth without Dios were used as a control. Data are presented as the mean ± standard deviation (n=5). *P<0.05, **P<0.01 vs. control group. Dios, diosgenin; OD, optical density; CHX, chlorhexidine; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*.

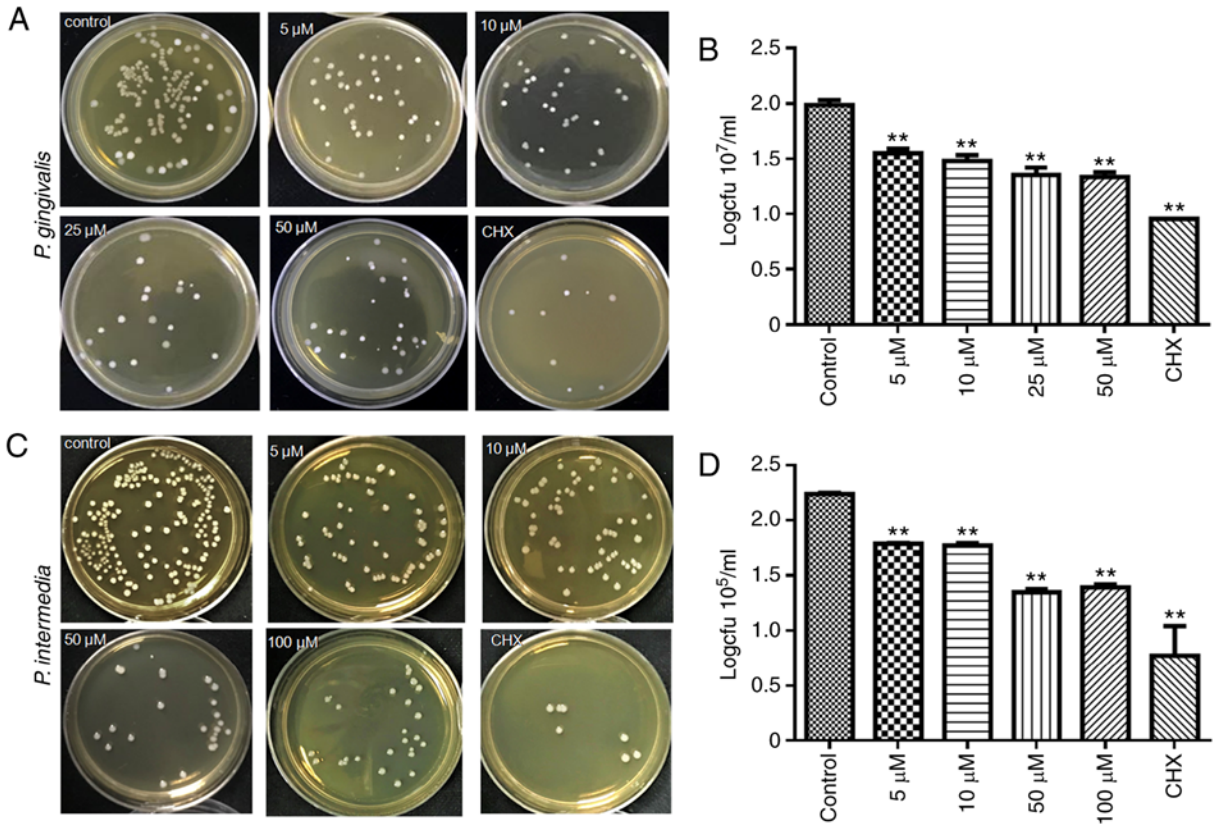

Figure 4. To determine the concentration-dependent anti-bacterial activity of Dios, CFU counts at 24 h were determined. (A) Images of colonies formed by *P. gingivalis* in the presence/absence of Dios and (B) quantified numbers of colonies in the different groups. (C) Images of colonies formed by *P. intermedia* in the presence/absence of Dios and (D) quantified numbers of colonies in the different groups. Bacterial suspensions in tryptic soy broth without Dios were used as a control. Quantified values are expressed as the mean ± standard deviation (n=3). **P<0.01 vs. control group. Dios, diosgenin; CFU, colony-forming units; CHX, chlorhexidine; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*.

the biovolume of interest in the biofilms. After the bacterial biofilms were treated for 24 h, the control group displayed mostly green fluorescence (Fig. 5A and B), with cell viabilities of 88.73 and 74.12% for the *P. intermedia* and *P. gingivalis* biofilms, respectively. Approximately 37.84 and 47.68% of the *P. intermedia* cells had died in the 25 and 50 μM groups, respectively, and 47.73 and 52.91% of the *P. gingivalis* cells had died in the 25 and 50 μM groups, respectively. The proportions of dead *P. intermedia* and *P. gingivalis* cells after incubation with Dios were lower than those after incubation with 0.2% CHX (68.83 and 56.68%, respectively), but were obviously higher than those in the control groups (Fig. 5C and D).

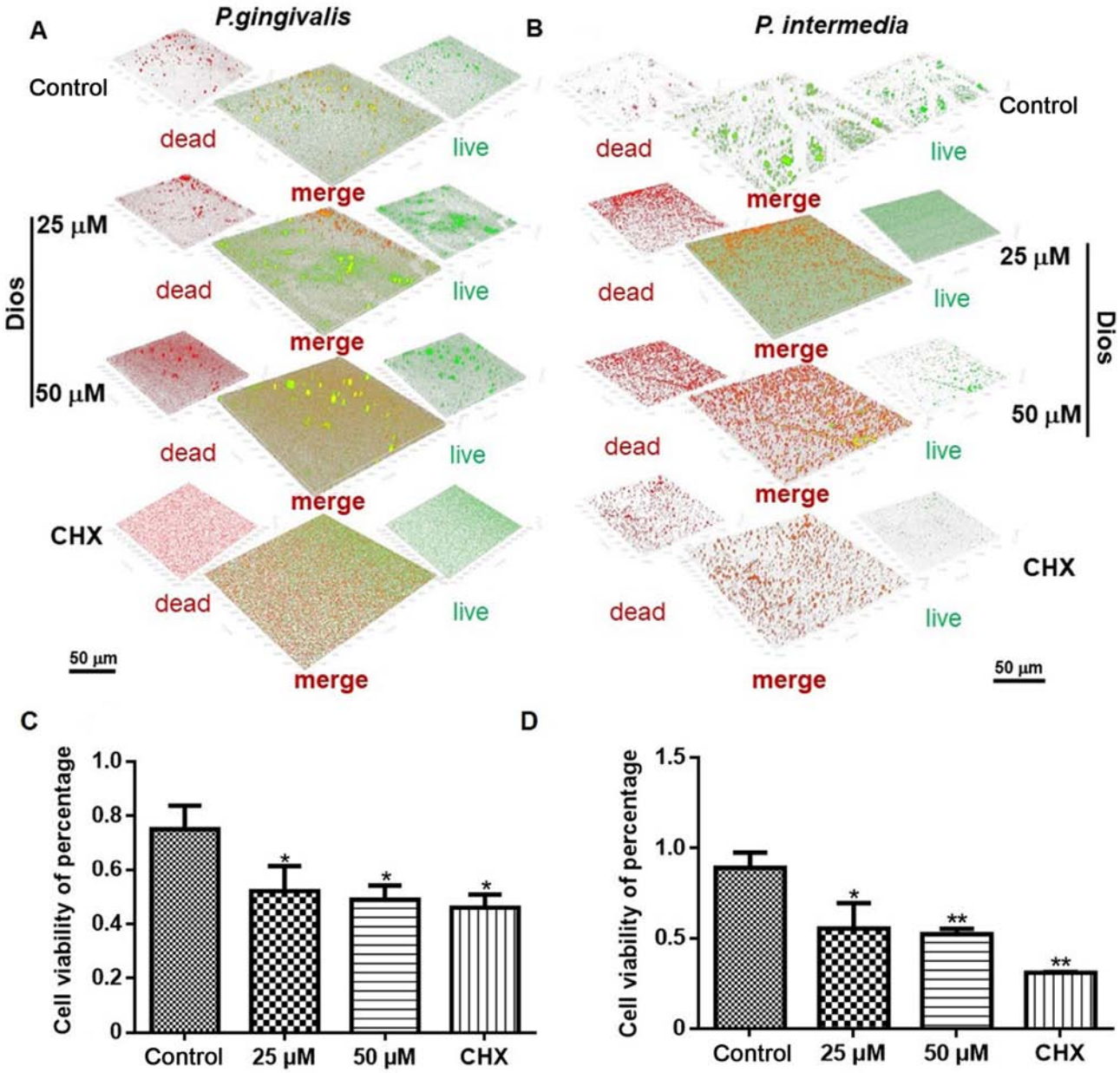

Figure 5. Viability of *P. gingivalis* and *P. intermedia* biofilms, as assessed by CLSM. (A and B) CLSM images of (A) *P. gingivalis* and (B) *P. intermedia* biofilms (scale bar, 50 μm). (C and D) Percentage of live cells on (C) *P. gingivalis* and (D) *P. intermedia* biofilms. Bacterial suspensions in tryptic soy broth without Dios were used as a control. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. control group. Dios, diosgenin; CHX, chlorhexidine; CLSM, confocal laser scanning microscopy. *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*.

According to the above results, the Dios treatment groups exhibited superior anti-bacterial effects against *P. intermedia* and *P. gingivalis* compared with those in the control group, demonstrating the anti-microbial activity of Dios.

Discussion

PD, induced by oral pathogenic bacteria, is a chronic inflammatory disease that leads to periodontal bone destruction and periodontal soft tissue loss (27). Dios is a natural steroid sapogenin obtained from Dioscorea and potato plants. Glycoside ligands, which are involved in the synthesis of steroids, have pharmacological effects, including anti-inflammatory, anti-tumor and anti-oxidant effects (28). In the present study, the effects of Dios on two key periodontal pathogens, *P. gingivalis* and *P. intermedia* were examined. The results suggested that Dios not only inhibited the planktonic growth of *P. gingivalis* and *P. intermedia* but also impaired *P. gingivalis* and *P. intermedia* biofilm viability, which suggested that Dios may be a novel effective agent for PD therapy in the future.

Dios, a well-known steroid sapogenin derived from plants, has been used as a starting material for the production of steroidal hormones. Dios exhibits a vast range of pharmacological activities, including cardioprotective, anti-diabetic, neuroprotective, immunomodulatory, estrogenic and skin protective effects (29), mainly by decreasing oxidative stress, preventing inflammatory events (30), promoting cellular differentiation/proliferation (31), and regulating the T-cell immune response (32). Dios inhibits the production of proinflammatory cytokines (33), enzymes and adhesion molecules (34).

Furthermore, Dios drives cellular growth/differentiation through the estrogen receptor cascade and transcriptional factor peroxisome proliferator-activated receptor γ (35). Dios is also able to reduce ovariectomy-induced bone loss by enhancing osteoblast genesis and inhibiting osteoclastogenesis (36) by downregulating Akt signaling cascades (37). More importantly, Dios is a naturally occurring steroidal sapogenin that easily combines with cholesterol in the cell membrane, resulting in destruction of cell membrane structure and function and promoting cell dissolution (38). In addition, this sapogenin is able to effectively prevent DNA replication and promote phagocytic clearance (39,40), change voltage-dependent ion channels and destroy the mitochondrial structure (41). All of these pharmacological roles are linked to the anti-bacterial effects of Dios.

Chronic inflammation in PD is difficult to control, as it is difficult to eliminate oral pathogenic bacteria. In the present study, it was hypothesized that Dios may be a potential anti-bacterial medicine for the treatment of PD. Considering the advantages of high efficiency, low toxicity and lack of microbial resistance (42), research has increasingly focused on Traditional Chinese Medicines as periodontal medications. Furthermore, several plant extracts used in Traditional Chinese Medicine, such as those of Platycodi Radix, Paeoniae Radix (43) and burdock roots (44), were recently demonstrated to have strong anti-bacterial effects. However, the impact of Dios on oral pathogens, particularly periodontal pathogens, has remained elusive. In the present study, the effects of Dios on two key periodontal pathogens, namely *P. gingivalis* and *P. intermedia*, were examined. The results of the DCT and CCK-8 assays demonstrated the anti-bacterial effects of Dios on *P. gingivalis* and *P. intermedia* at concentrations ranging from 5 to 100 μM *in vitro*. The CFU counting results further indicated the anti-bacterial effects of Dios against *P. gingivalis* and *P. intermedia in vitro*. Relative to planktonic bacteria, it is well known that bacterial biofilms are more challenging to eradicate. In the present study, bacterial biofilm models of *P. gingivalis* and *P. intermedia* were constructed *in vitro*. The CLSM results suggested that bacterial biofilm viability was much lower after treatment with an appropriate concentration of Dios. All of these results proved the anti-bacterial effects of Dios on *P. gingivalis* and *P. intermedia*. However, the mechanism underlying the anti-bacterial effects remains to be determined. Considering the complexity of bacterial biofilms in PD, further studies should be performed to investigate the influence of Dios on dental plaque biofilms, which contain numerous other microorganisms.

In conclusion, Dios inhibited the growth of *P. gingivalis* and *P. intermedia* as planktonic bacteria and in biofilms *in vitro*, which suggested that this compound may have potential applications in PD therapy. However, the anti-bacterial mechanisms and biocompatibility require further research prior to the clinical application of Dios in PD treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SQ and QT designed the experiments. SC performed the experiments and conducted the statistical analysis of the data. SQ, SC and XZ drafted the manuscript. XZ, TS and QP helped with the bacterial preparation, collected the data and performed statistical analyses. SQ, SC and YX acquired funding. YX analyzed and interpreted the data, and drafted and edited the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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