Synergistic effects of D-arginine, D-methionine and D-histidine on the inhibition and eradication of *Porphyromonas gingivalis* biofilm

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Abstract

Porphyromonas gingivalis (*P. gingivalis*) biofilm is involved in peri-implantitis and periodontitis. Whether a mixture of D-AAs has synergistic effects on *P. gingivalis* biofilm remains unknown. The aim of this study was to investigate the effects of multiple D-AAs on *P. gingivalis* biofilm. D-arginine (R), D-methionine (M) and D-histidine (H) were used. The bacterial growth activity and minimum inhibitory concentrations (MICs) were determined. The effects of D-AAs mixture on biofilm biomass, extracellular polymeric substances (EPS), biofilm morphology, cytotoxicity, biofilm structure and bacterial membrane integrity were determined. D-AAs mixture delayed the proliferation of *P. gingivalis* below MICs. Mixtures of D-AAs displayed definite effects on the inhibition and disassembly of biofilm than single D-AAs. The EPS content increased with D-AAs concentrations. D-AAs mixture damaged the integrity of biofilm, while RMH showed the best effects. 8 mM RH and 4 mM RMH had no cytotoxicity. 4 mM RMH decreased biofilm thickness and altered bacteria membranes structure. Hence, the combined mixture of 4 mM RMH presents the best synergistic effects on the inhibition and eradication of *P. gingivalis* biofilm. Our study provides new insights of application of D-AAs for the treatment of peri-implantitis and periodontitis.

Introduction

Peri-implantitis, characterized by bone resorption and peri-implant inflammation, is the major cause leading to implant failure. Among the etiologies for peri-implantitis, plaque biofilm is a definite and crucial factor¹. Similarly, periodontitis is also usually caused by the accumulation of plaque biofilm. Biofilm is formed by multiple bacterial strains, while bacteria are encapsulated in matrix in order to prevent antibacterial agents to penetrate the biofilm, leading to hundreds of times of tolerance to antibacterial agents². Hence antibiotic treatment is not effective to eliminate biofilm and embedded bacteria³. Other methods like mechanical treatment, laser therapy and photodynamic therapy also did not acquire ideal result yet, for either damaging the implant surface or causing substance residue. Therefore, finding an optimal method to eradicate plaque biofilm would be an innovation to treat peri-implantitis as well as periodontitis.

P. gingivalis is a gram-negative, black-pigmented anaerobic bacterium, which belongs to the red complex. Once incorporating into biofilm, *P. gingivalis* could colonize subgingivally and contact with surrounding tissues directly, then it secretes lipopolysaccharide, peptidoglycan, gingipain and other virulence factors to stimulate the host's innate immune response to produce inflammatory factors such as IL-6, IL-8, INF- γ and TNF- α , resulting in tissue necrosis^{4,5}. Furthermore, gingipain could enable *P. gingivalis* to escape the host defense system by degrading antimicrobial peptides⁶. In general, *P. gingivalis* has been proven to be an important pathogen causing peri-implantitis or periodontitis, eradicating its biofilm phenotype is crucial for the elimination of its toxicological effects.

In biofilm communities, the EPS matrix is mainly composed of polysaccharides, proteins, lipids, and extracellular DNA. In most cases, the EPS matrix presents around 90% of the total biofilm biomass⁷. Therefore, strategies focusing on disruption of the EPS matrix have attracted much attentions recent

years, which may increase bacterial susceptibility to antibacterial agents. Due to the characteristics of EPS matrix, those strategies can be classified into matrix disruptive agents, nanocarriers and biofilm physical removal technologies (magnetic field, photodynamic therapy, ultrasounds)⁸. But they usually have common disadvantages, such as low bioavailability at later stage⁹ or less effective during eradicating mature bacterial biofilms¹⁰. Additionally, these mentioned strategies mainly focus on how to eradicate existing mature bacterial biofilms, few studies concerned about preventing biofilm formation, which is equally important for combat biofilm infections.

In recent decades, D-amino acids (D-AAs) have been found in microorganisms, plants, and humans¹¹. Especially in microorganisms, D-AAs are essential components of cell wall peptidoglycan, within which D-AAs participate in synthesis and assembly process, as well as the metabolism of bacterium. Subsequently, D-AAs were found during the biofilm lifecycle, that was, D-AAs were produced and accumulated rapidly at the dispersion phase¹². Afterwards, several D-AAs were found effective in inhibiting biofilm formation of classic pathogenic bacteria^{12–14}. Our previous research revealed that D-arginine, D-valine, and several other D-AAs had the abilities of both inhibiting *P. gingivalis* biofilm formation and triggering the disassembly of mature *P. gingivalis* biofilm^{15,16}. Considering the cytotoxic effects of D-AAs at certain concentrations¹⁷, D-AAs should be used biocompatibly to combat biofilm and finding an ideal strategy to avoid the cytotoxicity of D-AAs is urgent. Whereas previous studies neither investigated the biocompatibility of different concentrations of D-AAs to mammalian cells nor the effects of a mixture of multiple D-AAs on *P. gingivalis* biofilm.

Therefore, the aim of this paper is to investigate the combined effects of different D-AAs on *P. gingivalis* biofilm and EPS matrix below the concentrations of inhibiting *P. gingivalis* viability, and the cytotoxicity of effective D-AAs mixture to mouse fibroblast L929. In order to provide an optimal strategy for combined application of D-AAs.

Results

Effects of D-AAs mixture on *P. gingivalis* bacterial growth activity and minimum inhibitory concentrations (MICs)

The bacterial growth activities of *P. gingivalis* within mixture of D-AAs were shown in Fig. 1. Different concentrations of D-AAs had the ability of delaying *P. gingivalis* proliferation. Particularly, RMH expressed the most notable effects at equal concentrations as RM, RH and MH. Nevertheless, *P. gingivalis* could proliferate in the form of logarithmic phase. The MICs of each group were: RM 20 mM, RM 20 mM, MH 16 mM, RMH 10 mM. Taking into consideration that completely inhibition of bacterial proliferation might causing bacterial resistance to biological agents¹⁸, the concentrations of D-AAs used in subsequent experiments were below the MICs.

Effects of D-AAs mixture on *P. gingivalis* biofilm formation and biofilm disassembly

The concentrations of D-AAs using for all groups in this part were below the MICs. This experiment was divided into two parts, namely biofilm formation and biofilm disassembly. As shown in Fig. 2a, b, c, d, the biomass of *P. gingivalis* biofilm formation displayed descending tendency with the increase of concentrations of RM/RH/MH/RMH, so as the same tendency in biofilm disassembly experiments (Fig. 2e, f, g, h). Whereas the exception was that 4 mM MH and 8 mM MH did not show statistical difference with 0 mM ($P > 0.05$). Hence, the minimum effective concentration of each group was used for subsequent experiments. Figure 2i, j, k showed that RM (4 mM), RH (4 mM), MH (12 mM) were more effective in inhibiting biofilm formation than the single use of corresponding D-AAs respectively ($P < 0.05$). Figure 2l showed the difference among different D-AAs mixture groups on inhibiting biofilm formation at equal concentration of 4 mM, which revealed that RM was more effective than RH/MH/RMH and RMH was more effective than RH/MH respectively ($P < 0.05$). Next, the effects of 4 mM D-AAs on disassembling mature biofilm were investigated, 4 mM of RM, RH and MH showed enhanced effects on disassembling *P. gingivalis* mature biofilm than the single use of corresponding D-AAs respectively (Fig. 2m, n, o). Figure 2p showed the different effects among mixtures of D-AAs: RH+MH+RM/RMH ($P < 0.05$).

Effects of D-AAs mixture on *P. gingivalis* biofilm EPS matrix

The concentrations of D-AAs that both below MICs and significantly affected biofilm were used in this part. As shown in Fig. 3a, at 4 mM and 8 mM, the mixture of RMH significantly inhibited EPS matrix production in biofilm formation phase than the mixture of RM or RH ($P < 0.05$). At 12 mM, the effects of RM seemed to attenuate than RH/MH. With the increase of concentration, RM displayed promoting effects on EPS matrix, while RH displayed reducing effects on the contrary. In biofilm disassembly experiment (Fig. 3b), the EPS matrix of RM/RH/MH/RMH raised with concentration consistently. When comparing with 0 group, MH/RMH did not show notable effects on EPS matrix, even resulted in more EPS matrix biomass. However, RH showed the most significant effects on reducing EPS matrix comparing with 0, RM, MH, RMH respectively at each concentration ($P < 0.05$).

Effects of D-AAs mixture on *P. gingivalis* biofilm morphology

The morphological characteristics of *P. gingivalis* biofilm treated with D-AAs mixture was further analyzed by SEM (Fig. 4). The left two columns represented the biofilm formation phase: 4 mM RM notably inhibited biofilm formation, the integrity of biofilm completely disappeared, while remaining few scattered cells. 4 mM RH did not present manifest difference with 0 group, but 8 mM RH evidently prevent the accumulation of cells, and furtherly, 12 mM RH completely inhibited biofilm formation. 4 mM and 8 mM MH did not wreck the junction among cells, but 12 mM MH seemed to reduce the EPS matrix, in which cells trended to separate with each other. There was also a similar tendency in group RMH, with the increase of concentrations, the adhesion became insecure and cells tended to sparse. The morphology change tendency was consistent with the experiment of biofilm biomass (Fig. 2). The right two columns represented the mature biofilm disassembly phase: without D-AAs, mature biofilm was compact and multilayer. 4 mM RM damaged the integrity, and the biofilm was thoroughly invisible under 8 mM RM. Despite the effects of 4 mM and 8 mM RH on the biofilm formation were not obvious, their effects on biofilm disassembly were notable, especially at 8 mM, only scattered EPS matrix and few cells could be

observed. With at 12 mM, the biofilm completely disappeared. 4 mM and 8 mM MH showed similar effects on disassembling mature biofilm, which the biofilm remained compact under SEM (5 k). While at 12 mM MH, biofilm separated into pieces, but cells remained adhering by EPS matrix. Low concentrations of RMH showed prominent effects on disassembling mature biofilm. Briefly, 2 mM and 4 mM RMH reduced the thickness and compactness of biofilm, additionally 6 mM and 8 mM RMH obviously eradicated biofilm with very few cells being observed. The morphology change tendency of biofilm disassembly was also consistent with the experiment of biofilm biomass (Fig. 2).

Cytotoxicity of D-AAs mixture

The cytotoxicity of D-AAs mixture was then measured. As shown in Fig. 5: 8 mM, 12 mM and 16 mM of RM were cytotoxic to L929 cells at day1, 3, 5, and 16 mM RH were cytotoxic to L929 cells at day 3 and day 5. Other concentrations of different D-AAs mixture did not show influence on L929 viability at day 1, 3, and 5.

Effects of 4 mM RMH on the thickness and density of *P. gingivalis* biofilm

The biofilm formation of *P. gingivalis* treated with 0 or 4 mM RMH were analyzed by confocal laser scanning microscopy. Figure 6 showed the whole state of *P. gingivalis* biofilm. After culture for 3 days without D-AAs, the biofilm formed a thick and dense mature structure, with dominant green (live) signals (Fig. 6a, b). While the biofilm green (live) signals decreased significantly (Fig. 6c, d) in the 4 mM RMH treated biofilm. The thickness of biofilms was then measured, which revealed that 4 mM RMH decreased the thickness of *P. gingivalis* biofilm compared with D-AAs untreated group ($P < 0.05$), and the density of biofilm was also decreased (Fig. 6e).

Effects of 4 mM RMH on the structure of *P. gingivalis* bacteria in biofilm

As shown in Fig. 7a-c, *P. gingivalis* bacteria in 0 group presented regular shapes, as either rod-like along the long axis or round-like along the transverse axis. The membranes were intact, and the intracellular structures could be clearly recognized. Moreover, there was no intracellular content overflowed and intracellular staining was few. On the contrary, however, 4 mM RMH significantly changed the cell structure (Fig. 7d-f). Briefly, the shapes tended to become irregular under low magnification, and the cell volume became larger. In addition, the membranes were damaged, and the contents of the bacteria obviously overflowed. Massive intracellular staining could be observed compared with 0 group.

Discussion

Herein, we investigated the combined effects of D-arginine, D-methionine, and D-histidine on *P. gingivalis* biofilm for the first time. As is known, peri-implantitis is one of the most important causes for implant failure, and plaque biofilm accumulation is a major initial factor for peri-implant mucositis and subsequent peri-implantitis^{19–22}. Likewise, periodontitis is also tightly related to subgingival plaque biofilm, which is mainly composed of anaerobes. Hence, the inhibition as well as the eradication of biofilm should be an ideal strategy for treating these biofilm-associated diseases. Antibiotics have always been the first choice for combating against infection by their bactericidal efficacy for centuries. However,

massive evidences have indicated that extensive use of antibiotics leads to bacterial resistance^{18,23,24}. Meanwhile, bacterial biofilms increase tolerance to antibiotics by preventing antibiotics penetrating biofilm matrix²⁵. Therefore, we conceived to damage biofilm either through inhibition or eradication by agents, and meanwhile not causing bacterial resistance, in order to increase bacterial susceptibility to additional acknowledged antibiotics.

We previously reported that D-arginine and D-valine were effective in inhibiting *P. gingivalis* biofilm formation and promoting the disassembly of mature *P. gingivalis* biofilm respectively^{15,16}. Considering that different D-AAs might be involved in different metabolic forms of bacteria, we speculated that combined application of D-AAs would present synergistic effects on biofilm. After confirming the effects of R/M/H on biofilm respectively, we evaluated the effects of D-AAs mixture on bacterial growth activity and MICs. Gradient concentrations of D-AAs mixture that below MICs merely delayed cell proliferation, but it finally grew as typical logarithmic growth form (Fig. 1). The growth curve of 20 mM D-Met on *V. cholerae* presented the similar performance²⁶, which inferred that D-Met regulated cell wall synthesis in *V. cholerae*. Hence, we supposed that the mixture of R/M/H might affect distinct aspects of *P. gingivalis* synthesis, which needs further research.

CV staining was used to determine the biofilm biomass combining with crystal violet. While SEM was used to observe *P. gingivalis* biofilm morphology more intuitively. The results in Fig. 2 were consistent with Fig. 4. For inhibiting biofilm formation, the minimum concentrations of obvious effects were: 4 mM RM, 8 mM RH and 4 mM RMH ($P < 0.05$). For triggering mature disassembly, the minimum concentrations of obvious effects were: 8 mM RM, 8 mM RH and 4 mM RMH. Interestingly, the mixture of MH seemed to have no significant effects either on inhibition or disassembly compared with other D-AAs mixture. But MH mixture still displayed enhanced effects against biofilm than single M or H respectively ($P < 0.05$). Confocal laser scanning microscopy (CLSM) results similarly showed that 4 mM RMH inhibit biofilm formation both by decreasing biofilm thickness and density (Fig. 6), consistent with the study of Shiwen²⁷, which revealed that the thickness of the DANA-treated *P. gingivalis* biofilm was significantly lower than that of the untreated group. Kinds of micromolecules may possess competitiveness for the target protein or binding sites with others^{28,29}. Therefore, we assumed that M and H were likely to act on the same site during *P. gingivalis* anabolism. Cells embedded in biofilm without D-AAs displayed oval or rod-like shape, whereas within the notable effects of D-AAs on biofilm formation, cells became elongated from 1 μm to 2 μm (Fig. 5). Likewise, the wild-type *Vibrio cholerae* could produce M, and 1 mM M did not alter morphology of cells. But 2 mM D-Ala stimulated the conversion of rod-shaped cells to spheres³⁰. Hence, we speculate that R/M/H may act on *P. gingivalis* cell membranes, resulting in the weaken of the scaffold function of cell membranes. Without the encapsulation of EPS matrix, cells become slender.

In order to verify the above presume, we further observed the bacterial structure by transmission electron microscopy. The results showed that 4 mM RMH obviously changed the normal shapes of *P. gingivalis* bacteria. Specifically, the cell volume became enlarged and membranes were disrupted resulting in the leakage of intercellular contents. Since the integrity of bacterial membranes were weakened, additional

staining easily permeated into intracellular environment. *S. gordonii* membranes were damaged when treated with TBP-1-GGG-hBD3-3 for 12 h, which indicated that TBP-1-GGG-hBD3-3 presented the antibacterial and anti-biofilm activities by acting on the *S. gordonii* membrane³¹. Amyl-1-18 had a bactericidal effect on *F. nucleatum* owing to its membrane-disrupting properties, resulting in membranes destruction and inner structures leaking out³². These previous studies prove our mentioned presume that D-R/M/H might misincorporate in *P. gingivalis* cell membrane synthesis or directly act on membrane, subsequently weakens the integrity or elasticity of membrane. Whereas, the mechanisms need further research.

EPS assay seemed not corresponding to the results of biofilm biomass (Fig. 2) or biofilm morphology (Fig. 4). In the biofilm formation experiment, RM promoted EPS matrix production, whereas RH reduced EPS matrix production on the contrary. Considering the mentioned speculation that M and H might act on the same site during *P. gingivalis* anabolism, we further deduce that M and H display the opposite effect on the production of EPS matrix (Fig. 3). 1 mM D-Alanine or 1 mM D-Serine strikingly reduced EPS production, but different concentrations of D-Glutamic acid did not have similar inhibitory effect on bacteria EPS production³³, which revealed that D-AAAs could reduce biofilm formation independent of affecting EPS production. In the biofilm disassembly experiment, the EPS matrix of all combinations of D-AAAs mixture increased with concentration. Interestingly, the EPS matrix of RH was significantly lower than other groups at each concentration ($P < 0.05$). Retrospecting that 4 mM RH notably disassembled biofilm under SEM, we put forward a point of view that after totally disassembling biofilm, bacteria lose the environment of forming biofilm. Contrarily, other D-AAAs triggered biofilm disassembly moderately, the dispersed planktonic cells rejoined the edges of intrinsic biofilm by secreting EPS matrix. Owing to the high molecular weight and viscosity of polysaccharides, the scattered polysaccharides still adhered to remaining biofilm or the bottom of plate. Therefore, the EPS content of RM/MH/RMH might be more than 0 group, consistent with our previous research¹⁶. In summary, there are multiple factors influencing the content of EPS matrix, hence, it is not an appropriate index to measure the effects of D-AAAs on *P. gingivalis* biofilm.

Comprehensively referring to the results of bacterial growth activity, biofilm biomass and biofilm morphology, the ideal concentrations for inhibiting biofilm formation were: 4 mM RM, 8 mM RH and 4 mM RMH. Meanwhile, the ideal concentrations for disassembling mature biofilm were: 8 mM RM, 8 mM RH and 4 mM RMH. Whether these mixtures are biocompatible should be determined. Figure 5 indicated that 4 mM, 8 mM RM and 16 mM RH were cytotoxic to L929 cells. Together with abovementioned ideal concentrations, 8 mM RH or 4 mM RMH should be selected for future research. In view of the fact that higher concentrations of D-AAAs are cytotoxic to HeLa cells and MCF-7 cells than lower ones¹⁷, safer strategy should be carried out. Comparing with 8 mM RM, 4 mM RMH should be given the priority for both treatment and prevention of *P. gingivalis* biofilm associated diseases. Whereas, further research is needed for the synergistic mechanism of D-AAAs mixture as well as the effects of D-AAAs mixture on biofilm *in vivo*.

Conclusions

Within the limitations of this study, this work has shown that the combined mixture of 4 mM RMH presents the best synergistic effects on the inhibition and eradication of *P. gingivalis* biofilm without inhibiting bacterial proliferation, which suggests that the combination application of D-AAs could be an optimal strategy for the treatment of peri-implantitis and periodontitis.

Materials And Methods

Bacterial culture and reagents

P. gingivalis ATCC 33277 was used in this study, which was subcultured on sterilized BHI (HopeBio) supplemented with hemin (5 µg/mL), menadione (0.5 µg/mL), and yeast extract (LP0021, Oxoid) and incubated at 37 °C under anaerobic conditions (80% N₂, 10% H₂, and 10% CO₂). D-AAs (Sigma-Aldrich) including D-arginine (D-Arg, R), D-methionine (D-Met, M) and D-histidine (D-His, H) were prepared as concentrated raw liquid in distilled water, then filtered and sterilized by 0.22 µm filter membrane. Then, it was applied to the experiments of gingival bacteria. D-AAs combination experiment, according to the MIC values of three D-AAs, the concentrations of double D-AAs in the mixed solution was determined to be 0/4/8/12/16/20 mM and RMH was 0/2/4/6/8/10 mM. The concentrations among R/M/H in all groups were equal in same solutions. Distilled water was used as control (0) group.

Bacterial growth activity and MICs

Single *P. gingivalis* colonies were inoculated into BHI bacterium liquid medium, then the inoculum was adjusted to 10⁷ colony-forming unit (CFU) counts (CFU/mL)¹⁵. Afterwards, the suspension was inoculated to 96-well plates (Nest) and mixed with a series of concentrations of D-AAs, consequently incubated for 72 hours at 37 °C under anaerobic conditions. Simultaneously, the OD 600 value was measured every 4 hours with a spectrophotometer (Bole Life)³⁴. The minimum concentration of sample showing no turbidity at 72 hours was recorded as the minimal inhibitory concentrations (MICs).

Biofilm biomass assay

This experiment was divided into two parts, namely biofilm formation and biofilm disassembly. The concentrations of D-AAs for all groups were below the MICs referred to above results.

Biofilm formation inhibition experiment: Different concentrations of D-AAs and their mixture were added in 96-well plates which inoculated with *P. gingivalis* at a concentration of 10⁸ CFU/mL for 72 hours.

Mature biofilm disassembly experiment: *P. gingivalis* were inoculated in 96-well plates at a concentration of 10⁸ CFU/mL for 72 hours to form mature biofilm, then different concentrations of D-AAs and their mixture were added in each well, subsequently cultured for 72 hours.

At the end of above experiments, planktonic bacterial supernatant was removed and the culture plate was rinsed with phosphate-buffered saline (PBS) (Boster), carefully air-dried for 20 min, and then fixed with

2.5% (v/v) glutaraldehyde for 20 minutes. 0.1% Crystal violet (CV) added subsequently. Then the excess CV was rinsed with PBS. The CV bounded with biofilms were decolorized by 95% ethanol, then transferred to another 96-well plate. Finally, the biofilm biomass was evaluated at OD 600 nm using a microplate reader (Synergy HT, BioTek)³⁵.

EPS matrix detection

The concentrations of D-AAs mixture that both below MICs and significantly affected biofilm biomass were used in this part. The extraction of EPS from biofilm based on the method of Albuquerque³⁶. Briefly, biofilms were sequentially rinsed with PBS, distilled water, 6% phenol solution and 97% sulfuric acid. Afterwards, samples were air-dried for 20 min, followed by incubation for 20 min at room temperature. Then, the amount of polysaccharides in the biofilm was determined by recording the absorbance at OD 490 nm with glucose as standard³⁷.

SEM analysis of biofilms

The concentrations of D-AAs mixture used here were based on MICs, biofilm biomass and EPS matrix. The morphological characteristics of *P. gingivalis* biofilms treated with D-AAs mixture were observed by Scanning electron microscopy (SEM) (Hitachi S-4800). In general, 24-well plates were rinsed three times using PBS, then fixed overnight in 2.5% (v/v) glutaraldehyde at 4 °C, and dehydrated by gradient ethanol solutions (30%-100%). After completely oven drying, the biofilm was observed under the SEM after the process of sputter coating with gold.

Cytotoxicity assay

The concentrations of D-AAs mixture used here were based on above experiments. Mouse fibroblast L929 was resuscitated and then cultured in DMEM solution, placed in an incubator of 5% CO₂ at 37°C. Cells were counted when the bottom of culture bottle was covered up to 80%-85% by cells. Logarithmic growth phase cells were selected to prepare single cell suspension, and inoculated in 96-well plates for 24 hours. Afterwards, D-AAs mixture was added. After 1, 3 and 5 days of culture at 37°C, 10 µL CCK-8 (Beyotime) was added to each well, and went on culture for 2 hours at 37°C. The absorbance value was measured by recording the absorbance at OD 450 nm.

CLSM

The biofilm formation of *P. gingivalis* treated with 0 or 4 mM RMH were analyzed by confocal laser scanning microscopy. The method of culture of *P. gingivalis* biofilm was identical with biofilm biomass. After 3 days culture, the samples were gently washed twice with distilled water to eradicate planktonic bacteria on the surface of 24-well plate. Then using the Live/Dead backlight bacterial viability kit (Invitrogen, Eugene, OR) to fluorescence-stained the biofilm for 30 minutes. The whole experiment was operated in dark area. Finally, the samples were observed under a confocal laser scanning microscope (FV3000, Olympus, Japan). Dual-channel scanning observations were performed with a red channel (490/635 nm for propidium iodide) and a green channel (480/500 nm for SYTO 9 stain). and. Images

were drawn using Imaris software (Zeiss, Germany), the thicknesses of biofilm were calculated using ImageJ software (NIH, USA).

Transmission electron microscopic imaging (TEM)

The bacterial morphology in 4 mM RMH showed prominent change under SEM, hence we further studied the structural details under transmission electron microscope. The groups were the same as CLSM, the samples were gently washed three times with distilled water to eradicate planktonic bacteria on the surface of 24-well plate. The collection of biofilm was referred to Dejana' methods³⁸. Briefly, the plates were vortexed with 0.9% NaCl for 3 min to remove the biofilm from the bottom, and collected into 5 mL centrifuge tube respectively. The specimens were centrifuged at 4000 rpm for 10 minutes (4°C) and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH, 7.4) at room temperature for 1 h, subsequently post-fixed with 1% osmium tetroxide for 1 h. Then, the specimens were rinsed with 0.1 M phosphate buffer (pH, 7.4) for 4 times (5 minutes per time). Graded ethanol series (50%-70%-90%-100%) and acetone were used for dehydration. Then the specimens were embedded for resin penetration and ultrathin section (90 nm). The sections were then double stained by 3% uranium acetate and lead citrate. Finally the sections were observed under a transmission electron microscope (JEM 1400 PLUS, JEOL, US).

Statistical analysis

Data are expressed as mean $\pm$ SD. Dunnett's t-test was applied to compare each test group with the control group, and one-way ANOVA with appropriate post-tests was applied for multiple groups. Data analysis was performed using the SPSS 24.0 software package. Values of $P < 0.05$ were considered statistically significant. All experiments were performed in triplicate and repeated three times.

Declarations

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Author contributions

Zhenyang Zhang and Baosheng Li have equally performed the experiments and wrote the manuscript; Qing Cai, Shuwei Qiao and Dan Wang have performed the experiments and analyzed the data; Heling Wang, Huiyan Zhang and Yalan Yang have collected materials and data; Weiyan Meng have designed the experiments and revised the manuscript.

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Figures

Figure 1
Effects of D-AAs on bacterial growth activity and MICs. (a) Different concentrations of mixture of RM. (b) Different concentrations of mixture of RH. (c) Different concentrations of mixture of MH. (d) Different concentrations of mixture of RMH. MICs were determined with no turbidity at the time of 72 hours.

Figure 2

Effects of D-AAs on biofilm formation and biofilm disassembly. (a-d) Series of concentrations of D-AAs on biofilm formation. (e-h) Series of concentrations of D-AAs on biofilm disassembly. (i) 4 mM of R/M/RM on biofilm formation. (j) 4 mM of R/H/RH on biofilm formation. (k) 12 mM of M/H/MH on biofilm formation. (l) 4 mM of RM/RH/RMH on biofilm formation. (m) 4 mM of R/M/RM on biofilm disassembly. (n) 4 mM of R/H/RH on biofilm disassembly. (o) 4 mM of M/H/MH on biofilm disassembly. (p) 4 mM of RM/RH/RMH on biofilm disassembly. Measured at OD 600nm. *P< 0.05

Figure 3

Effects of D-AAs on *P. gingivalis* biofilm EPS matrix. (a) Effects of gradient concentrations of D-AAs mixture on EPS matrix of *P. gingivalis* biofilm formation. (b) Effects of gradient concentrations of D-AAs mixture on EPS matrix of *P. gingivalis* mature biofilm disassembly. Measured at OD 490nm. *P< 0.05

Figure 4

Effects of D-AAs mixture on *P. gingivalis* biofilm morphology under SEM ($\times 1k$, $\times 5k$). The left two columns represented the biofilm formation phase. The right two columns represented the mature biofilm disassembly phase. Bar, 50 μm in $\times 1k$, 10 μm in $\times 5k$

Figure 5

Cytotoxicity of D-AAs mixture. (a) Cell viability at day 1. (b) Cell viability at day 3. (c) Cell viability at day 5. Measured at OD 450nm. *P < 0.05, versus 0 group

Figure 6

Effects of 4 mM RMH on the thickness and density of *P. gingivalis* biofilm. (a-b) The biofilm formed a thick and dense mature structure, with dominant green (live) signals. (c-d) The biofilm green (live) signals decreased significantly in the 4 mM RMH treated biofilm. (e) The thickness of biofilms 4 mM RMH

decreased the thickness of *P. gingivalis* biofilm compared with 0 D-AAs group ($P<0.05$), and the density of biofilm was also decreased.

Figure 7

. Effects of 4 mM RMH on the structure of *P. gingivalis* bacteria in biofilm. *P. gingivalis* bacteria in 0 group presented regular shapes, as either rod-like or round-like. The membranes were intact and intracellular staining was few. (d-f) 4 mM RMH significantly changed the cell structure. Cell shapes tended to become irregular and the cell volume became larger. Cell membranes were damaged (black arrow) and the contents of the bacteria released (black triangle).