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Antibacterial Activity of Capsaicin against Sectional Cariogenic Bacteria

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ABSTRACT

Caries can cause defect of dental tissue, mainly attributed to *Streptococcus mutans*, *Actinomyces viscosus*, *Lactobacillus* and *Streptococcus sanguis*. Capsaicin extracted from chili peppers were selected to explore the antibacterial activity against the four cariogenic bacteria in terms of acid producing and biofilm. The antibacterial activity of capsaicin was evaluated by the minimal bactericidal concentration, change of pH in the first 12 h and OD values of biofilms. Besides, fluorescent images of exopolysaccharides and living bacteria of *Streptococcus mutans* were measured with confocal laser scanning microscopy (CLSM). In our study, capsaicin showed an outstanding performance in the inhibition of acid producing and biofilm of the four strains of cariogenic bacteria. Meanwhile, low values of MIC of capsaicin on four sectional cariogenic bacteria indicated high efficiency of antibacterial activity of capsaicin. Antibacterial activity of capsaicin on *Streptococcus mutans, Actinomyces viscosus, Lactobacillus and Streptococcus sanguis* were proved in this study. In vivo experiments of capsaicin should be conducted later via establishing animal model of caries simulating human oral environment. After the complete experiments, capsaicin is expected to be used as adjuvant medicine to treat caries.

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Authors' Contribution
YF conceived the study and helped in data analysis. HG and ZY performed the experiments, analyzed the data and wrote the article. WY helped in data analyses and preparation of the manuscript. KX helped in data and constructive discussion.

Key words
Antibacterial activity, Biofilm, Capsaicin, pH.

INTRODUCTION

Caries, a common oral disease, can cause defect of dental tissue, mainly attributed to *Streptococcus mutans*, *Actinomyces viscosus*, *Lactobacillus* and *Streptococcus sanguis* (Caufield and Griffen, 2005). In the case of caries, as a result of ferment of dietary carbohydrates rapidly and reduced pH, accelerating tooth demineralization, frequent acid environment attracts an acidogenic and aciduric microflora (Burne *et al*., 2009; Liu *et al*., 2012). Sugar in food acts as substrate for acid and exopolysaccharides produced by these bacteria, and the acid and EPS provide important components and suitable environment for biofilms (Flemming and Wingender, 2010). Dental caries is a dynamic pathologic process, in which adherence and accumulation of biofilms is usually the first step (Gutt *et al*., 2018). Besides, acid produced by bacteria could cause demineralization of enamel and the stable acid environment provided by biofilms would make dental tissues more vulnerable (Hajishengallis, 2015). Thus, caries could be controlled effectively via modulating pH environment and inhibiting biofilms of bacteria theoretically. At present, many antibacterial agents have been used to inhibit the growth and adhesion of bacteria plaque, which usually comprise fluorides, alcohols and other antibiotic constituents (Sharma *et al*., 2017). However, more and more attention has been paid to the toxicological and ecotoxicological properties of these antibacterial agents (Singletary, 2011).

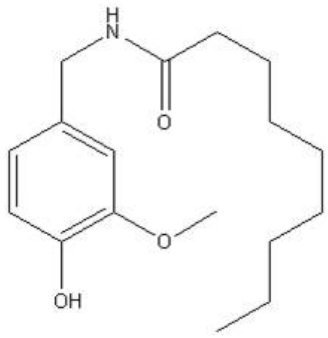

Fig. 1. Chemical structure and molecular properties of capsaicin.

Chili peppers have appeared in daily life used as a food additive or spice. Chili peppers comprise a group of fat-soluble phenols described as 'capsaicinoids', including capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the main active composition extracted from chili peppers (Fig. 1). In

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addition to its pungent taste, the medical use of capsaicin cannot be ignored. Capsaicin has various biological effects on controlling and reducing diseases, which were proved by a series of related experiments. Treatments of chronic neuropathic pain, chronic cough and rhinitis involve capsaicin (Sommer and Cruccu, 2016; Satia *et al*., 2016; Fokkens *et al*., 2016; Ekeh *et al*., 2018). As an agonist, capsaicin has the potential to modulate immune factors via activation of transient receptor potential vanilloid 1 (TRPV1) receptors (Srinivasan, 2016). Calcium influx induced by TRPV1 activation may cause activation or expression of key proteins such as endothelial nitric oxide synthase (eNOS), uncoupling protein 2 (UCP2), KLF2, PPARdelta, PPARgamma, and LXRα (McCarty *et al*., 2015). Presently, capsaicin is tried as a drug in Phase III clinical trials for treating rheumatoid arthritis, postoperative pain, acute/chronic neuropathic and musculoskeletal pain (Nalini *et al*., 2010). Moreover, capsaicin targets multiple signaling pathways, oncogenes and tumor-suppressor genes in various types of cancer models, expected to be a new therapy for cancer patients (Clark and Lee, 2016). Furthermore, capsaicin has gotten widespread attention in terms of its antibacterial activity. Yamasaki *et al*. (2011) proved that capsaicin inhibited the growth and virulence genes transcription of Vibrio cholerae. Ozçelik *et al*. (2011) verified the antibacterial activity of capsaicin on *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Bacillus subtilis*. The formation of biofilm and adherence to oral epithelial cells can be affected negatively by capsaicin on *Porphyromonas gingivalis* (La *et al*., 2010; Gerits *et al*., 2017). Besides, capsaicin exhibited antibacterial activity and modulate pH of *Staphylococcus aureus* (Koffi-Nevry *et al*., 2012). *These* bacteriostatic actions above may have the same effect in *Streptococcus mutans*, *Actinomyces viscosus*, *Lactobacillus* and *Streptococcus sanguis*.

Therefore, based on the above, it is reasonable to postulate the potential antibacterial activity on the sectional cariogenic bacteria to control and prevent caries. In this study, we investigated the antimicrobial activity of capsaicin against the growth and biofilm formation of *Streptococcus mutans*, *Actinomyces viscosus*, *Lactobacillus* and *Streptococcus sanguis*, to expect to provide a promising method and idea for clinical caries prevention.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The clinically isolated strains of *Streptococcus mutans*, *Actinomyces viscosus*, *Lactobacillus acidophilus*, *Lactobacillus* and *Streptococcus sanguis* were obtained from State Key Laboratory of Oral Diseases, Sichuan University. The bacteria were inoculated into BHI flat medium and cultivated in the anaerobic incubator (80% N_2, 10% CO_2, 10% $10H_2$, 37°C). Microbial inoculums were adjusted to final concentrations of 0.5 (10^8 CFU/ml) on the McFarland scale.

Minimum inhibitory concentration (MIC)

The 0.4 mg/ml capsaicin solution (capsaicin +95% BHI+5% alcohol) were diluted on a 1:2 basis from the initial concentration to 1:32, and added 100 µl into each well with 40 µl microbial inoculums(10^7 CFU/ml) in the 96-well plates according to different concentration. The inoculated plates were incubated in the anaerobic incubator for 24 h at 37°C.

Measurement of pH

The 1/2 MIC experimental capsaicin solution and the contrast solution (95% BHI+5% alcohol) were respectively added 2 ml into 12-well plates after adjusted the pH value to 7. Subsequently, the plates with 40 µl microbial inoculums each well were placed in the anaerobic incubator. The values of pH were recorded per hour in the first 12 h.

Biofilm growth in glass tubes

The 1/2 MIC experimental capsaicin solution and the contrast solution were separately added 200 µl into 96-well plates. The experimental groups and the contrast groups were inoculated by adding 40 µl of an overnight culture of bacteria which were in the logarithmic phase of growth, and the tubes were incubated statically in the anaerobic incubator at 37°C for two days. The blank groups were constructed by adding capsaicin solution without microbial inoculums. After discarding the supernatant in the tubes, the adhered cells were rinsed twice with sterile PBS solution. Afterwards, the cells were fixed by 200 µl methanol for 15min and the tubes were patted dry on a paper towel. 100 µl of a 0.1% CV solution was added to each tube to stain the adhered biomass and the tubes incubated for 20 min at room temperature. The CV dye was discarded and the tubes were rinsed three times with distilled water until they were colorless. Each tube which was patted dry was added 200 µl 75% ethanol to release the bound CV dye from the biofilm. The 96-well plates were vibrated for 30 min and the values of OD were measured at an absorbance of 595 nm (A_{595}).

Confocal laser scanning microscopy (CLSM)

Fluorescent images were measured with a confocal laser scanning microscope (LeicaTCSSP2). The 6-well

plates with a cover glass at the bottom were added the 1/2 MIC experimental capsaicin solution, 1 µl dyes Alexa Fluor 647 and 100 µl microbial inoculums and then were incubated in the anaerobic incubator at 37°C for 24 h. The cover glasses were dyed by SYTO9 after surface washing with distilled water for 15 min in a dark place. Alexa Fluor 647 was excited with a He–Ne laser at 633 nm, and the emission was measured with a long-pass filter with a cutoff wavelength of 650 nm. SYTO9 was excited at 485nm, and the emission was at 498 nm.

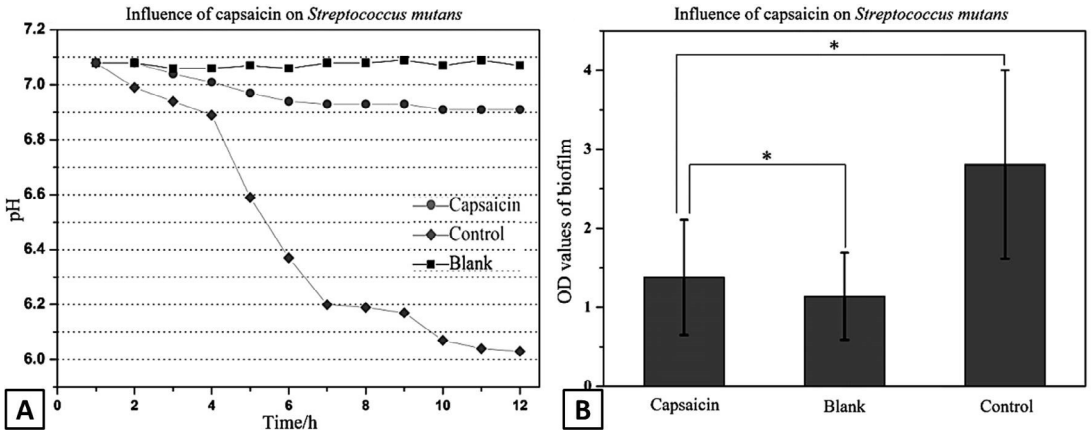

Fig. 2. Antibacterial activity of capsaicin on *Streptococcus mutans* and biofilms: **A**, the trend of pH values of *Streptococcus mutans* effected by capsaicin in 12h. **B**, OD values of biofilm of *Streptococcus mutans* affected by capsaicin. The capsaicin group consisted of 1/2 MIC experimental capsaicin solution (95% BHI+5% alcohol) and *Streptococcus mutans* microbial inoculums. The control group consisted of the solution (95% BHI+5% alcohol) and microbial inoculums without capsaicin. The blank group consisted of 1/2 MIC experimental capsaicin solution (95% BHI+5% alcohol) without microbial inoculums. The data are presented as the mean ± SD (n>3). The symbol * means statistic difference between the two groups (p<0.05).

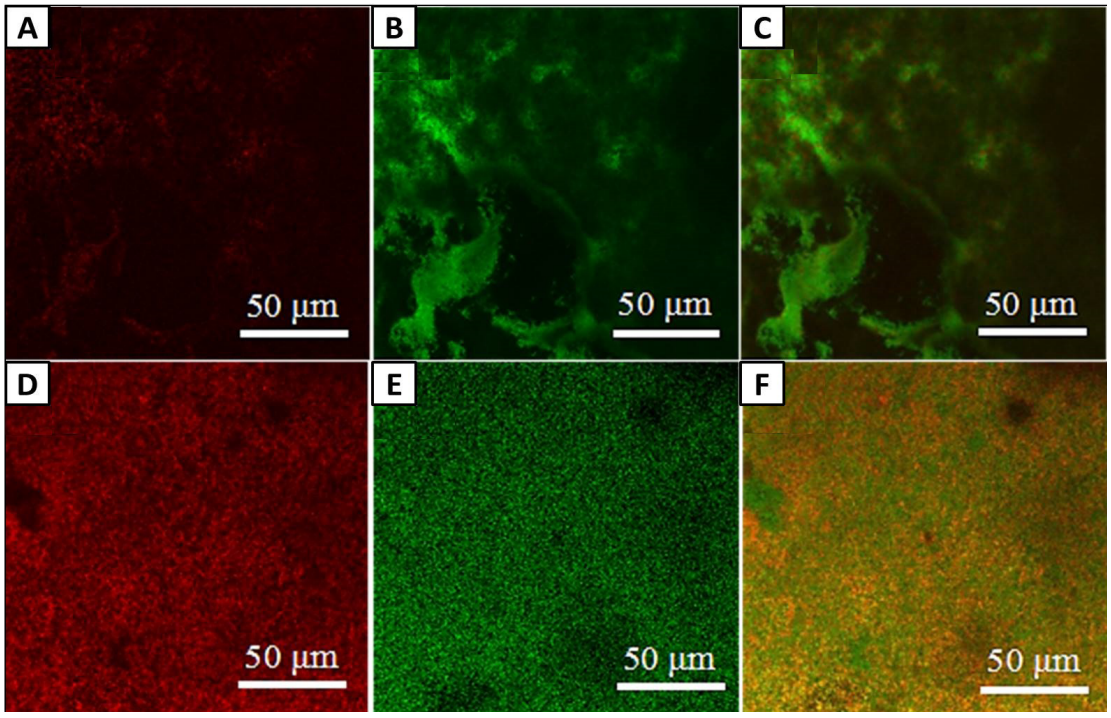

Fig. 3. CLSM images of *Streptococcus mutans*: exopolysaccharides (**A**) and living bacteria (**B**) of *Streptococcus mutans* effected by capsaicin. **C**, merged image of (A) and (B). Exopolysaccharides (**D**) and living bacteria (**E**) in the control group without capsaicin. **F**, merged image of (D) and (E).

RESULTS

Effect of Capsaicin on Streptococcus mutans *and biofilms*

The MIC value of capsaicin on *Streptococcus mutans* was 50 μg/ml. Figure 2A shows that the pH values of *Streptococcus mutans* cultivated in the culture medium (95% BHI+5% alcohol) decreased continuously to nearly 6 in the first 12 h. Compared with the control group, the capsaicin group showed a flat downslope trend of pH values from 7.09 to 6.91. As a result of the lack of bacteria, the blank group did not have difference in the pH values. In addition, there were differences of the OD values of *Streptococcus mutans* biofilm among the three groups (Fig. 2B). The average OD value of the capsaicin group was 1.38±0.38 and obviously lower than that of the control group (2.81±1.19). Difference between the two groups had statistical significance (P<0.05). As shown in Figure 3, compared with the capsaicin group, the control group displayed strong signals, indicating the existence of more exopolysaccharides and living bacteria. Besides, the average area ratio of exopolysaccharides and living bacteria was 0.745±0.060 and 1.200±0.099 in the capsaicin group and the control group, respectively (P<0.05).

Effect of Capsaicin on Actinomyces viscosus *and biofilms*

The MIC of capsaicin on *Actinomyces viscosus* was 50μg/ml. Figure 4A shows that the trend of pH values of *Actinomyces viscosus* was descending apparently in the 1/2 MIC experimental capsaicin solution and the contrast solution. However, the general pH values of the capsaicin group from 7.08 to 6.82 were higher than those of the control one from 7.08 to 6.79. The observation indicated the inhibition of acid producing on *Actinomyces viscosus* affected by capsaicin, though it was not as obvious as it was for *Streptococcus mutans*. Figure 4B shows various OD values of *Actinomyces viscosus* biofilm of the capsaicin group, the control group and the blank group. The average OD value of the capsaicin group was 0.97±0.14, while that of the control group was 1.35±0.28 (P<0.05).

Effect of Capsaicin on Lactobacillus *and biofilms*

The MIC of capsaicin on *Lactobacillus* was 50μg/ml. Figure 5A shows the change of pH values of *Lactobacillus* in the three groups. The pH values descended from 7.05 to 6.67 in the capsaicin group. Similarly, the pH values of the control group decreased from 7.05 to 6.4 approximately. The changing curve of the capsaicin was over that of the control group. The OD values of *Lactobacillus* are shown in Figure 5B. The capsaicin group revealed lower average OD value (0.53±0.17) than the control one (1.10±0.30). Difference between the two groups has statistical significance (P<0.05).

Effect of Capsaicin on Streptococcus sanguis *and biofilms*

The MIC of capsaicin on *Streptococcus sanguis* was 25μg/ml. As shown in Figure 6A, the pH of *Streptococcus sanguis* in the capsaicin group and the control group declined. The pH values of the capsaicin group decreased from 7.05 to 6.65, while those of the control group were from 7.05 to 6.37. Figure 6B shows the OD values of the three groups separately. Compared with the control group (2.12±1.07), the capsaicin group had lower average OD value (1.47±0.62).

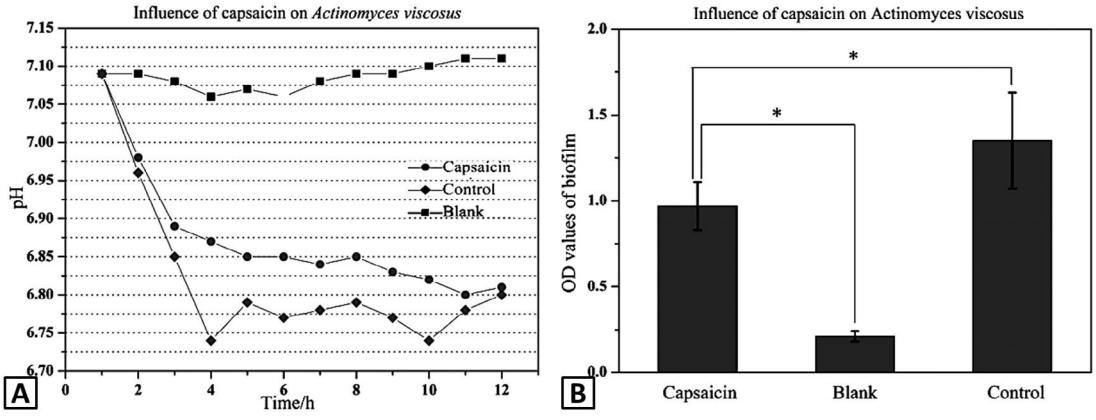

Fig. 4. Antibacterial activity of capsaicin on *Actinomyces viscosus* and biofilms: **A**, the trend of pH values of *Actinomyces viscosus* effected by capsaicin in 12h. **B**, OD values of biofilm of *Actinomyces viscosus* affected by capsaicin. The capsaicin group consisted of 1/2 MIC experimental capsaicin solution (95% BHI+5% alcohol) and *Actinomyces viscosus* microbial inoculums. The control group consisted of the solution (95% BHI+5% alcohol) and microbial inoculums without capsaicin. The blank group consisted of 1/2 MIC experimental capsaicin solution (95% BHI+5% alcohol) without microbial inoculums. The data are presented as the mean ± SD (n>3). The symbol * means statistic difference between the two groups (p<0.05).

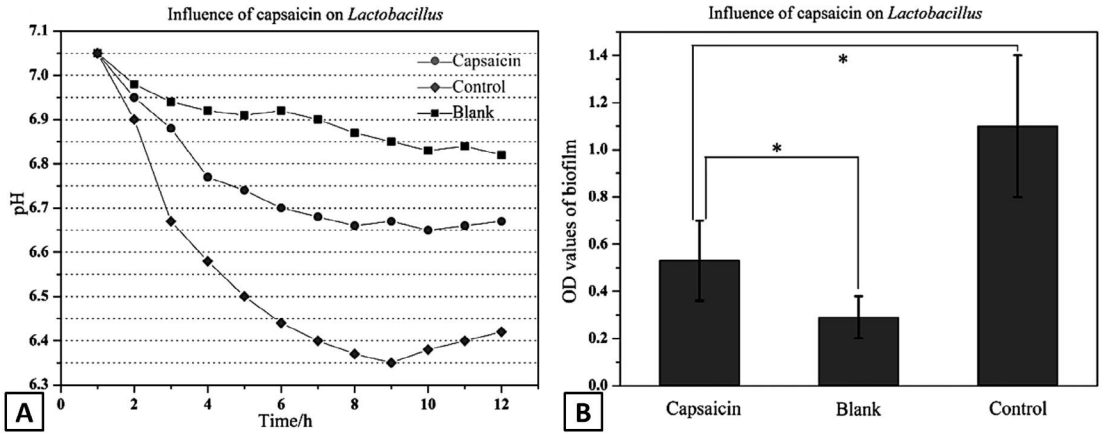

Fig. 5. Antibacterial activity of capsaicin on *Lactobacillus* and biofilms: **A**, the trend of pH values of *Lactobacillus* effected by capsaicin in 12h. **B**, OD values of biofilm of *Lactobacillus* affected by capsaicin. The capsaicin group consisted of 1/2 MIC experimental capsaicin solution (95% BHI+5% alcohol) and *Lactobacillus* microbial inoculums. The control group consisted of the solution (95% BHI+5% alcohol) and microbial inoculums without capsaicin. The blank group consisted of 1/2 MIC experimental capsaicin solution (95% BHI+5% alcohol) without microbial inoculums. The data are presented as the mean ± SD (n>3). The symbol * means statistic difference between the two groups (p<0.05).

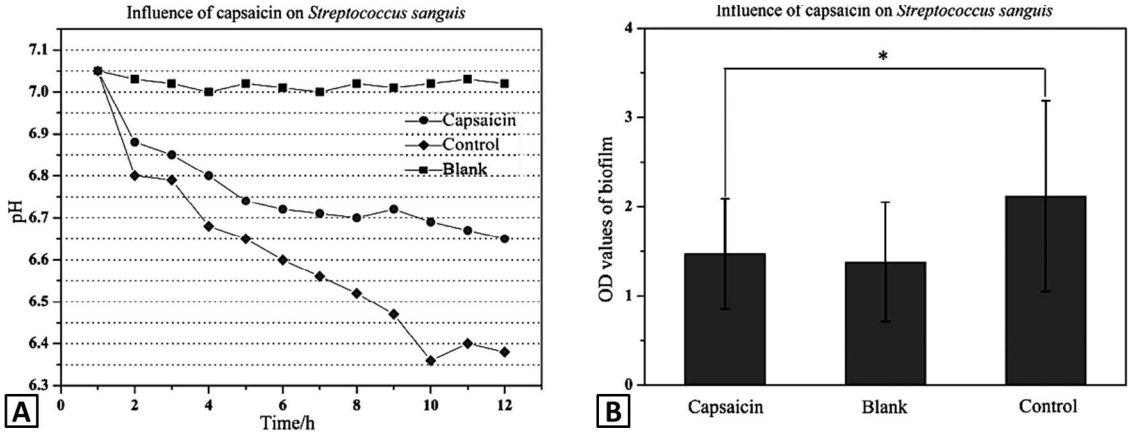

Fig. 6. Antibacterial activity of capsaicin on *Streptococcus sanguis* and biofilms: **A**, the trend of pH values of *Lactobacillus* effected by capsaicin in 12h. **B**, OD values of biofilm of *Streptococcus sanguis* affected by capsaicin. The capsaicin group consisted of 1/2 MIC experimental capsaicin solution (95% BHI+5% alcohol) and *Streptococcus sanguis* microbial inoculums. The control group consisted of the solution (95% BHI+5% alcohol) and microbial inoculums without capsaicin. The blank group consisted of 1/2 MIC experimental capsaicin solution (95% BHI+5% alcohol) without microbial inoculums. The data are presented as the mean ± SD (n>3). The symbol * means statistic difference between the two groups (p<0.05).

DISCUSSION

In our study, capsaicin was used to evaluate the antimicrobial property on the inhibition of acid producing and biofilm of *Streptococcus mutans*, *Actinomyces viscosus*, *Lactobacillus* and *Streptococcus sanguis,* which were sectional cariogenic bacteria, in order to search for new antimicrobial agents or treatment methods to cure or prevent dental caries. Acid producing is a key link during the process of caries caused by bacteria. Acid condition could cause demineralization of teeth and provide a good living environment for bacteria. Therefore, inhibiting bacteria from producing acid is an effective method to control the incidence of dental caries. Biofilms are known to be significant part of adhesion and growth of bacteria, surrounded by extracellular polymeric substances (EPS). EPS intersect to form a network to maintain and transport nutrition for bacteria. Biofilms can effectively prevent bacteria from conventional antimicrobial compounds due to the existence of EPS. Destroying biofilms or inhibiting

their formation can impede the growth of bacteria greatly. Thus, it can be seen that the most effective treatment against dental caries is not only to prevent early biofilms, but also to inhibit or destroy the formed biofilms.

Capsaicin is a kind of alkaloid extracted from chili peppers and has multiple pharmacological and physiological properties (Yeung and Tang, 2015). Alkalinity of capsaicin can damage the original acid environment to inhibit growth of bacteria. To avoid the influence of alkalinity on bacteria, the initial pH values of experimental capsaicin solution and microbial inoculums were rectified to 7. Interestingly, capsaicin showed abilities to inhibit acid producing of *Streptococcus mutans*, *Actinomyces viscosus*, *Lactobacillus* and *Streptococcus sanguis*, except the effect of alkalinity. The trend of pH values of the capsaicin group became flatter than that of the control, indicating that duration and amount of acid producing both decreased. The changes between the capsaicin group and the control group of these four kinds of bacteria could be observed apparently. In addition, biofilms of these bacteria in the 1/2 MIC experimental capsaicin solution reduced compared with the control group. The inhibition of biofilm formation could be on account of the reduction of living bacterial and EPS, which may lead to lower biofilm thickness and increased cell death. This theory could be proved by the weak red and green staining signal, which represented the amount of EPS and living bacteria. Compared with the control group, the capsaicin group showed weaker signals of EPS and living bacteria, and owned less average area ratio of EPS and living bacteria, indicating that capsaicin inhibited not only the growth of *Streptococcus mutans* but also the formation of EPS produced by *Streptococcus mutans*. The thin and disintegrated biofilm may be more vulnerable to capsaicin treatment, leading to the further destruction of biofilm owing to the lack of EPS. Moreover, the inhibition of the growth of these bacteria and the formation of acid, EPS and biofilms could be available at a low concentration of capsaicin about 25 to 50μg/ml, which reflected the high efficiency of antibacterial activity of capsaicin on these bacteria and biofilms. Similar results were obtained by Marini *et al*. (2015), that capsaicin could inhibit bacterial activity, cell-invasion and infection spread to deep tissues of erythromycin-resistant, cell-invasive pharyngeal GAS isolates. Toyoda *et al*. (2016) proved the direct antibacterial effects of capsaicin on Helicobacter pylori and the potential for use in the chemoprevention of H. pylori-associated gastric carcinogenesis. Furthermore, capsaicin could activate the vanilloid receptor subtype 1 (TRPV1), and change the cytokine framework to protect periodontal tissues (Zhou *et al*., 2014). Alcohol was added in order to dissolve capsaicin in this study due to the slight solubility of capsaicin. However, differences were obvious between capsaicin and non-capsaicin group, emphasizing the effects of capsaicin, as well as, microscale alcohol had no apparent influence on these bacteria, according to the data analysis of the control group.

Although antibacterial activity of capsaicin on *Streptococcus mutans, Actinomyces viscosus, Lactobacillus and Streptococcus sanguis* were proved in this study, the effects of capsaicin on internal relation among these bacteria should be lucubrated in later investigations, because that caries are not caused by single bacteria but multiple effects of various bacteria (Yu *et al*., 2017). Also, the related mechanism of capsaicin on the formation of acid and EPS, and biofilms of these bacteria should be further researched. In addition, *in vivo* experiments of capsaicin should be conducted via establishing animal model of caries simulating human oral environment. After the complete experiments, capsaicin is expected to be used as adjuvant medicine to treat caries.

CONCLUSION

Capsaicin may offer a new way to inhibit or prevent caries as a natural extract. The present work selected four strains of cariogenic bacteria (*Streptococcus mutans*, *Actinomyces viscosus*, *Lactobacillus* and *Streptococcus sanguis*) as research objects. In our study, capsaicin showed an outstanding performance in the inhibition of acid producing and biofilm of those four strains of cariogenic bacteria. Meanwhile, low values of MIC of capsaicin on four sectional cariogenic bacteria indicated high efficiency of antibacterial activity of capsaicin.

Statement of conflict of interest

We declare no conflicts of interest in this study.

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