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Original Article

Combination effects of baicalin with levofloxacin against biofilm-related infections

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Abstract: It is important to improve the existing techniques and develop new strategies to prevent bacterial biofilm formation. In this in vitro study, biofilms were established by a clinically isolated strain of *Staphylococcus aureus* 17546 (t037). Different concentrations of baicalin were added to 3- and 7-day biofilms. Based on colony counts and quantitative analysis of the biomass, sub-minimum inhibitory concentrations (sub-MICs) (1024, 512 or 256 μg/mL) of baicalin clearly decreased the number of bacterial colonies and biomass in vitro. Fluorescence microscopy revealed that sub-MICs (1024, 512, or 256 μg/mL) of baicalin inhibited bacterial adherence to the carrier surface and decreased polysaccharide production. Moreover, baicalin disrupted biofilms and exhibited synergistic effects with levofloxacin. Virulence factors were assessed by western blotting and real-time quantitative polymerase chain reaction, confirming that staphylococcal enterotoxin A, α-haemolysin and coagulase production decreased after baicalin treatment. Additionally, baicalin increased production of thermonuclease in *S. aureus*, and baicalin at 1024 and 512 μg/mL downregulated agrA expression. Based on these findings, the combination of baicalin with levofloxacin might be a new, feasible strategy for treating *S. aureus* biofilm-related infections. Baicalin may serve as a new inhibitor that modulates *S. aureus* virulence factors.

Keywords: Biofilm, *staphylococcus aureus*, baicalin, virulence factors

Introduction

Antimicrobial efficacy *in Staphylococcus aureus* biofilm-related infections is decreasing because due to the high tolerance of biofilms to antibiotics. Biofilm formation is a protective mechanism that prevents bacteria from eradication. Indwelling medical devices [1-3] and infected lung, trachea, and urinary tract tissues serve as suitable sites for biofilm formation, and once established, the dose of antibiotic required increases multifold [4]. Novel strategies are therefore required to address such biofilm-related infections.

The formation of biofilm by *S. aureus* is regulated by the quorum sensing (QS) system [5], a universal mechanism for the transmission of information between bacterial cells [6-8]. Bacteria can regulate the density and behaviours of the entire colony by synthesizing and secreting signalling molecules (also called self-inducers) [9]. When these signalling molecules accumulate to a certain threshold, expression of certain specific genes is activated, and some regulatory proteins are secreted [10]. The Agr system is a major density signal induction system in *S. aureus* [11] that regulates not only the growth of the entire biofilm but also the production and release of virulence factors, such as polysaccharide intercellular adhesin (PIA), phenol-soluble modulin peptide, and enterotoxin. Therefore, the status of the regulatory QS system in *S. aureus* is indirectly reflected by the levels of virulence factors produced.

Baicalin (a major constituent of the roots of *Scutellaria baicalensis*) is widely used clinically for treating fever, bronchitis and upper respiratory tract infections [12, 13]. Baicalin also exerts antifungal activity against *Candida albicans*, antiviral activity against enteroviruses and antibacterial activity against methicillin-resistant *S. aureus*. In addition, baicalin was reportedly inhibits acyl homoserine lactone (AHL)-based QS-regulated gene expression in

Burkholderia cenocepacia [14]. However, there is no specific evidence to date demonstrating the effects of baicalin on *S. aureus* biofilm morphology or combinatorial effects with other classes of antibiotics, such as fluoroquinolones. Furthermore, data regarding the effects of baicalin on QS-controlled virulence and gene expression in *S. aureus* are still lacking. Thus, the aim of our research was to establish the antibiofilm effect of baicalin on *S. aureus* and determine its effects on virulence factor and agr gene expression.

Materials and methods

Bacterial strains and reagents

S. aureus 17546 (t037) and standard *S. aureus* ATCC 29213 (supplied by The First Affiliated Hospital of GuangXi Medical University) strains were selected for this in vitro study. Baicalin, clarithromycin (CLR) and levofloxacin (LEV) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in dimethyl sulfoxide (DMSO, Amresco, Solon, OH, USA).

Detection of MICs and growth curve assay

The minimum inhibitory concentrations (MICs) of baicalin, levofloxacin and clarithromycin were determined using the microtitre broth dilution method according to the Clinical and Laboratory Standards Institute (CLSI, 2012), and growth curves were generated based on spectrophotometry. Briefly, *S. aureus* was cultured overnight in tryptic soy broth (TSB) supplemented with 0.5% glucose (TSB-G, glucose is a known additive that positively impacts *S. aureus* biofilm formation [15, 16]) and diluted to an absorbance of OD600 = 0.05. Baicalin at sub-MICs (1024, 512, 256 or 128 μg/mL) were added to the cultures, followed by incubation at 37°C (200 rpm), absorbance at OD600 was measured every 2 hours.

Antibiofilm effect assay in vitro

Colony counts assay: The inhibitory and synergistic effects of the combination of baicalin with levofloxacin were evaluated using the plate counting method. A bacterial culture at an absorbance of OD600 = 0.1 was prepared, and 2 mL of the bacterial suspension with baicalin (final concentrations of 1024, 512, 256 or 128 μg/mL) was added to 24-well plates. A polystyrene carrier (1 × 1 cm^2) was placed in each well. Planktonic bacteria were removed using sterile saline, and the medium (TSB-G and baicalin) was refreshed every other day until the 3rd and 7th days. To examine synergistic effects, a bacterial suspension without drugs was added to 24-well plates at the beginning of biofilm cultivation. Antibiotics were added on the 3rd and 7th days according to the following groups: control group, 256 μg/mL baicalin group, 16 μg/mL clarithromycin group, 32 μg/mL levofloxacin group, 256 μg/mL baicalin + 32 μg/mL levofloxacin group, and 16 μg/mL clarithromycin + 32 μg/mL levofloxacin group. All drugs were applied for 12 hours, and the carriers were washed with sterile saline to remove planktonic bacteria. The bacteria in the biofilm were enumerated using the plate counting method. The experiment was repeated three times.

Semiquantitative analysis of biofilm

A crystal violet staining protocol was applied to quantify the mass of biofilms. Biofilms were prepared as inhibitory effects assay, and planktonic bacteria on the surface were removed using phosphate-buffered saline (PBS). The carriers were placed at room temperature for 15 min, stained with 0.5% crystal violet for 20 min, washed three times with PBS and incubated in 2 mL 95% ethanol. Quantitative analysis of biomass was performed by measuring OD595 using a microplate reader (Multiskan, Thermo Scientific, USA). The experiments were performed three times in parallel.

Observations of biofilm morphology

Fluorescence microscopy assay: Biofilms were constructed as inhibitory effects assay, and planktonic bacteria were removed by washing three times with PBS. The carriers were stained with 2 mL SYTO-9 (Sigma, USA) in the dark for 15 min, and unbound dye was removed with PBS. Fluorescent images were acquired under a fluorescence microscope (Olympus, Japan).

Scanning electron microscopy (SEM)

S. aureus biofilms were grown as synergistic effects. After treatment with baicalin and antibiotics, carriers were washed with sterile saline and with 2.5% glutaraldehyde and then dehydrated in increasing concentrations of ethanol (70%, 80%, 90% and 100%). The carriers were dried at room temperature, coated with gold, and subsequently analysed by SEM (TM-1000, Hitachi, Tokyo, Japan) at 30 kV.

Table 1. The primers for Real-time quantitative PCR

Primers	Sequence	Length
16sRNA forward	CCATAAAGTTGTTCTCAGTT	83 bp
16sRNA reverse	CATGTCGATCTACGATTACT	
Hla forward	ATGGCTCTATGAAAGCAGCAGA	105 bp
Hla reverse	AAGTCTGGTGAAAACCCTGAAGA	
agrA forward	ACG TGG CAG TAA TTC AGT GTA TGT T TAA TTC AGT GTA TGT T	82 bp
agrA reverse	GGC AAT GAG TCT GTG AGA TTT TGT	

Confocal laser scanning microscopy (CLSM)

Synergistic bactericidal effects were also observed by CLSM. Biofilms were established as above, and after incubation, each biofilm carrier was washed with sterile saline to remove planktonic bacteria. The carriers were stained with 2 mL of LIVE/DEAD Viability kit reagent (Invitrogen, MA, USA) in saline (0.9% NaCl) containing a mixture of SYTO-9 and propidium iodide (Sigma, USA, 1.5 μL/mL of each) and incubated for 15 min in the dark. A confocal laser scanning microscope (Nikon A1, Japan) was used to observe the biofilm, with green representing a live biomass and red a dead biomass.

Detection of virulence factors

Suspension separation: S. aureus 17546 was cultivated overnight, and the bacterial suspension was diluted to an absorbance of OD600 = 0.1, after which baicalin was added to final concentrations of 0, 1024, 512, 256 or 128 μg/mL. The suspensions were continuously cultured for 5 hours and centrifuged at 12,000 rpm for 5 min. The supernatants and pellets were separated for subsequent experiments.

Detection of staphylococcal enterotoxin A (SEA)

The supernatant obtained above containing 30 mg protein was mixed 3:1 with sulface polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and denatured by boiling for 5 min. Then the samples were electrophoresed (constant voltage of 110 V for 2 hours) in a Mini-Protean II vertical dual-cell apparatus (Bio-Rad). The protein bands were stained with Coomassie brilliant blue R (Sigma, USA) and imaged. The gel was incubated with Towbin transfer buffer, and the proteins were transferred to a membrane (Pall Corporation USA), followed by blocking non-specific proteins. The membrane was probed with rabbit polyclonal antibodies and secondary antibodies (Sigma, USA) and rinsed three times with TBST for 10 min each. The immunoreactive bands were exposed to X-ray film, and densitometric analysis of the bands was performed using Quantity One (Bio-Rad, USA). The experiments were repeated three times.

Detection of α-haemolysin (hla) and agrA genes

Real-time quantitative polymerase chain reaction (RTQ-PCR) was used to determine the expression levels of the genes hla and agrA. The primers used are shown in **Table 1**.

RNA extraction and reverse transcription reaction

The bacterial pellet was washed with diethylpyrocarbonate (DEPC)-treated water and centrifuged at 10,000 rpm for 1 min (twice) at 4°C. The supernatant was discarded, and the bacterial pellet was suspended in 60 μL of lysozyme (10 mg/L, Sigma, USA) containing 5 μL of lysostaphin (Sigma, USA) + TE buffer (pH 8.0) and incubated for 40 min at 37°C. The suspension was centrifuged at 12,000 rpm for 1 min at 4°C, and the supernatant was discarded. One millilitre of Trizol was added, and the pellet was homogenized (5 min) with vigorous shaking for 15 seconds after the addition of 200 μL of chloroform. Next, the mixture was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was transferred to a new tube, thoroughly mixed with 480 μL of isopropanol and shaken vigorously. The mixture was incubated at 4°C for 20 min and centrifuged at 12,000 rpm for 15 min. The supernatant was discarded, and the white pellet at the bottom of the tube was considered RNA. One millilitre of 75% ethanol was added to resuspend the RNA, followed by centrifugation at 12,000 rpm for 4 min and drying at room temperature. The concentration and purity of the total RNA sample were measured spectrophotometrically by calculating the 260/280 nm ratio (1.9-2.1 target), and the RNA was reverse transcribed into cDNA. A mixture of 2 μL of RNA treated with RNase-free DNase (Roche), 1 μL of oligo (dT) 18 primers and DEPC water in a final volume of 12 μL was incubated at 65°C for 5 min and at 4°C for 5 min. Next, 4

Table 2. MICs of baicalin, levofloxacin and clarithromycin against ATCC 29213

Antimicrobial	MICs (μg/ml)	
	S. aureus 17546	ATCC 29213
Baicalin	2048	2048
Levofloxacin	32	32
Clarithromycin	128	128

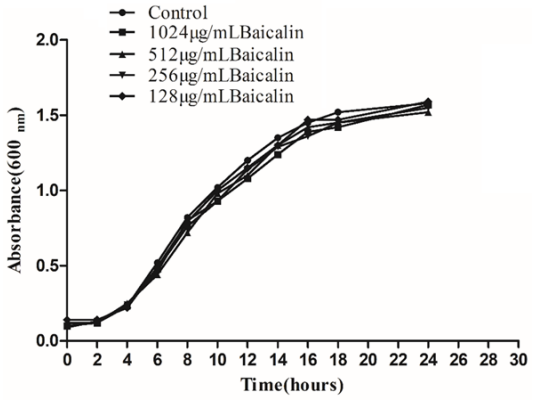

Figure 1. Growth curves of *S. aureus* at sub-MIC concentrations of baicalin. *S. aureus* with different concentrations of baicalin (1024-128 μg/ml) or without baicalin (control) was incubated at 37°C for 24 hours, and OD600 was measured every 2 hours. Experiments were performed in triplicate. Mean values are shown. Baicalin concentrations < 1024 μg/mL did not inhibit *S. aureus* growth.

μL of 5 × reaction buffer (40 mg/L), 1 μL of RNase inhibitor (20 U/μL), 2 μL of 10 mM dNTP mix, 1 μL of reverse transcriptase (200 U/μL) and DEPC water to a final volume of 20 μL were added, and the mixture was incubated at 42°C for 60 min and 70°C for 5 min and then cooled at 4°C. The RevertAid First Strand cDNA synthesis kit was used (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA).

RTQ-PCR

The total reaction mixture of 25 μL contained 12.5 μL of SYBR® Premix Ex Taq™ (2 ×) (Sigma, USA), 0.5 μL of the forward primer, 0.5 μL of the reverse primer, 2 μL of cDNA templates and 9.5 μL of sterile DEPC water. The PCR programme involved pre-denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 58°C for 1 min and extension at 60°C for 1 min. FastStart Universal SYBR Green Master (Roche) and StepOne Plus Real-Time PCR System (Applied Biosystems, USA) were employed for amplification and detection. The results were analysed using the relative quantitative ($2^{-\Delta\Delta Ct}$) method. The experiments were repeated three times.

Detection of S. aureus thermonuclease

Toluidine blue nucleic acid agar was prepared, and a puncher was used to generate a 3-mm hole. The supernatant was boiled for 15 min and incubated in the hole at 37°C for 16 hours. The diameter was measured for comparison (a diameter >1 mm indicates a positive result).

Detection of S. aureus coagulase

Four millilitres of fresh rabbit blood [17] was centrifuged at 3,000 rpm for 10 min; the cells pelleted were discarded, and the plasma was diluted with PBS (1:4). A bacterial culture was diluted to different concentrations with TBS-G, and 100 μL of medium and 0.5 mL of rabbit blood plasma were mixed and incubated at 37°C until coagulation was observed. The largest fold dilution of the bacterial culture that resulted in coagulation was determined as the coagulase valency.

Statistical analysis

All experiments were repeated at least three times in duplicate to validate reproducibility. All values are presented as the mean ± standard error. Graphs were constructed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). A one-way ANOVA with Games-Howell post-hoc test was employed to examine differences between groups for statistical significance. *P*-values of less than 0.05 were considered statistically significant.

Results

Determination of MICs and the effects of baicalin on S. aureus growth in vitro

The MICs of baicalin, clarithromycin and levofloxacin against *S. aureus* 17546 are shown in **Table 2**. *S. aureus* was grown in the presence of baicalin (1024, 512, 256 or 128 μg/mL) for 24 hours to examine the antibacterial activity of sub-inhibitory concentrations of baicalin. As indicated by the growth curves (**Figure 1**), sub-inhibitory concentrations of baicalin had no inhibitory effect on the growth of *S. aureus*.

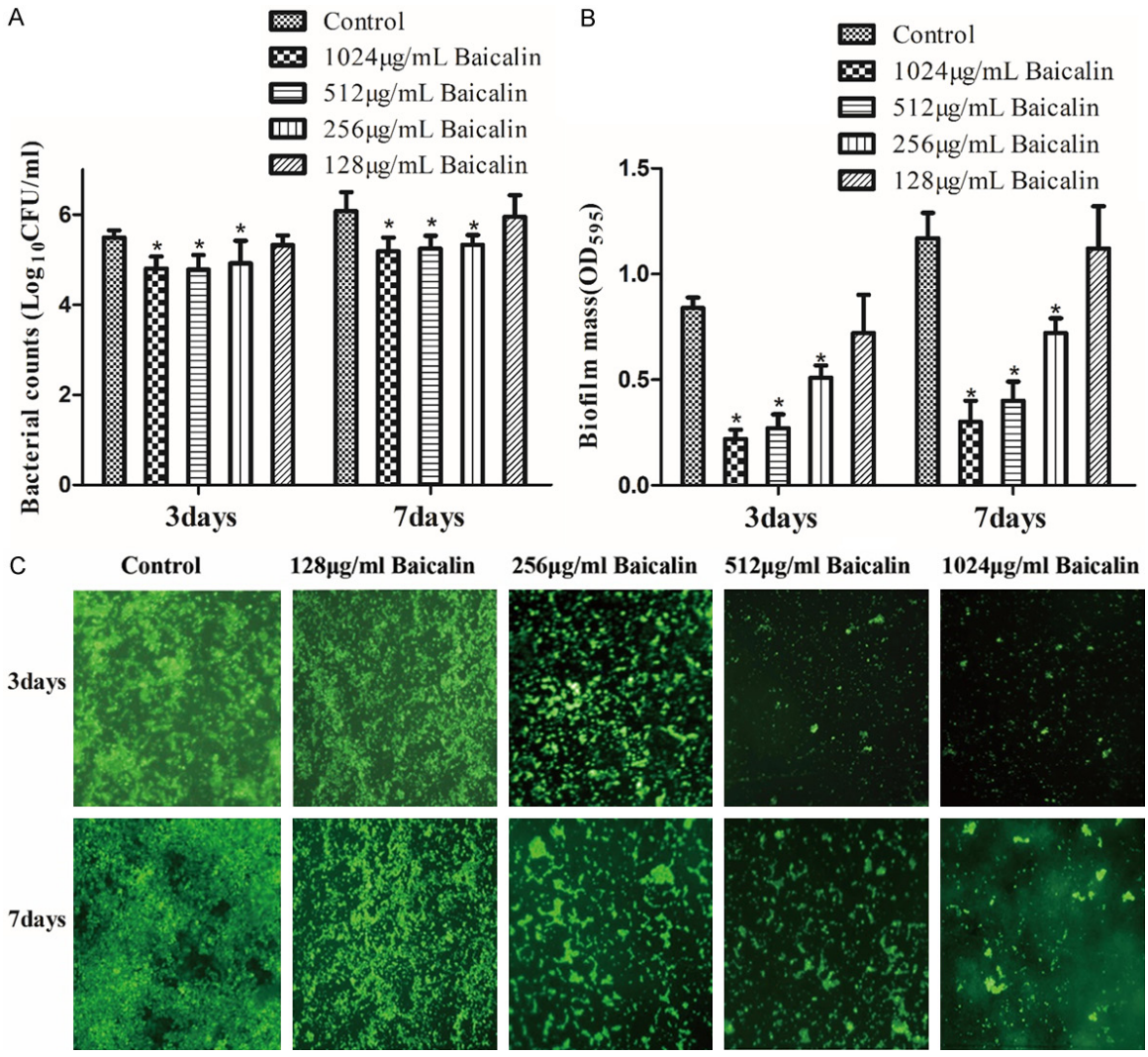

Figure 2. Inhibitory effects of baicalin on *S. aureus* biofilm formation. *S. aureus* was grown on polystyrene carriers with baicalin (1024 μg/mL, 512 μg/mL, 256 μg/mL and 128 μg/mL) for 3 days and 7 days. A. Biofilm bacterial counts were obtained after exposure to baicalin. Biofilm mass formation and bacterial counts were quantified in triplicate ($P < 0.05$; *compared to the control group). B. Biofilms were stained with crystal violet, and OD595 was measured ($P < 0.05$; *compared to the control group). C. Static biofilms after exposure to baicalin for 3 days (top) and 7 days (bottom) were stained with SYTO-9. *S. aureus* within biofilms on polystyrene carriers display green fluorescence (200 ×).

Baicalin inhibits S. aureus biofilm formation

The antibiofilm activity of baicalin was examined using the plate counting method and crystal violet staining. *S. aureus* 17546 was grown in the presence of baicalin (1024, 512, 256 or 128 μg/mL) on polystyrene carriers for 3 and 7 days, and the biofilm mass was stained with crystal violet and measured spectrophotometrically (at OD595); the number of bacteria in biofilms was calculated by the colony count assay. Sub-MICs (1024, 512, or 256 μg/mL) of baicalin significantly reduced cell attachment and biofilm biomass development, but concentrations less than 128 μg/mL did not exert an effect (**Figure 2A** and **2B**). Fluorescence microscopy revealed a similar finding: sub-MICs (1024, 512, or 256 μg/mL) of baicalin inhibited adhesion of the bacteria to the carrier surface and decreased polysaccharide production compared to the control treatment, whereas 128 μg/mL baicalin did not have an effect (**Figure 2C**).

Synergistic effects of baicalin and levofloxacin on S. aureus biofilm formation

Biofilms were established as described above, and baicalin alone or in combination with levofloxacin was applied on the 3rd and 7th days

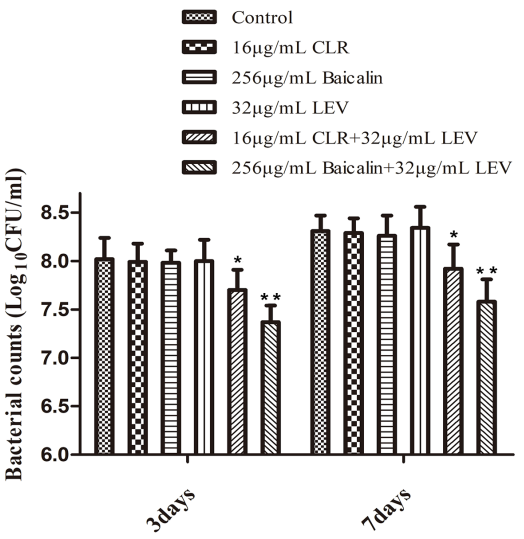

Figure 3. Quantitative analysis of the effects of baicalin with levofloxacin on 3- and 7-day-old biofilms. 3- and 7-day-old biofilms were established on polystyrene carriers by growing *S. aureus* in TSB-G and then treating with CLR, baicalin, or LEV alone or LEV in combination with baicalin or CLR for 12 h (*compared to the control, $P < 0.05$; **compared to the LEV, $P < 0.05$; average of 3 experiments).

followed by incubation for 12 hours; clarithromycin was used as a positive control. Treatment with baicalin, clarithromycin and levofloxacin alone did not reduce the number of bacteria within the biofilm regardless of whether the compounds were added on the 3rd or 7th day of biofilm formation. However, treatment with baicalin + levofloxacin or clarithromycin + levofloxacin reduced the number of bacteria, with fewer viable counts in these groups than in the control group and the levofloxacin group. Furthermore, bacterial counts for biofilms treated with baicalin + levofloxacin were lower than for those treated with clarithromycin + levofloxacin (**Figure 3**).

SEM

Biofilms were treated with baicalin, clarithromycin, levofloxacin, baicalin + levofloxacin, or clarithromycin + levofloxacin on days 3 and 7, and SEM (**Figure 4**) revealed bacterial adhesion to the carriers as well as secretion of an extracellular matrix to form a biofilm. The structure of the 7-day biofilm was denser than that of the 3-day biofilm, and more extracellular matrix was observed. Regardless of whether a 3-day or 7-day biofilm was examined, baicalin or antibiotics alone did not eradicate *S. aureus* from pre-existing biofilms. However, combined treatment with baicalin and antibiotics was effective at disrupting biofilms: very few *S. aureus* cells and extracellular matrix remained, and those that were present exhibited a scattered distribution.

CLSM

Biofilm was established as prepared for SEM and visualized by CLSM (**Figure 5**; green fluorescence represents a live biomass). Similar to the SEM results, *S. aureus* formed biofilms on the surface of the carrier after 3 and 7 days. The 7-day biofilm was denser than was the 3-day biofilm. Regardless of whether a 3-day or 7-day biofilm was examined, baicalin, clarithromycin and levofloxacin alone did not eradicate the bacteria within the biofilm, and little red fluorescence (representing dead cells) was observed. However, red fluorescence was primarily observed in the baicalin + levofloxacin and clarithromycin + levofloxacin groups, indicating that baicalin disrupted the biofilm and promoted penetration of levofloxacin to kill the bacteria.

Western blotting

Western blot analyses were performed to examine *S. aureus* exotoxin A (SEA) expression. The SEA band for cells treated with baicalin (1024, 512 or 256 μg/mL, respectively) was less intense than that in the control group (**Figure 6A**). Moreover, SEA was expressed at lower levels in the treated group than in the control group (**Figure 6B**).

Relative expression of the Hla and agrA genes

Expression of hla and agrA was quantified by RTQ-PCR. After treatment with baicalin (concentrations ranging from 1024 to 128 μg/mL), lower relative expression of the hla gene was observed in the baicalin group than in the control group, and the 512 μg/mL baicalin group exhibited the lowest expression (**Figure 7**). Levels of the agrA transcript were also significantly decreased ($P < 0.05$) (**Figure 8**).

S. aureus thermonuclease

The diameters of the different baicalin groups were larger than those in the control group after 16 hours of incubation with toluidine blue nucleic acid agar (**Figure 9**).

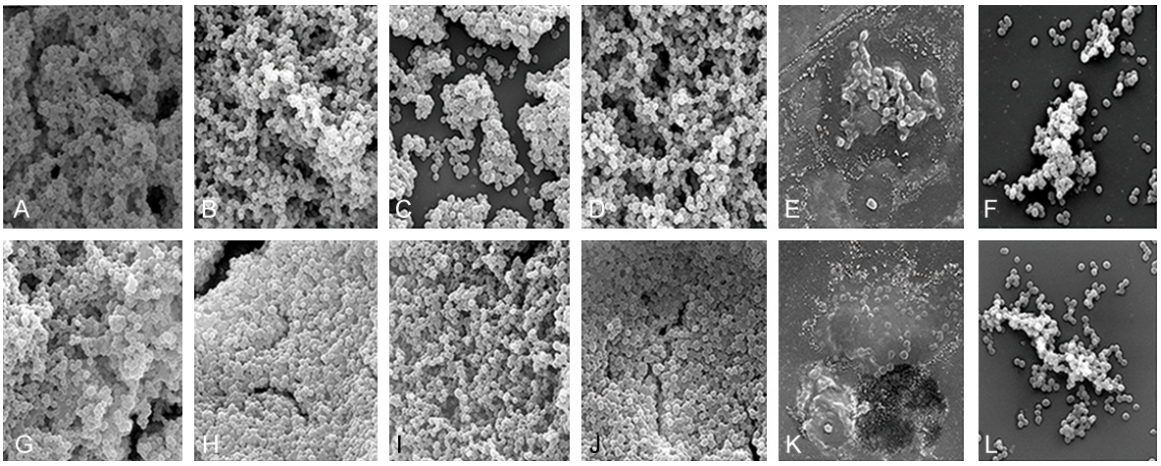

Figure 4. Synergistic effects of baicalin with levofloxacin on 3- (A-F) and 7-day (G-L)-old biofilms, as determined by SEM (8000 ×). 3- and 7-day-old biofilms were established on polystyrene carriers by growing *S. aureus* in TSB-G and treating with CLR, baicalin, and LEV alone or LEV in combination with baicalin or CLR for 12 h. Static biofilms were dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, and 100%) and coated with gold. (A/G) control group, (B/H) 16 μg/mL CLR, (C/I) 256 μg/mL baicalin, (D/J) 32 μg/mL LEV, (E/K) 16 μg/mL CLR + 32 μg/mL LEV, (F/L) 256 μg/mL baicalin + 32 μg/mL LEV.

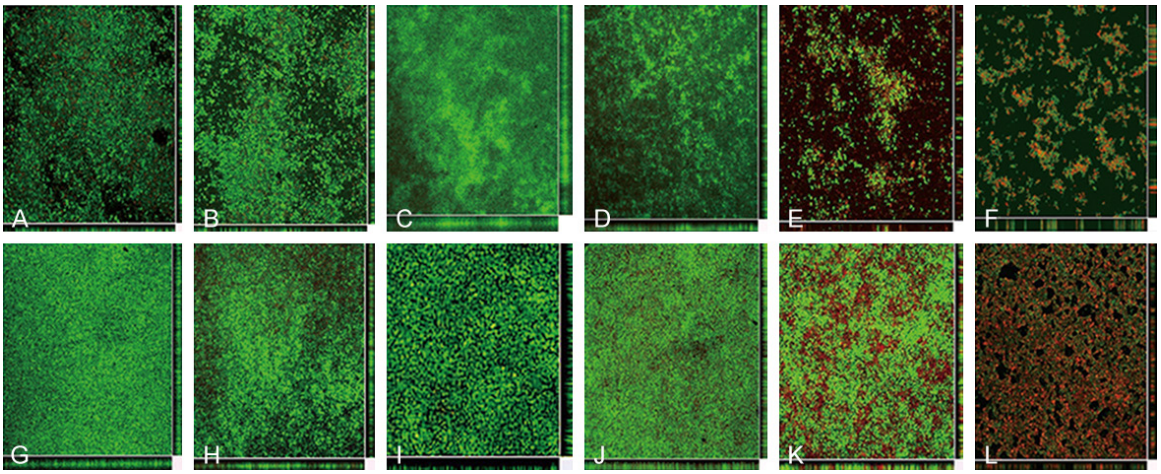

Figure 5. Synergistic effects of baicalin with levofloxacin on 3- (A-F) and 7-day (G-L)-old biofilms, as determined by CLSM (400 ×). 3- and 7-day-old biofilms were established on polystyrene carriers by growing *S. aureus* in TSB-G and treating with CLR, baicalin, and LEV alone or LEV combination with baicalin or CLR for 12 h. Static biofilms were stained with SYTO-9 and propidium iodide. (A/G) control group, (B/H) 16 μg/mL CLR, (C/I) 256 μg/mL baicalin, (D/J) 32 μg/mL LEV, (E/K) 16 μg/mL CLR + 32 μg/mL LEV, (F/L) 256 μg/mL baicalin + 32 μg/mL LEV. Green represents live biomass; red represents dead biomass.

S. aureus coagulase

In contrast to the results from the experiments described above, treatments with higher concentrations of baicalin (1024 and 512 μg/mL) inhibited the activity of *S. aureus* coagulase (**Figure 10**).

Discussion

The difficulty in controlling *S. aureus* infections is not only related to the improper use of antibiotics but is also closely related to the production of bacterial biofilm (BF) [18, 19]. Biofilms comprise a very dynamic system, and their formation is a layer delivery process [20, 21]. First, the bacteria must be close to a solid surface to form a mature biofilm, accordingly, attachment to the surface is the key step in the process by which a biofilm forms from single cells in suspension [22]. Zeng *et al*. and Luo *et al*. reported the ability of baicalin to inhibit *Pseudomonas aeruginosa* attachment to a

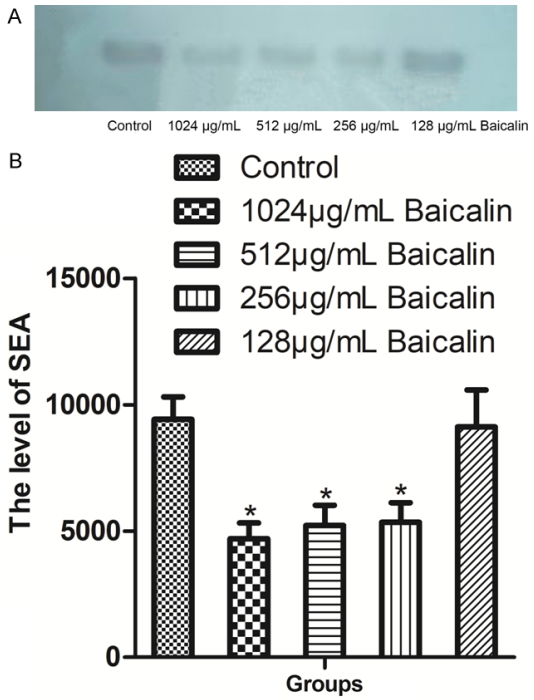

Figure 6. Detection of SEA. A. Western blot analysis of SEA for control group and groups treated with 1024, 512, 256 and 128 μg/ml baicalin. B. Quantitative analysis of SEA expression. (*P < 0.05, compared to the control). Data shown are the average of 3 experiments.

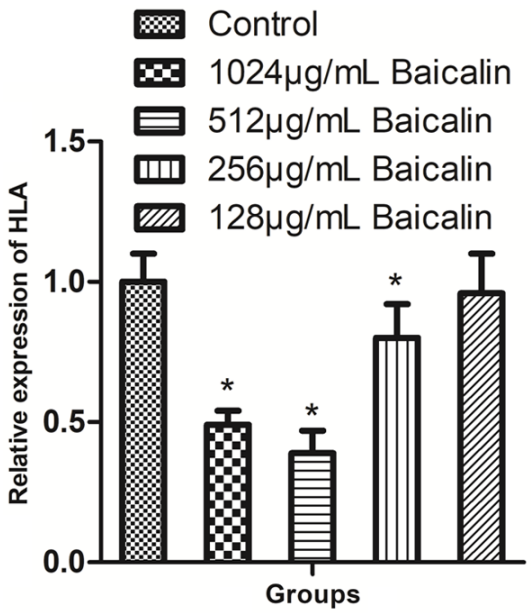

Figure 7. Effect of sub-MIC concentration of baicalin on HLA. After treatment of bacterial suspensions with 1024-128 μg/mL baicalin, the levels of specific RNA transcripts in different samples were measured. Total RNA extracts were used as a template for cDNA synthesis, and cDNA was assessed by RTQ-PCR. Relative gene expression levels of hla were calculated. Comparative measurements of relative expression are shown as an average of 3 experiments. *P < 0.05, compared to the control.

glass or polystyrene microtiter plate [23, 24]. In our study, baicalin effectively prevented *S. aureus* biofilm formation at sub-inhibitory concentrations. Fluorescence microscopy revealed that fewer bacteria had attached to the carrier surface after baicalin treatment, an observation that may be considered an extension of the research of Zeng *et al*. and Luo *et al*. We speculate that baicalin inhibits *S. aureus* adhesion, disrupting the biofilm formation. Therefore, baicalin shows promise for prophylactic treatment of early-stage *S. aureus* biofilm-related infections.

The treatment of mature bacterial biofilm infections in the clinical environment is mainly dependent on a large number of combined antibiotics. According to Jorge Parra-Ruiz *et al.*, a combination of linezolid plus daptomycin is a more effective treatment for *S. aureus* biofilms than is either agent alone [25]. In addition, Dong Chai *et al*. reported that the combination of linezolid and fosfomycin has good therapeutic effects on biofilm-embedded MRSA infections both in vitro and in vivo [26]. Nonetheless, the combined use of antibiotics is easily circumvented by antibiotic resistance. Baicalin is a major component of *Scutellaria* extracts [27]; it has the advantage of low toxicity and is not associated with drug resistance. Based on our current SEM and CLSM results, baicalin destroyed mature biofilms, as the biofilm structure was looser and the amount of extracellular matrix was significantly reduced. Moreover, according to colony counts, the combination of baicalin and levofloxacin effectively killed bacteria within biofilms, and this combination resulted in a greater reduction in viable count than did levofloxacin alone. Our data indicate a potential application of baicalin as an adjuvant therapeutic agent for preventing *S. aureus* mature biofilm-related infections.

The pathogenicity of *S. aureus* mainly depends on its virulence factors [28]. Therefore, treatments that target bacterial virulence factors are being increasingly studied. SEA causes food poisoning, shock, and allergy [29], and Shimamura Y *et al*. demonstrated that plant-

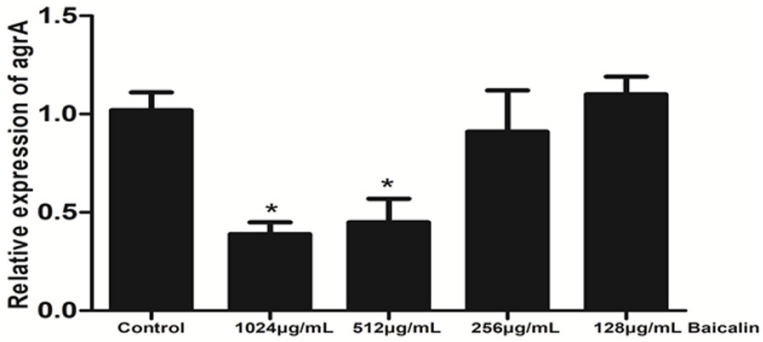

Figure 8. Effect of sub-MIC concentration of baicalin on agrA gene expression. After *S. aureus* 17546 was cultured with different sub-MIC concentrations of baicalin, the levels of specific RNA transcripts in different samples were measure. Total RNA extracts were used as a template for cDNA synthesis, and cDNA was assessed by RTQ-PCR. Data shown are the average of 3 experiments, and relative quantitative values were calculated using the $2^{-\Delta\Delta Ct}$. *$P < 0.05$, compared to the control.

derived polyphenols interact with SEA and inhibit toxin activity [30]. In the present study, sub-MICs of baicalin decreased SEA production in a dose-dependent manner.

Hla forms a micro-channel in the hydrophobic region of the cell membrane, resulting in an imbalance in intracellular and extracellular ions and subsequent cell necrosis [31-33]. In a study involving in vitro biofilm formation on polystyrene, an *S. aureus* hla mutant exhibited dramatically reduced biofilm formation based on a standard microtiter plate assay and under flow conditions. Initial attachment to the surface was also decreased, indicating that an inability to bind to the surface contributes to decreased formation of a mature biofilm. However, the exact mechanism remains unclear. Based on our RTQ-PCR results, baicalin (1024, 512 and 256 μg/mL) inhibited expression of hla compared with the control treatment.

Nucleic acids degrades by *S. aureus* thermonuclease when a tissue or the cell infected by *S. aureus* undergoes apoptosis, promoting a diffuse infection [34], and activity of this enzyme has been shown to correlate with biofilm formation. Extracellular DNA (eDNA) [35] is one of most important components of the biofilm matrix: it promotes the connection between cells and helps to stabilize the mature biofilm. *S. aureus* with a Sig B mutation rarely forms biofilms because it secretes thermonuclease to degrade eDNA [36]. Therefore, we postulate that *S. aureus* thermonuclease exerts negative effects on biofilms. Our research revealed that sub-MICs of baicalin increased thermonuclease production in a dose-dependent manner, indicating that baicalin may be considered a secretagogue that enhances thermonuclease production.

When *S. aureus* invades the human body, coagulase causes fibrin in the blood or plasma to deposit or coagulate on the surface of the bacteria, hindering phagocytosis and causing abscess formation and adhesion of *S. aureus* to catheters during biofilm-associated infections [37-39]. *S. aureus* also produces toxic shock syndrome toxin-1, gelatinase, protease, lipase, peptidase and other proteins. Based on the results of our assays, coagulase was inhibited by baicalin at concentrations of 1024 and 512 μg/mL.

The QS system not only plays a regulatory role in the formation of *S. aureus* biofilms at various stages but is also important for coordinating the production of virulence factors [5]. Such regulatory networks are composed of various systems and pathways that are both independent of and interact with each other. The accessory gene regulator (agr) system is considered a QS system that plays a key role in *S. aureus* [40], and Qiu *et al*. confirmed that thymol significantly inhibited agrA transcription [41]. The agr system, which is activated by self-encoded polypeptides, has an important role in regulating many extracellular proteins and cytoplasmic protein genes. The agr locus consists of two divergent transcriptional loci, RNAII and RNAIII, driven by promoters P2 and P3, respectively. RNA II contains four open reading frames, agrA, agrB, agrC and agrD, encoding four proteins, AgrA, AgrB, AgrC and AgrD, respectively. AgrD is the precursor of autoinducing polypeptide (AIP), which is processed by AgrB to generate mature AIP and then transported to the extracellular space. When AIP accumulation reaches a certain threshold, AgrA is activated by agrC-dependent phosphorylation, significantly accelerating the transcriptional efficiency of P2 and P3. P3 initiates the transcription of RNA III, a regulatory RNA molecule that controls

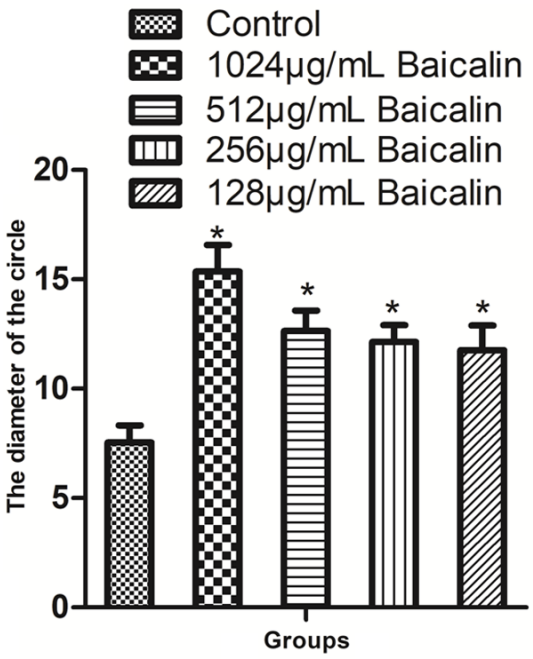

Figure 9. Detection of *S. aureus* thermonuclease. *S. aureus* 17546 was cultured with 1024-128 μg/mL baicalin. The bacterial suspension was centrifuged, and the supernatants were incubated for 16 hours. The diameters of the different baicalin groups were larger than those of the control group. *P < 0.05, compared to the control.

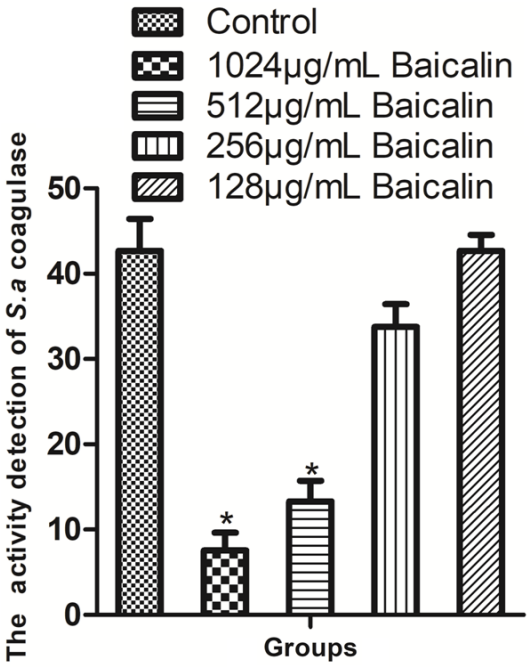

Figure 10. Detection of *S. aureus* coagulase. *S. aureus* 17546 was cultured with 1024-128 μg/mL baicalin; rabbit blood plasma was mixed with diluted bacterial suspensions and incubated. Coagulation indicates a positive effect. Treatments with higher concentrations of baicalin (1024 and 512 μg/mL) showed inhibited activity of *S. aureus* coagulase. *P < 0.05, compared to the control.

expression of many virulent factors. Although the agr system is considered to play a leading role, many genes interact with it to form a complex regulatory network that coordinates the regulation of virulence factor expression. For example, the dual regulation system of ArlS/ArlR regulates expression of beta-hemolysin, lipase, coagulase and other virulence factors, and The SAR system is activated by AgrA, upregulating expression of various toxins. Jia, P *et al*. confirmed that clindamycin differentially inhibits the transcription of exoprotein genes in *S. aureus*, partly through sar [42]. In the present study, 1024 and 512 μg/mL baicalin downregulated agrA expression. However, the mechanisms by which *S. aureus* controls virulence gene expression are fairly intricate. We hypothesize that baicalin regulates expression of virulence factors by inhibiting the agr system, though the specific mechanism needs further in-depth research.

In conclusion, baicalin inhibits *S. aureus* biofilm formation and exerts a synergistic effect with levofloxacin. Baicalin might be considered to act as a QS inhibitor that in vitro regulates *S. aureus* virulence factors by inhibiting the agr system.

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Disclosure of conflict of interest

None.

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References

[1] Nickel JC, Gristina AG and Costerton JW. Electron microscopic study of an infected Foley catheter. Can J Surg 1985; 28: 50-51, 54.

[2] Peters RG, Jones WC and Cromartie TH. Inactivation of L-lactate monooxygenase with 2,3-butanedione and phenylglyoxal. Biochemistry 1981; 20: 2564-2571.

[3] Tenney JH, Moody MR, Newman KA, Schimpff SC, Wade JC, Costerton JW and Reed WP. Adherent microorganisms on lumenal surfaces of long-term intravenous catheters. Importance of staphylococcus epidermidis in patients with cancer. Arch Intern Med 1986; 146: 1949-1954.

[4] Parvizi J, Alijanipour P, Barberi EF, Hickok NJ, Phillips KS, Shapiro IM, Schwarz EM, Stevens MH, Wang Y and Shirtliff ME. Novel developments in the prevention, diagnosis, and treatment of periprosthetic joint infections. J Am Acad Orthop Surg 2015; 23 Suppl: S32-43.

[5] Xu L, Li H, Vuong C, Vadyvaloo V, Wang J, Yao Y, Otto M and Gao Q. Role of the luxS quorum-sensing system in biofilm formation and virulence of staphylococcus epidermidis. Infect Immun 2006; 74: 488-496.

[6] Banerjee G and Ray AK. Quorum-sensing network-associated gene regulation in gram-positive bacteria. Acta Microbiol Immunol Hung 2017; 64: 439-453.

[7] March Rossello GA and Eiros Bouza JM. Quorum sensing in bacteria and yeast. Med Clin (Barc) 2013; 141: 353-357.

[8] Myszka K and Czaczyk K. Quorum sensing mechanism as a factor regulating virulence of Gram-negative bacteria. Postepy Hig Med Dosw (Online) 2010; 64: 582-589.

[9] Barnard AM, Bowden SD, Burr T, Coulthurst SJ, Monson RE and Salmond GP. Quorum sensing, virulence and secondary metabolite production in plant soft-rotting bacteria. Philos Trans R Soc Lond B Biol Sci 2007; 362: 1165-1183.

[10] Maddula VS, Zhang Z, Pierson EA and Pierson LS 3rd. Quorum sensing and phenazines are involved in biofilm formation by pseudomonas chlororaphis (aureofaciens) strain 30-84. Microb Ecol 2006; 52: 289-301.

[11] Qiu J, Zhang X, Luo M, Li H, Dong J, Wang J, Leng B, Wang X, Feng H, Ren W and Deng X. Subinhibitory concentrations of perilla oil affect the expression of secreted virulence factor genes in Staphylococcus aureus. PLoS One 2011; 6: e16160.

[12] Zhang F, Sun L, Gao SH, Chen WS and Chai YF. LC-MS/MS analysis and pharmacokinetic study on five bioactive constituents of Tanreqing injection in rats. Chin J Nat Med 2016; 14: 769-775.

[13] Zhang H, Chen Q, Zhou W, Gao S, Lin H, Ye S, Xu Y and Cai J. Chinese medicine injection shuanghuanglian for treatment of acute upper respiratory tract infection: a systematic review of randomized controlled trials. Evid Based Complement Alternat Med 2013; 2013: 987326.

[14] Brackman G and Coenye T. Comment on: synergistic antibacterial efficacy of early combination treatment with tobramycin and quorum-sensing inhibitors against pseudomonas aeruginosa in an intraperitoneal foreign-body infection mouse model. J Antimicrob Chemother 2013; 68: 2176-2177.

[15] Boles BR and Horswill AR. Agr-mediated dispersal of staphylococcus aureus biofilms. PLoS Pathog 2008; 4: e1000052.

[16] Chen Y, Liu T, Wang K, Hou C, Cai S, Huang Y, Du Z, Huang H, Kong J and Chen Y. Baicalein inhibits staphylococcus aureus biofilm formation and the quorum sensing system in vitro. PLoS One 2016; 11: e0153468.

[17] Kuroda H, Kuroda M, Cui L and Hiramatsu K. Subinhibitory concentrations of beta-lactam induce haemolytic activity in staphylococcus aureus through the SaeRS two-component system. FEMS Microbiol Lett 2007; 268: 98-105.

[18] Hoiby N, Ciofu O, Johansen HK, Song ZJ, Moser C, Jensen PO, Molin S, Givskov M, Tolker-Nielsen T and Bjarnsholt T. The clinical impact of bacterial biofilms. Int J Oral Sci 2011; 3: 55-65.

[19] Wu H, Moser C, Wang HZ, Hoiby N and Song ZJ. Strategies for combating bacterial biofilm infections. Int J Oral Sci 2015; 7: 1-7.

[20] York A. Biofilms: the architect of the biofilm. Nat Rev Microbiol 2017; 15: 642-643.

[21] de la Fuente-Nunez C, Reffuveille F, Fernandez L and Hancock RE. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. Curr Opin Microbiol 2013; 16: 580-589.

[22] Mack D. Molecular mechanisms of Staphylococcus epidermidis biofilm formation. J Hosp Infect 1999; 43 Suppl: S113-125.

[23] Zeng Z, Qian L, Cao L, Tan H, Huang Y, Xue X, Shen Y and Zhou S. Virtual screening for novel quorum sensing inhibitors to eradicate biofilm formation of Pseudomonas aeruginosa. Appl Microbiol Biotechnol 2008; 79: 119-126.

[24] Luo J, Dong B, Wang K, Cai S, Liu T, Cheng X, Lei D, Chen Y, Li Y, Kong J and Chen Y. Baicalin inhibits biofilm formation, attenuates the quorum sensing-controlled virulence and enhances pseudomonas aeruginosa clearance in a mouse peritoneal implant infection model. PLoS One 2017; 12: e0176883.

[25] Parra-Ruiz J, Bravo-Molina A, Pena-Monje A and Hernandez-Quero J. Activity of linezolid

and high-dose daptomycin, alone or in combination, in an in vitro model of staphylococcus aureus biofilm. J Antimicrob Chemother 2012; 67: 2682-2685.
[26] Chai D, Liu X, Wang R, Bai Y and Cai Y. Efficacy of linezolid and fosfomycin in catheter-related biofilm infection caused by methicillin-resistant staphylococcus aureus. Biomed Res Int 2016; 2016: 6413982.
[27] Zhao Q, Chen XY and Martin C. Scutellaria baicalensis, the golden herb from the garden of Chinese medicinal plants. Sci Bull (Beijing) 2016; 61: 1391-1398.
[28] Lewandowski T, Huang J, Fan F, Rogers S, Gentry D, Holland R, Demarsh P, Aubart K and Zalacain M. Staphylococcus aureus formyl-methionyl transferase mutants demonstrate reduced virulence factor production and pathogenicity. Antimicrob Agents Chemother 2013; 57: 2929-2936.
[29] Schelin J, Wallin-Carlquist N, Cohn MT, Lindqvist R, Barker GC and Radstrom P. The formation of staphylococcus aureus enterotoxin in food environments and advances in risk assessment. Virulence 2011; 2: 580-592.
[30] Shimamura Y, Aoki N, Sugiyama Y, Tanaka T, Murata M and Masuda S. Plant-derived polyphenols interact with staphylococcal enterotoxin A and inhibit toxin activity. PLoS One 2016; 11: e0157082.
[31] Kaneko J and Kamio Y. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci Biotechnol Biochem 2004; 68: 981-1003.
[32] Vandenesch F, Lina G and Henry T. Staphylococcus aureus hemolysins, bi-component leukocidins, and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors? Front Cell Infect Microbiol 2012; 2: 12.
[33] Wiseman GM. The hemolysins of Staphylococcus aureus. Bacteriol Rev 1975; 39: 317-344.
[34] Suarez MJ, Arias ML and del Mar Gamboa M. Staphylococcus aureus enterotoxin A detection using the polymerase chain reaction (PCR) and its correlation with coagulase and thermonuclease tests. Arch Latinoam Nutr 2008; 58: 59-63.
[35] Whitchurch CB, Tolker-Nielsen T, Ragas PC and Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science 2002; 295: 1487.
[36] Liu H, Shang W, Hu Z, Zheng Y, Yuan J, Hu Q, Peng H, Cai X, Tan L, Li S, Zhu J, Li M, Hu X, Zhou R, Rao X and Yang Y. A novel SigB(Q225P) mutation in Staphylococcus aureus retains virulence but promotes biofilm formation. Emerg Microbes Infect 2018; 7: 72.
[37] Peetermans M, Verhamme P and Vanassche T. Coagulase activity by staphylococcus aureus: a potential target for therapy? Semin Thromb Hemost 2015; 41: 433-444.
[38] McAdow M, DeDent AC, Emolo C, Cheng AG, Kreiswirth BN, Missiakas DM and Schneewind O. Coagulases as determinants of protective immune responses against staphylococcus aureus. Infect Immun 2012; 80: 3389-3398.
[39] Thomer L, Schneewind O and Missiakas D. Multiple ligands of von Willebrand factor-binding protein (vWbp) promote staphylococcus aureus clot formation in human plasma. J Biol Chem 2013; 288: 28283-28292.
[40] Chen J and McClane BA. Role of the Agr-like quorum-sensing system in regulating toxin production by clostridium perfringens type B strains CN1793 and CN1795. Infect Immun 2012; 80: 3008-3017.
[41] Qiu J, Wang D, Xiang H, Feng H, Jiang Y, Xia L, Dong J, Lu J, Yu L and Deng X. Subinhibitory concentrations of thymol reduce enterotoxins A and B and alpha-hemolysin production in Staphylococcus aureus isolates. PLoS One 2010; 5: e9736.
[42] Jia P, Xue YJ, Duan XJ and Shao SH. Effect of cinnamaldehyde on biofilm formation and sarA expression by methicillin-resistant Staphylococcus aureus. Lett Appl Microbiol 2011; 53: 409-416.