Effects of stigmata maydis on the methicillin resistant *Staphylococus aureus* biofilm formation (#30690)

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Effects of stigmata maydis on the methicillin resistant *Staphylococcus aureus* biofilm formation

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Background Mastitis is an inflammatory reaction of the mammary gland tissue, which causes huge losses to dairy farms throughout the world. *Staphylococcus aureus* is the most frequent agent associated with this disease. *S. aureus* isolates, which have the ability to form biofilms, usually lead to chronic mastitis in dairy cows.Moreover, methicillin resistance of the bacteria further complicates the treatment of this disease. Stigmata maydis (corn silk), one kind of traditional Chinese medicine, possess many biological activities. **Methods** In this study, we performed antibacterial activity assays, biofilm formation assays and real-time reverse transcription PCR (RT-PCR) experiments to investigate the effect of stigmata maydis (corn silk) on biofilm formation and vancomycin susceptibility of methicillin-resistant *S. aureus* (MRSA) strains isolated from dairy cows with mastitis. **Results** In this study, the aqueous extracts of stigmata maydis inhibited the biofilm formation ability of MRSA strains and increased the vancomycin susceptibility of the strains under biofilm-cultured conditions. **Conclusion** This study proves that the aqueous extracts of stigmata maydis inhibit the biofilm formation ability of MRSA strains and increase the vancomycin susceptibility of the MRSA strains under biofilm-cultured conditions.

RUNNING TITLE: Stigmata maydis affects MRSA biofilms

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ABSTRACT

Background Mastitis is an inflammatory reaction of the mammary gland tissue, which causes huge losses to dairy farms throughout the world. *Staphylococcus aureus* is the most frequent agent associated with this disease. *S. aureus* isolates, which have the ability to form biofilms, usually lead to chronic mastitis in dairy cows. Moreover, methicillin resistance of the bacteria further complicates the treatment of this disease. Stigmata maydis (corn silk), one kind of traditional Chinese medicine, possess many biological activities.

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KEYWORDS: MRSA, bovine mastitis, biofilm, vancomycin, stigmata maydis

INTRODUCTION

Staphylococcus aureus is a major pathogen that can cause a series of infections in both hospital and community environments (Archer & Climo 2001; F D 1998). The infections caused by this bacterium are complicated by frequent and multiple antibiotic use in medical treatment in the past several decades (Lowy 2003; Queck et al. 2009). Methicillin-resistant *Staphylococcus aureus* (MRSA) arose in the 1960's (9), after methicillin became the antibiotic of first choice for *S. aureus* infections because of the wide spread of penicillin-resistant strains (Richmond 1979). In the first few years, MRSA strains only affected people who were associated with risk factors, such as surgery, recent admittance, or long-term residence in care facilities. However, community-associated MRSA affections are now prevalent in the general population and pose a serious threat to public health worldwide (Chambers 2001; Health et al. 1999; Hiramatsu et al. 2001; Naimi et al. 2001). In addition, the treatment for these infections becomes more difficult and complicated due to the development of biofilms (Kiedrowski & Horswill 2011).

The formation of a biofilm is characterized by the structure of a population of bacteria encased within a self-produced extracellular matrix of exopolysaccharide, proteins and some micromolecules, such as DNA (O'Gara 2007). It is well known that the properties of biofilm populations are largely different from planktonic cell populations, and these contribute to better adaptation to the host environment. The presence of glycocalyx layers protects the enclosed bacteria from host defenses and resists the access of antibiotics (Atshan et al. 2012b; Fedtke et al. 2004; Joh et al. 1999). It has been reported that biofilms can resist antibiotic concentration 10-10,000 fold higher than those required to inhibit the growth of their planktonic counterparts (Atshan et al. 2015; Jefferson et al. 2005). Indeed, the ability of biofilm formation in MRSA can

lead to resistance to most currently used antibiotics (Ando et al. 2004). Therefore, biofilm formation brings great challenges for the infection treatment, eventually leading to chronic infections, which can be difficult to eradicate (Kiedrowski & Horswill 2011; Petrelli et al. 2008; Pozo & Patel 2007).

Bovine mastitis is a disease causing substantial economic loss in the dairy industry worldwide (Hillerton & Berry 2005; Huijps et al. 2008; Szweda et al. 2014). Although many species of etiological microorganisms have been isolated from bovine mastitis (Watts 1988), *S. aureus* is a frequent cause that is responsible for the main loss (Kozytska et al. 2010; Malinowski & Kłossowska 2010; Piepers et al. 2007). Since *S. aureus* has the ability to form biofilms and is resistant to many antibiotics, it causes chronic bovine mastitis, which is difficult to treat (Cramton et al. 1999). Moreover, methicillin resistance of *S. aureus* could further complicate the treatment of this disease (Joshi et al. 2018; Lowy 2003).

Many plants have been used as traditional Chinese medicine for the treatment of various diseases in China. The medicinal value of plants lies in some constituents that have definite biological functions. In recent years, many Chinese medicines have been reported to have antimicrobial effects.

Stigmata maydis (corn silk) refers to the stigmas of the female flowers of maize, which contain proteins, carbohydrates, vitamins, Ca, K, Mg and Na salts, fixed and volatile oils, steroids, such as sitosterol and stigmasterol, alkaloids, saponins, tannins, and flavonoids (Bhaigyabati et al. 2011; Hasanudin et al. 2012). Many biological activities of corn silk constituents have been reported. Extracts of corn silk inhibited TNF and LPS-induced cell

adhesion, but not cytotoxic activity or TNF production(Habtemariam 1998). Moreover, volatiles from corn silk showed antifungal activity (Jr 2000). In addition, extracts of corn silk displayed antioxidant activity on the level of lipid peroxidation (Bhaigyabati et al. 2011). Corn silk has also been used as a remedy for acute inflammation of the urinary system, such as urethritis, cystitis and prostatitis in many parts of the world, and it has also been used as an oral antidiabetic agent in China for decades (Hasanudin et al. 2012). However, whether stigmata maydis is associated with biofilm formation and antibiotic resistance in bacteria has not been reported.

In this study, we aim to investigate the effect of stigmata maydis aqueous extracts on growth and biofilm formation of MRSA strains isolated from dairy cows with mastitis. The new finding in this study may provide new clues or potential methods to the efficient antibiotic treatment of this disease.

MATERIALS AND METHODS

Bacterial strain and growth condition

Staphylococcus aureus MRSA strains SA2 and SA3 used in this study were isolated from dairy cows with mastitis. The two MRSA strains are *mecA* positive and susceptible to vancomycin (vancomycin minimum inhibitory concentration (MIC) SA2 0.5 mg/L; SA3: 1 mg/L), and sensitive to chloromycetin, and resistant to ampicillin, erythromycin, and oxacillin. The strains were grown at 37 °C in tryptic soy broth (TSB) containing 0.25% glucose media (Oxoid, Basingstock, UK).

106 ***Aqueous extraction of stigmata maydis***

107 One gram of stigmata maydis powder was suspended in 10 mL water and soaked for 24 h. The
108 supernatant was dried by vacuum freeze drying and then supernatants were mixed into a 100
109 mg/mL extract.

110

111 ***Antimicrobial activity assay***

112 The method was performed as described previously as follows (Chen et al. 2015). Colonies of
113 MRSA strains were picked into 2 mL of TSB medium and cultivated at 37 °C with shaking at
114 200 rpm for 16 h. Then the overnight cultures were inoculated into fresh TSB medium and this
115 was diluted to a final optical density (600 nm) of 0.05, which was dispensed into 96-well plates
116 (Costar, Corning, Steuben, NY) containing serial dilutions of aqueous extracts of stigmata
117 maydis (with appropriate vancomycin, if needed). Plates were incubated at 37 °C for 12 h and
118 then 10-fold serial dilutions of cultures were performed by successive transfer (0.1 mL) through
119 seven microfuge tubes containing 0.9 mL of TSB. The 100 μL dilutions were dropped onto LB
120 agar plates and viable colonies were counted via their colony-forming units (CFU) on TSB agar
121 plates after incubation at 37 °C for 24 h. The survival rate of the control group without exposure
122 to *S. officinalis L.* was designated as 100 percent. CFU of the test groups were all compared with
123 that of the control group. Experiments were repeated three times with four parallels. Experiments
124 were repeated three times with four parallels.

125

126 ***Biofilm assays***

127 The method for biofilm quantification was performed as described previously and modified as
128 follows (Chen et al. 2015; Xue et al. 2014). MRSA strains were grown in TSB (containing 0.5%
129 glucose) for 16 h and diluted 1:100 into fresh TSB. The diluted cultures were transferred into
130 sterile 96 well flat-bottomed tissue culture plates and incubated at 37 °C for 24 h. Aqueous
131 extracts of stigmata maydis was added to the TSB media with diluted cultures at different
132 concentrations. The adherent bacteria were stained with crystal violet, and the excess stain was
133 washed off gently with slowly running water. The biomass of the biofilm was determined using a
134 MicroELISA auto-reader (Bio-Rad Co.) at a wavelength of 560 nm under single-wavelength
135 mode (Pozzi et al. 2012; Ziebuhr et al. 1997).
136
137 ***Total RNA isolation, cDNA generation, and real-time PCR processing***
138 Overnight cultures of MRSA strains were diluted 1:100 in TSB medium and the aqueous extracts
139 of stigmata maydis was added to the experimental group at different concentration (0.5mg/mL,
140 1mg/mL and 2mg/mL, respectively). The method used for the total RNA isolation of the treated
141 group is same with that of the non-treated group. The cells were grown to the late exponential
142 phase in 24 well plates (Costar, Corning, Steuben, NY). Subsequently, they were collected and
143 resuspended in TE (Tris-EDTA) buffer (pH 8.0) containing 10 g/L of lysozyme and 40 mg/L of
144 lysostaphin. After incubation at 37 °C for 5 min, cells were prepared for total RNA extraction
145 using the Trizol method (Invitrogen), and residual DNA was removed with DNase (RNase free;
146 TaKaRa). RT real-time PCR was performed with a PrimeScript 1st Strand cDNA synthesis kit
147 and SYBR Premix Ex Taq (TaKaRa) using a StepOne real-time PCR system (Applied

148 Biosystems). The quantity of cDNA measured by real-time PCR was normalized to the
149 abundance of 16S cDNA (Chen et al. 2000). All real-time RT-PCR assays were repeated at least
150 three times with similar results. The primers used in this study were listed in Table 1.
151
152 ***Statistical analysis***
153 The data was analyzed using statistical software SPSS by a one-way ANOVA method, the test
154 results were (mean ± standard deviation). The paired *t*-test was used for statistical comparisons
155 between groups. The level of statistical significance was set at a P value of ≤0.01.
156
157 **RESULTS**
158 ***Effects of stigmata maydis on the growth curve of MRSA strain***
159 The growth rates of the cells were tested when they were grown in TSB medium with different
160 concentrations of aqueous extract of stigmata. The results showed that the growth rates of the
161 bacteria did not change when the external concentration of stigmata maydis was 2.5 mg/mL, 5
162 mg/mL or 10 mg/mL, but when the concentration of stigmata maydis was 25 mg/mL, the growth
163 of the bacteria was a little inhibited (Fig. 1A). These data indicate that aqueous extracts of
164 stigmata maydis did not affect the growth curves of the MRSA strains.
165 Furthermore, in order to examine the antibacterial activity of the aqueous extracts of stigmata
166 maydis against *S. aureus in vitro*, antibacterial assays were performed. After exposure to extract
167 of stigmata maydis at different concentrations for 12 h at 37 °C, the cells of MRSA strains were
168 inoculated into fresh TSB and then spread onto the TSB agar plates. After cultivating for 24 h at

169 37 °C, the colony forming units of the bacteria were counted and compared. As is shown in Fig.
170 1B, the survival rate of the control group without exposure to stigmata maydis was designated as
171 100%. With the increase of the concentration of stigmata maydis, the survival rates of the MRSA
172 strain SA2 and SA3 also did not change significantly. These data confirmed that stigmata maydis
173 does not have antibacterial activity against the MRSA strains
174
175 ***Effects of stigmata maydis on the biofilm formation of MRSA strain***
176 To examine whether an aqueous extract of stigmata maydis would affect the biofilm formation of
177 *S. aureus*, we performed biofilm assays. As shown in Fig. 2A, strains of the control group
178 without aqueous extract of stigmata maydis formed obvious biofilms, when stigmata maydis at
179 different concentration was added, biofilm formation of the bacteria significantly decreased.
180 When the concentration of stigmata maydis reached 2 mg/mL, no biofilm was observed. In
181 addition, the quantity of biofilm formation was further tested using a MicroELISA autoreader.
182 We found that biofilm quantity decreased with the increase of stigmata maydis concentration
183 (Fig. 2B).
184
185 ***Effects of stigmata maydis on the biofilm genes expression***
186 The transcript levels of biofilm-associated genes were determined by performing real-time RT-
187 PCR experiments. As is shown in Fig. 3, the transcript levels of *icaA*, *icaB*, *icaC*, and *icaD* were
188 significantly decreased upon the addition of stigmata maydis. Moreover, with increased of
189 stigmata maydis concentrations, the inhibitory effect of stigmata maydis on the *ica* operon was

stronger, indicating that stigmata maydis affected the transcription of the *ica* operon in a concentration-dependent manner. To further investigate how stigmata maydis regulates the *ica* operon, we examined the transcript level of *icaR*, which has been identified as the repressor of the *ica* operon. Results showed that the transcript level of *icaR* increased by adding stigmata maydis to the culture medium with *S. aureus*, confirming that stigmata maydis influences the *ica* operon through the transcriptional regulator *icaR*.

Effects of stigmata maydis on the vacomycin susceptibility in the planktonic MRSA growth

To examine the effect of stigmata maydis on vacomycin susceptibility of the MRSA strains, the antibacterial assays were performed in the planktonic cultured MRSA strains. As is shown in Fig. 4A, in the presence of a low concentration of vancomycin (1/4 MIC concentration), with increased concentrations of stigmata maydis, the survival rates of MRSA strain SA2 and SA3 did not apparently change. As is shown in Fig. 4B, similar results were also observed in the planktonic cultured MRSA strains in the presence of a low concentration of vancomycin (1/2 MIC concentration). These data confirmed that stigmata maydis does not affect the vacomycin susceptibility of the planktonic cultured MRSA strains.

Effects of stigmata maydis on the vacomycin suspectibility in the biofilm MRSA growth

However, the results of the antibacterial assays performed in the biofilm-condition cultured MRSA strains were different with those performed in the planktonic-cultured MRSA strains. As shown in Fig. 5, the survival rates of MRSA strain SA2 and SA3 were all decreased with the

211 increased concentrations of stigmata maydis. When the 2 mg/mL stigmata maydis concentration
212 was added, the survival rates of strains SA2 and SA3 were decreased to about 50% in the
213 presence of 1/4 MIC concentration vancomycin (Fig. 5A), and the survival rates of strains SA2
214 and SA3 were decreased to about 30% in the presence of 1/2 MIC concentration vancomycin
215 (Fig. 5B). These results confirmed that, in the biofilm-cultured condition, the aqueous extract of
216 stigmata maydis could enhance the vancomycin susceptibility of the MRSA strains.
217

218 **DISCUSSION**

219 The data in this study showed that stigmata maydis aqueous extract did not affect the growth
220 of MRSA strains SA2 and SA3, and has no apparent antibacterial activity against these strains,
221 however, it significantly inhibited the biofilm formation of these strains only at a low
222 concentration. This is consistent with the previous findings by Lin et al. reported inhibitory
223 effects of 1,2,3,4,6-Penta-O-galloyl--D-glucopyranose (an active ingredient in plants) on biofilm
224 formation by *S. aureus* (Lin et al. 2011). Moreover, some kinds of Chinese medicine were
225 reported that both inhibited the growth and biofilm formation of *S.aureus* (Chen et al. 2015; Fan
226 et al. 2014).

227 According to previous work, biofilm formation in *S. aureus* includes two steps: attachment to
228 the material surface and then the formation of microcolonies and multilayered cell clusters
229 surrounded by a slimy matrix, which has been characterised as polysaccharide intercellular
230 adhesin (PIA). PIA is produced by the enzymes coded in an operon composed of four open
231 reading frames (ORFs) *icaA*, *icaD*, *icaB* and *icaC*. ~~e attachment process and accumulation of~~

bacteria is associated with several adhesion genes such as *fnbpA*, *fnbpB* (encoding fibronectin binding proteins A and B), *fib* (encoding fibrinogen binding protein), *clfA* (encoding clumping factors A), *clfB* (encoding clumping factor B), *aap* (accumulation-associated protein), *ssp1* (staphylococcal surface protein), *atlE* (major autolysin) and *bap* (biofilm-associated protein), etc (Atshan et al. 2012a). In this study, we tested the transcript levels of these genes by performing real-time RT-PCR experiments. The results showed that only the transcription of *ica* operon and its regulatory gene *icaR* changed with the addition of stigmata maydis. The transcript levels of the adhesion genes exhibited no apparent change. A previous study also reported the similar results indicating that lipoteichoic acid inhibited *S. aureus* biofilm formation through inhibiting *ica* gene expression, but not through the adhesive matrix molecules (MSCRAMMs) genes, such as clfA, clfB, cna (encoding collagen binding protein), and eno (encoding laminin binding protein) (Ahn et al. 2018).

Previous studies indicated that biofilms promote antibiotic resistance of many *Staphylococcus* strains. The antibiotic resistance of biofilm cells is up to 1000-fold greater than the free-living planktonic bacterial cell (Ceri et al. 1999; Costerton et al. 1995; Larsen & Fiehn 1996; Wentland et al. 1996). Since the MRSA strains used in this study are sensitive to vancomycin, we attempted to determine, in the presence of vancomycin (below the MIC concentration), whether or not the addition of stigmata maydis aqueous extract would affect the survival of MRSA strains cultured under different conditions. The results showed that the stigmata maydis aqueous extracts cannot affect the vancomycin-sensitivity of MRSA bacteria grown in planktonic culture, but can significantly increase the sensitivity of MRSA bacteria grown in biofilm. Because the

MRSA bacteria grown in biofilm have higher vancomycin resistance compared with that grown in planktonic culture, and stigmata maydis inhibited the biofilm formation of the MRSA bacteria, it indicated that the effect of stigmata maydis on vancomycin-sensitivity of MRSA bacteria grown in biofilm is through the inhibition of biofilm formation. These findings corroborated the previous study that reported that MRSA in planktonic growth was susceptible to vancomycin, however, the MIC of vancomycin for ica-positive MRSA tremendously increased (Shivani et al. 2015).

Since stigmata maydis is an inexpensive and easily available Chinese medicine, it could be used as an ancillary aid to vancomycin treatment of MRSA, providing new clues for the prevention and control of bovine mastitis caused by MRSA strains.

CONCLUSIONS

The aqueous extracts of stigmata maydis inhibit the biofilm formation ability of MRSA strains isolated from dairy cows with mastitis by down-regulating the transcription of the *ica* operon. Moreover, the effects of stigmata maydis on vancomycin-sensitivity of MRSA in biofilm was because inhibited the biofilm formation of the MRSA bacteria.

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Figure 1

Effect of stigmata maydis aqueous extract on growth of MRSA strains.

(A) The growth curves of MRSA strains SA2 and SA3 cultured in tryptic soy broth medium with or without specific concentrations of stigmata maydis extract. The results represent a mean of three independent experiments. (B) Colony-forming unit assays of MRSA strains SA2 and SA3. Colony counts of strains SA2 and SA3 were compared after 12 h of incubation at 37 °C with or without addition of stigmata maydis. The colony counts of the test group cultured with different concentrations of stigmata maydis were all compared with that of the control group (without stigmata maydis), the survival rate of which was designated as 100%. (* represents $P < 0.05$).

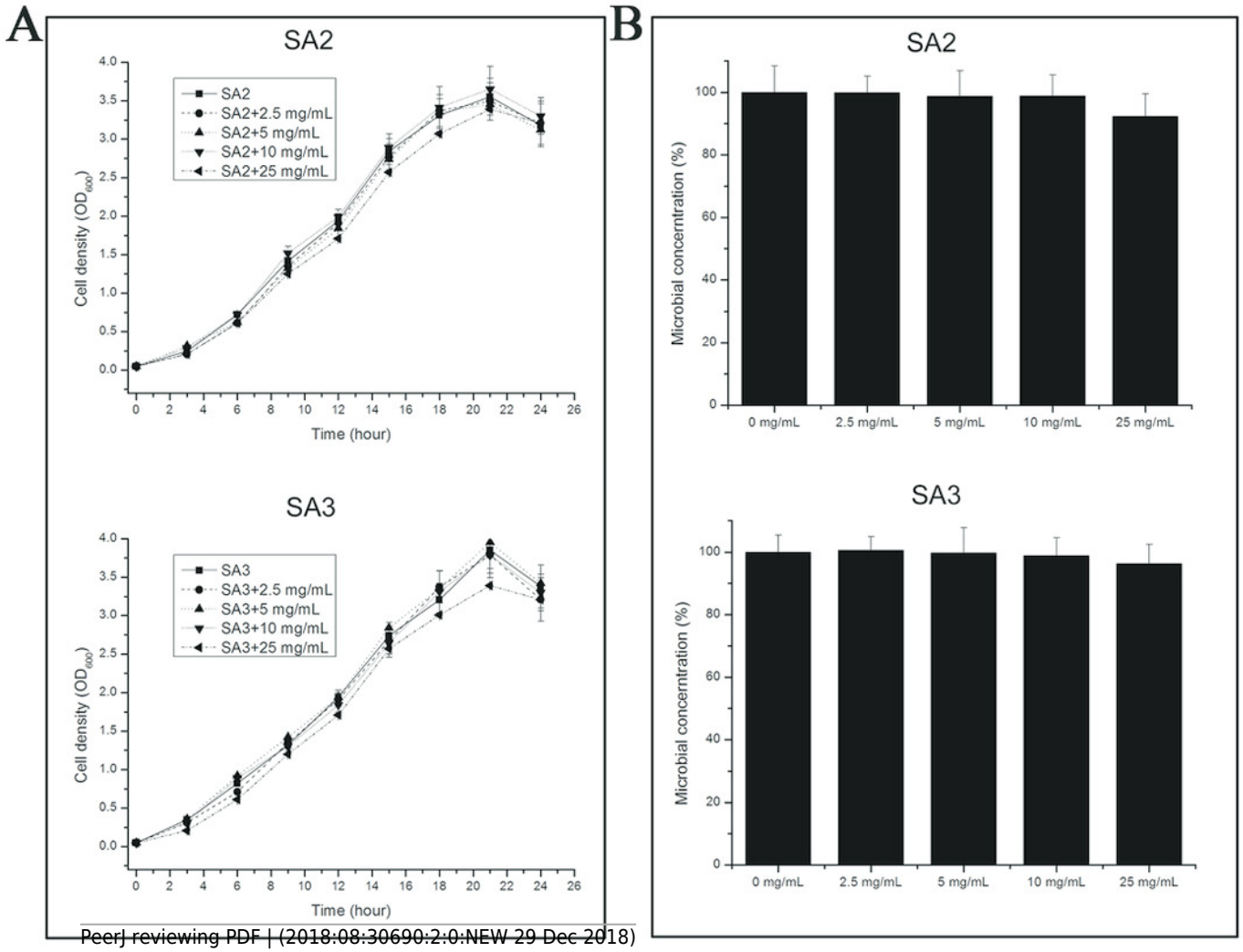


Figure 2

Effect of stigmata maydis aqueous extract on biofilm formation of MRSA strains.

The cells of strains SA2 and SA3 were cultured in 96 well plates for 24 h at 37 °C, and the tigmata maydis extract was added in the tryptic soy broth at concentrations of 0 mg/mL, 0.5 mg/mL, 1 mg/mL and 2 mg/mL, respectively. (A) Photographs of the 96 well plates were taken after staining with crystal violet. (B) The biomass that adhered to the plate after staining with crystal violet was measured by a MicroELISA auto-reader at a wavelength of 560 nm. The results represent a mean of three independent experiments. (** represents $P < 0.01$).

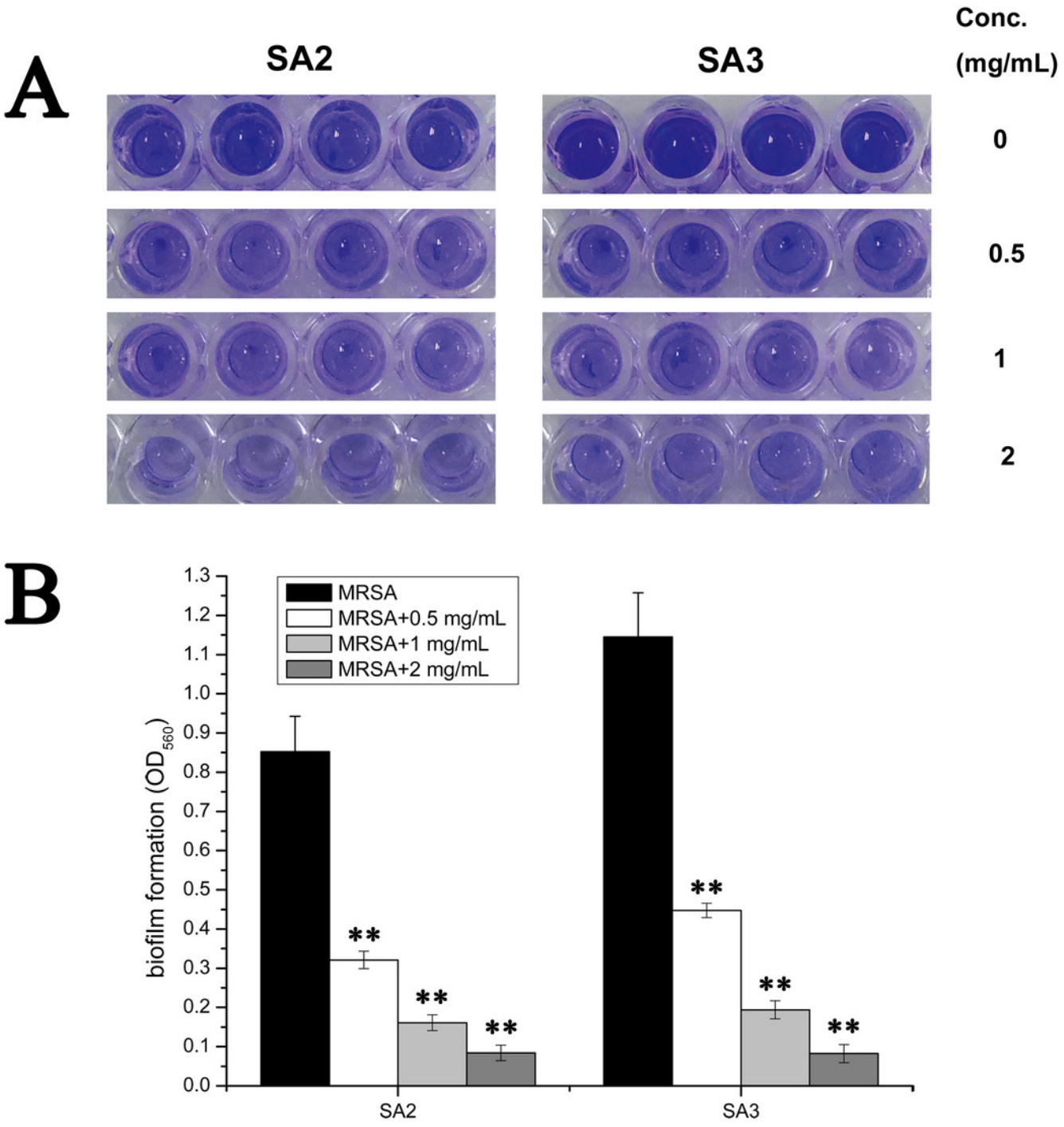
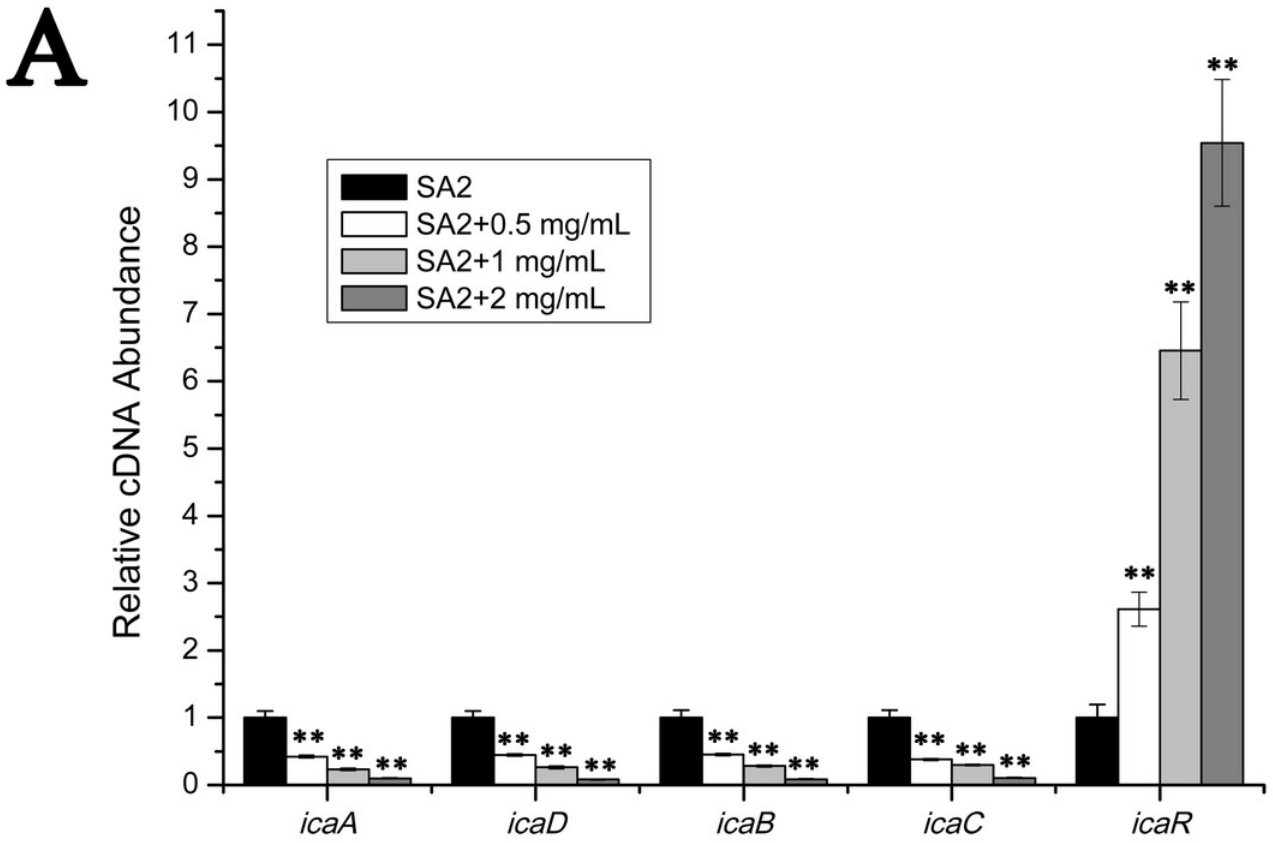

A
SA2
SA3
Conc. (mg/mL)
0
0.5
1
2
B
MRSA
MRSA+0.5 mg/mL
MRSA+1 mg/mL
MRSA+2 mg/mL
biofilm formation (OD560)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
SA2
SA3

Figure 3

Comparison of the relative transcript levels of several biofilm-associated genes.

The transcript levels of *icaA*, *icaD*, *icaB*, *icaC* and *icaR* were measured by performing real-time reverse transcription-PCR in strains SA2 (A) and SA3 (B). The stigmata maydis extract was added to the culture medium at concentrations of 0 mg/mL, 0.5 mg/mL, 1 mg/mL and 2 mg/mL, respectively.


A
Relative cDNA Abundance
SA2
SA2+0.5 mg/mL
SA2+1 mg/mL
SA2+2 mg/mL
icaA
icaD
icaB
icaC
icaR

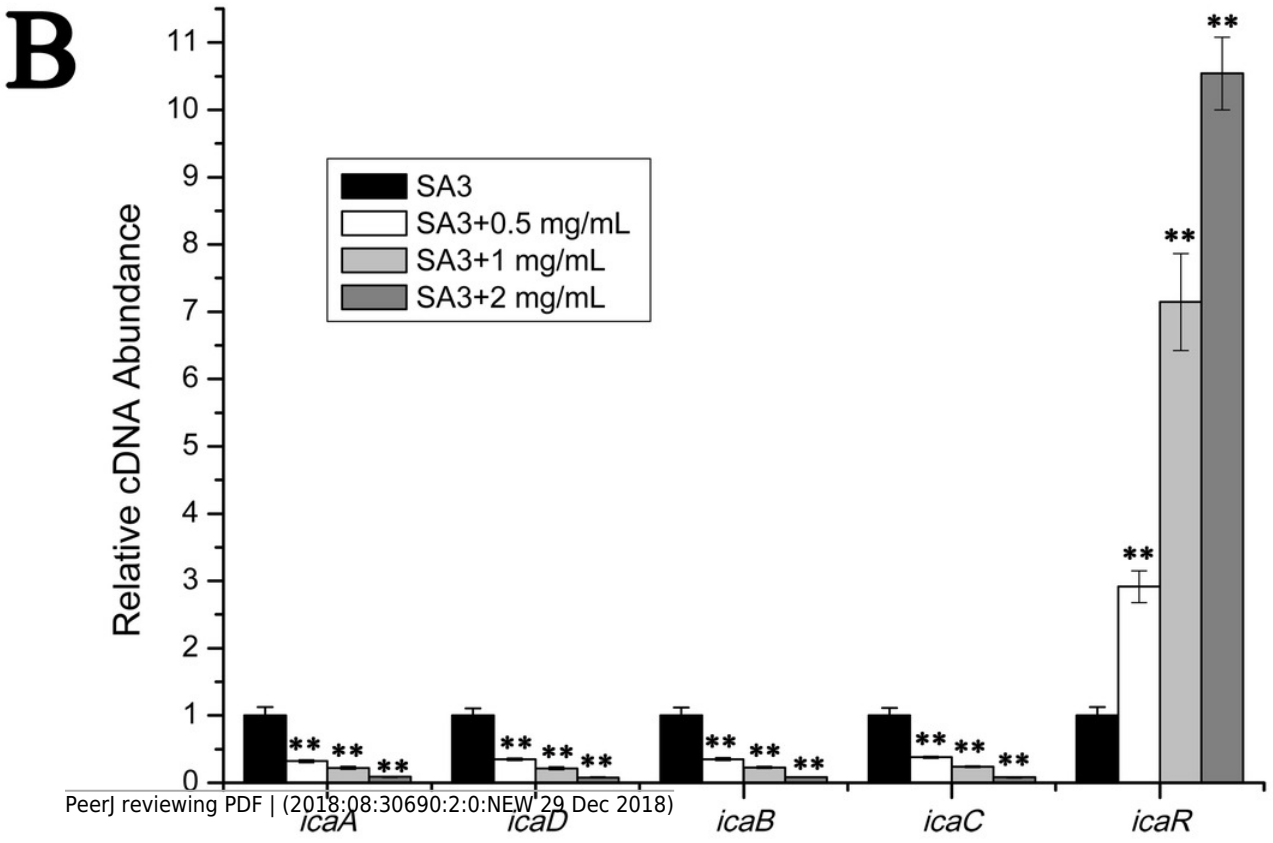
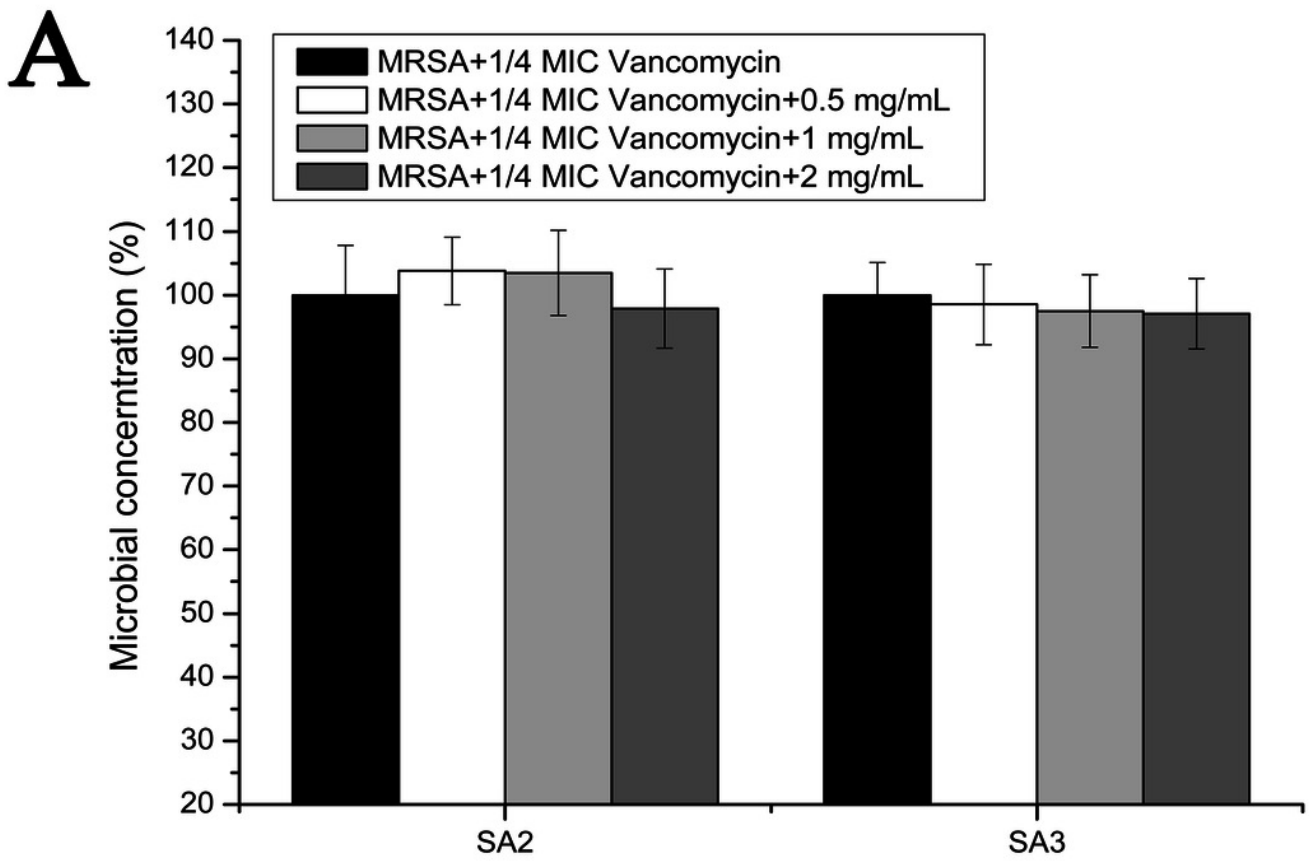

B
Relative cDNA Abundance
SA3
SA3+0.5 mg/mL
SA3+1 mg/mL
SA3+2 mg/mL
icaA
icaD
icaB
icaC
icaR

Figure 4

Colony-forming unit assays of the planktonic-cultured MRSA strains SA2 and SA3 in the presence of vancomycin.

The colony counts of the test group cultured with different concentrations of stigmata maydis were all compared with that of the control group (without stigmata maydis), the survival rate of which was designated as 100%. (A) MRSA strains were cultured with 1/4 MIC concentration of vancomycin (SA2: 0.125 μg/mL, SA3: 0.25 μg/mL). (B) MRSA strains were cultured with 1/2 MIC concentration of vancomycin (SA2: 0.25 μg/mL, SA3: 0.5 μg/mL).


A
MRSA+1/4 MIC Vancomycin
MRSA+1/4 MIC Vancomycin+0.5 mg/mL
MRSA+1/4 MIC Vancomycin+1 mg/mL
MRSA+1/4 MIC Vancomycin+2 mg/mL
Microbial concerntration (%)
140
130
120
110
100
90
80
70
60
50
40
30
20
SA2
SA3

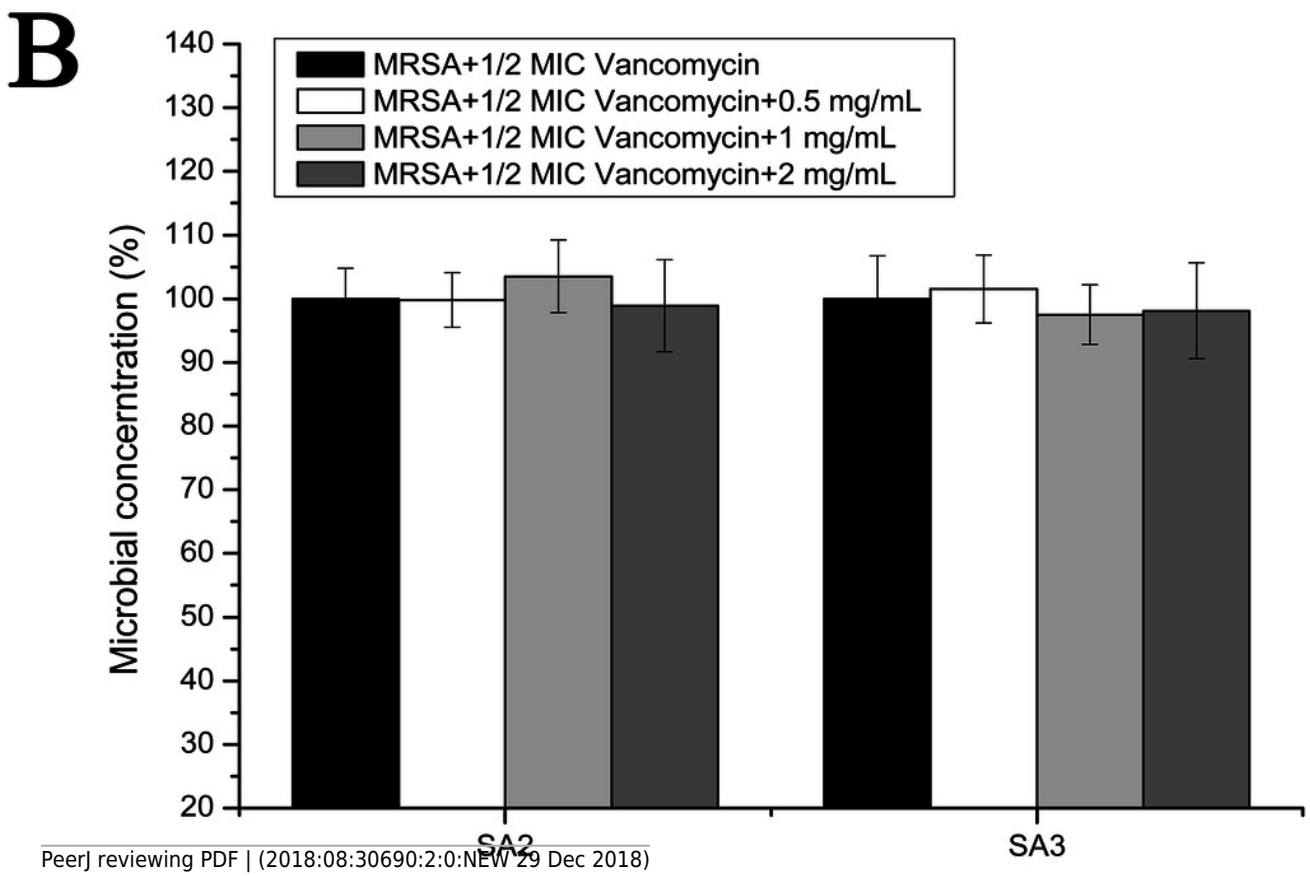
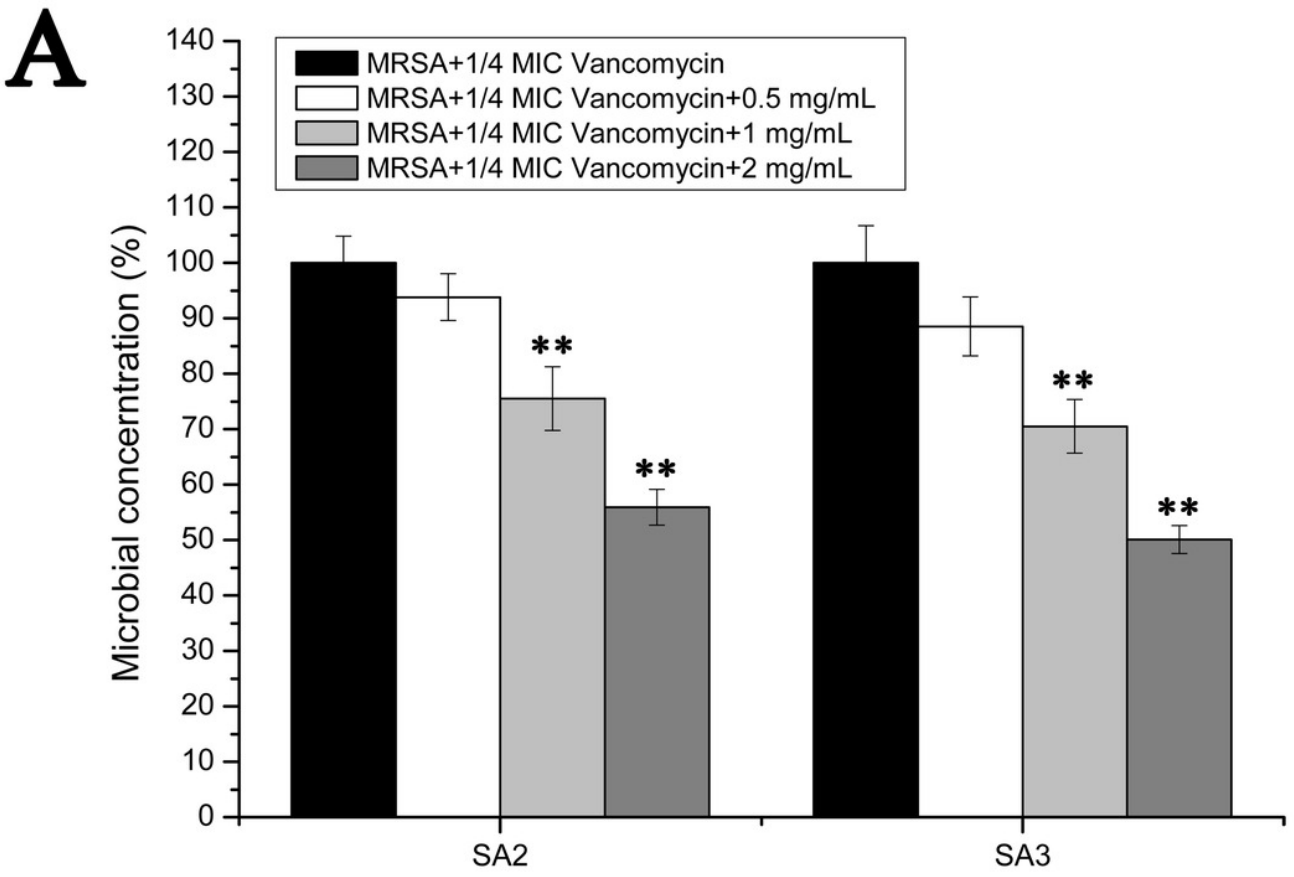

B
MRSA+1/2 MIC Vancomycin
MRSA+1/2 MIC Vancomycin+0.5 mg/mL
MRSA+1/2 MIC Vancomycin+1 mg/mL
MRSA+1/2 MIC Vancomycin+2 mg/mL
Microbial concerntration (%)
140
130
120
110
100
90
80
70
60
50
40
30
20
SA2
SA3

Figure 5

Colony-forming unit assays of the biofilm-condition cultured MRSA strains SA2 and SA3 in the presence of vancomycin.

The colony counts of the test group cultured with different concentrations of stigmata maydis were all compared with that of the control group (without stigmata maydis), the survival rate of which was designated as 100%. (A) MRSA strains were cultured with 1/4 MIC concentration of vancomycin (SA2: 0.125 μg/mL, SA3: 0.25 μg/mL). (B) MRSA strains were cultured with 1/2 MIC concentration of vancomycin (SA2: 0.25 μg/mL, SA3: 0.5 μg/mL).


A
MRSA+1/4 MIC Vancomycin
MRSA+1/4 MIC Vancomycin+0.5 mg/mL
MRSA+1/4 MIC Vancomycin+1 mg/mL
MRSA+1/4 MIC Vancomycin+2 mg/mL
Microbial concerntration (%)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
**
**
**
**
SA2
SA3

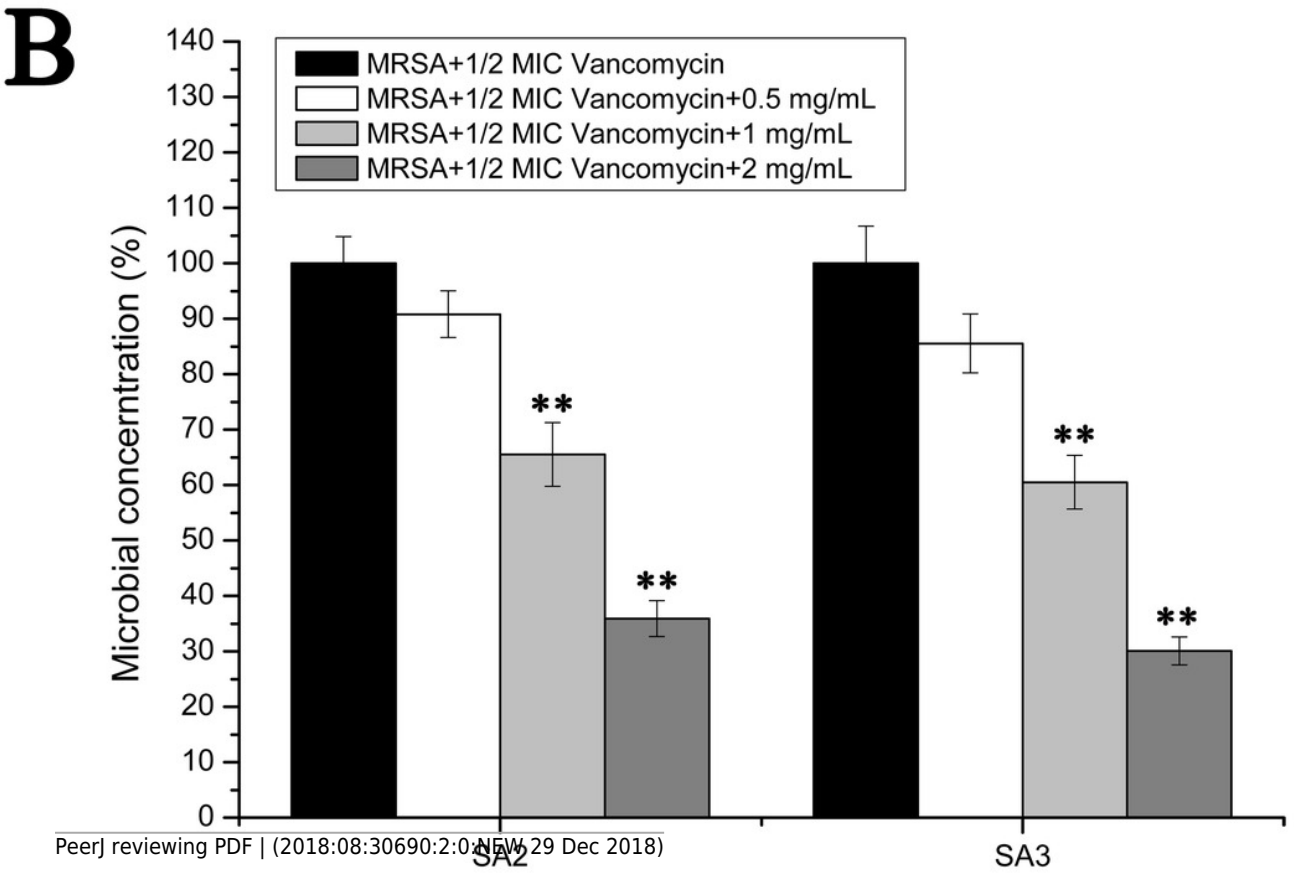

B
MRSA+1/2 MIC Vancomycin
MRSA+1/2 MIC Vancomycin+0.5 mg/mL
MRSA+1/2 MIC Vancomycin+1 mg/mL
MRSA+1/2 MIC Vancomycin+2 mg/mL
Microbial concerntration (%)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
**
**
**
**
SA2
SA3

Table 1(on next page)

Oligonucleotide primers used in this study.

Primer name	Oligonucleotide (5’-3’)
rt-icaA-f	TTTCGGGTGTCTTCACTCTAT
rt-icaA-r	CGTAGTAATACTTCGTGTCCC
rt-icaB-f	CCTATCCTTATGGCTTGATGA
rt-icaB-r	CATTGGAGTTCGGAGTGA
rt-icaC-f	TACTGACAACCTTGAATTACCA
rt-icaC-r	AATAGCCATACCATTGACCTAA
rt-icaD-f	CCAGACAGAGGGAATACC
rt-icaD-r	AAGACACAAGATATAGCGATAAG
rt-icaR-f	TTATCTAATACGCCTGAGGAAT
rt-icaR-r	GGATGCTTTCAAATACCAACT

1