Stigmata maydis increases bacterial vancomycin susceptibility by down-regulating biofilm formation in methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from dairy cows with mastitis (#30690)

First revision

Editor guidance

Please submit by **18 Dec 2018** for the benefit of the authors (and your $200 publishing discount).

Structure and Criteria
Please read the 'Structure and Criteria' page for general guidance.

Raw data check
Review the raw data. Download from the materials page.

Image check
Check that figures and images have not been inappropriately manipulated.

Privacy reminder: If uploading an annotated PDF, remove identifiable information to remain anonymous.

Files

Download and review all files from the materials page.

1 Tracked changes manuscript(s)
1 Rebuttal letter(s)
5 Figure file(s)
1 Table file(s)
1 Raw data file(s)

Structure and Criteria

Structure your review

The review form is divided into 5 sections. Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

You can also annotate this PDF and upload it as part of your review

When ready submit online.

Editorial Criteria

Use these criteria points to structure your review. The full detailed editorial criteria is on your guidance page.

BASIC REPORTING

- Clear, unambiguous, professional English language used throughout.
- Intro & background to show context. Literature well referenced & relevant.
- Structure conforms to PeerJ standards, discipline norm, or improved for clarity.
- Figures are relevant, high quality, well labelled & described.
- Raw data supplied (see PeerJ policy).

EXPERIMENTAL DESIGN

- Original primary research within Scope of the journal.
- Research question well defined, relevant & meaningful. It is stated how the research fills an identified knowledge gap.
- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- Impact and novelty not assessed. Negative/inconclusive results accepted. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- Data is robust, statistically sound, & controlled.
- Speculation is welcome, but should be identified as such.
- Conclusions are well stated, linked to original research question & limited to supporting results.

Standout reviewing tips

The best reviewers use these techniques

Tip	*Example*
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
Give specific suggestions on how to improve the manuscript	*Your introduction needs more detail. I suggest that you improve the description at lines 57- 86 to provide more justification for your study (specifically, you should expand upon the knowledge gap being filled).*
Comment on language and grammar issues	*The English language should be improved to ensure that an international audience can clearly understand your text. Some examples where the language could be improved include lines 23, 77, 121, 128 – the current phrasing makes comprehension difficult.*
Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Stigmata maydis increases bacterial vancomycin susceptibility by down-regulating biofilm formation in methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from dairy cows with mastitis

Fei Shang [1] , **Long Li** [1] , **Lumin Yu** [1] , **Jingtian Ni** [1] , **Xiaolin Chen** [1] , **Ting Xue** Corresp. [1]

[1] School of Life Sciences, Anhui Agricultural University, Hefei, Anhui, China

Corresponding Author: Ting Xue
Email address: xuet@ahau.edu.cn

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. **Discussion** 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. This indicates that stigmata maydis might be used as an adjuvant ingredient in the vancomycin treatment of MRSA infections.

RUNNING TITLE: Stigmata maydis affects MRSA biofilms

~~Stigmata maydis increases bacterial vancomycin susceptibility by down-regulating biofilm formation in methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from dairy cows with mastitis~~

Fei Shang, Long Li, Lumin Yu, Jingtian Ni, Xiaolin Chen, Ting Xue[1]

School of Life Sciences, Anhui Agricultural University, Hefei, Anhui 230036, China

[1]Corresponding author:

Ting Xue; School of Life Sciences, Anhui Agricultural University, Hefei, Anhui 230036, China;

Tel: (86) 551 65787380; Fax: (86) 551 65787380; E-mail: xuet@ahau.edu.cn

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.

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.

Discussion 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. This indicates that stigmata maydis might be used as an adjuvant ingredient in the vancomycin treatment of MRSA infections.

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. 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, considering that MRSA bacteria that form a biofilm structure usually cause chronic mastitis in cows, we aim at investigating 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 chloromycetin and vancomycin (vancomycin minimum inhibitory concentration (MIC) SA2 0.5 mg/L; SA3: 1 mg/L), 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).

Aqueous extraction of stigmata maydis

106 One gram of stigmata maydis powder was suspended in 10 mL water and soaked for 24 h. The
107 supernatant was dried by vacuum freeze drying and then supernatants were mixed into a 100
108 mg/mL extract.
109
110 ***Antimicrobial activity assay***
111 The method was performed as described previously as follows (Chen et al. 2015). Colonies of
112 MRSA strains were picked into 2 mL of TSB medium and cultivated at 37 °C with shaking at
113 200 rpm for 16 h. Then the overnight cultures were inoculated into fresh TSB medium and this
114 was diluted to a final optical density (600 nm) of 0.05, which was dispensed into 96-well plates
115 (Costar, Corning, Steuben, NY) containing serial dilutions of aqueous extracts of stigmata
116 maydis (with appropriate vancomycin, if needed). Plates were incubated at 37 °C for 12 h and
117 then 10-fold serial dilutions of cultures were performed by successive transfer (0.1 mL) through
118 seven microfuge tubes containing 0.9 mL of TSB. The 100 μL dilutions were dropped onto LB
119 agar plates and viable colonies were counted via their colony-forming units (CFU) on TSB agar
120 plates after incubation at 37 °C for 24 h. The survival rate of the control group without exposure
121 to *S. officinalis L.* was designated as 100 percent. CFU of the test groups were all compared with
122 that of the control group. Experiments were repeated three times with four parallels.
123 ***Biofilm assays***
124 The method for biofilm quantification was performed as described previously and modified as
125 follows (Chen et al. 2015; Xue et al. 2014). MRSA strains were grown in TSB (containing 0.5%
126 glucose) for 16 h and diluted 1:100 into fresh TSB. The diluted cultures were transferred into

127 sterile 96 well flat-bottomed tissue culture plates and incubated at 37 °C for 24 h. Aqueous
128 extracts of stigmata maydis was added to the TSB media with diluted cultures at different
129 concentrations. The adherent bacteria were stained with crystal violet, and the excess stain was
130 washed off gently with slowly running water. The biomass of the biofilm was determined using a
131 MicroELISA auto-reader (Bio-Rad Co.) at a wavelength of 560 nm under single-wavelength
132 mode (Pozzi et al. 2012; Ziebuhr et al. 1997).
133
134 ***Total RNA isolation, cDNA generation, and real-time PCR processing***
135 Overnight cultures of MRSA strains were diluted 1:100 in LB medium and grown to the late
136 exponential phase in 24 well plates (Costar, Corning, Steuben, NY). Cells were collected and
137 resuspended in TE (Tris-EDTA) buffer (pH 8.0) containing 10 g/L of lysozyme and 40 mg/L of
138 lysostaphin. After incubation at 37 °C for 5 min, cells were prepared for total RNA extraction
139 using the Trizol method (Invitrogen), and residual DNA was removed with DNase (RNase free;
140 TaKaRa). RT real-time PCR was performed with a PrimeScript 1st Strand cDNA synthesis kit
141 and SYBR Premix Ex Taq (TaKaRa) using a StepOne real-time PCR system (Applied
142 Biosystems). The quantity of cDNA measured by real-time PCR was normalized to the
143 abundance of 16S cDNA (Chen et al. 2000). All real-time RT-PCR assays were repeated at least
144 three times with similar results. The primers used in this study were listed in Table 1.
145
146 ***Statistical analysis***
147 The data was analyzed using statistical software SPSS by a one-way ANOVA method, the test

results were (mean ± standard deviation). The paired *t*-test was used for statistical comparisons between groups. The level of statistical significance was set at a P value of ≤0.01.

RESULTS

High concentration of aqueous extract of stigmata maydis did not affect the growth curves of MRSA strains

The growth rates of the cells were tested when they were grown in TSB medium with different concentrations of aqueous extract of stigmata. The results showed that the growth rates of the bacteria did not change when the external concentration of stigmata maydis was 2.5 mg/mL, 5 mg/mL or 10 mg/mL, but when the concentration of stigmata maydis was 25 mg/mL, the growth of the bacteria was a little inhibited (Fig. 1A). These data indicate that aqueous extracts of stigmata maydis did not affect the growth curves of the MRSA strains.

Furthermore, in order to examine the antibacterial activity of the aqueous extracts of stigmata maydis against *S. aureus in vitro*, antibacterial assays were performed. After exposure to extract of stigmata maydis at different concentrations for 12 h at 37 °C, the cells of MRSA strains were inoculated into fresh TSB and then spread onto the TSB agar plates. After cultivating for 24 h at 37 °C, the colony forming units of the bacteria were counted and compared. As is shown in Fig. 1B, the survival rate of the control group without exposure to stigmata maydis was designated as 100%. With the increase of the concentration of stigmata maydis, the survival rates of the MRSA strain SA2 and SA3 also did not change significantly. These data confirmed that stigmata maydis does not have antibacterial activity against the MRSA strains

169

170 ***Low concentration of aqueous extract of stigmata maydis inhibited the biofilm formation of***

171 ***MRSA strains***

172 To examine whether an aqueous extract of stigmata maydis would affect the biofilm formation of

173 *S. aureus*, we performed biofilm assays. As shown in Fig. 2A, strains of the control group

174 without aqueous extract of stigmata maydis formed obvious biofilms, when stigmata maydis at

175 different concentration was added, biofilm formation of the bacteria significantly decreased.

176 When the concentration of stigmata maydis reached 2 mg/mL, no biofilm was observed. In

177 addition, the quantity of biofilm formation was further tested using a MicroELISA autoreader.

178 We found that biofilm quantity decreased with the increase of stigmata maydis concentration

179 (Fig. 2B). These data revealed an interesting phenomenon that, although the aqueous extract of

180 stigmata maydis has no apparent antibacterial activity, it affects biofilm formation significantly.

181

182 ***The aqueous extract of stigmata maydis inhibited MRSA biofilm formation by decreasing the***

183 ***transcription of the icaADBC operon***

184 The transcript levels of biofilm-associated genes were determined by performing real-time RT-

185 PCR experiments. As is shown in Fig. 3, the transcript levels of *icaA*, *icaB*, *icaC*, and *icaD* were

186 significantly decreased upon the addition of stigmata maydis. Moreover, with increased of

187 stigmata maydis concentrations, the inhibitory effect of stigmata maydis on the *ica* operon was

188 stronger, indicating that stigmata maydis affected the transcription of the *ica* operon in a

189 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*.

The aqueous extract of stigmata maydis did not affect the vacomycin susceptibility of the planktonic-cultured MRSA strains

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.

The aqueous extract of stigmata maydis enhanced the vacomycin suspectibility of the biofilm-condition cultured MRSA strains

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 In our previous work, we reported that the ethanol extract of *Sanguisorba officinalis* L. both
220 inhibited growth and biofilm formation of MRSA strains isolated from dairy cows infected with
221 mastitis (Chen et al. 2015). The data in this study showed that although high concentrations of
222 stigmata maydis aqueous extract did not affect the growth of MRSA strains, a low concentration
223 significantly inhibited the biofilm formation of these strains.

224 According to previous work, biofilm formation in *S. aureus* includes two steps: attachment to
225 the material surface and then the formation of microcolonies and multilayered cell clusters
226 surrounded by a slimy matrix, which has been characterised as polysaccharide intercellular
227 adhesin (PIA). PIA is produced by the enzymes coded in an operon composed of four open
228 reading frames (ORFs) *icaA*, *icaD*, *icaB* and *icaC*. The attachment process and accumulation of
229 bacteria is associated with several adhesion genes such as *fnbpA*, *fnbpB* (encoding fibronectin
230 binding proteins A and B), *fib* (encoding fibrinogen binding protein), *clfA* (encoding clumping
231 factors A), *aap* (encoding accumulation-associated protein), *ssp1* (encoding staphylococcal

surface protein), *atlE* (encoding major autolysin) and *bap* (encoding 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 (Supplemental File 6).

Previous work indicated that biofilms promote antibiotic resistance of many *Staphylococcus* strains. 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.

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. However, the aqueous extracts of stigmata maydis increase the vancomycin susceptibility of the MRSA strains under biofilm-cultured conditions.

REFERENCES

Ando E, Monden K, Mitsuhata R, Kariyama R, and Kumon H. 2004. Biofilm formation among methicillin-resistant Staphylococcus aureus isolates from patients with urinary tract infection. *Acta Medica Okayama* 58:207-214.

Atshan SS, Nor Shamsudin M, Sekawi Z, Lung LT, Hamat RA, Karunanidhi A, Mateg Ali A, Ghaznavi-Rad E, Ghasemzadeh-Moghaddam H, Chong Seng JS, Nathan JJ, and Pei CP. 2012a. Prevalence of adhesion and regulation of biofilm-related genes in different clones of Staphylococcus aureus. *J Biomed Biotechnol* 2012:976972. 10.1155/2012/976972

Atshan SS, Shamsudin MN, Lung LT, Sekawi Z, Ghaznavi-Rad E, and Pei CP. 2012b. Comparative characterisation of genotypically different clones of MRSA in the production of biofilms. *J Biomed Biotechnol* 2012:417247. 10.1155/2012/417247

Atshan SS, Shamsudin MN, Sekawi Z, Thian Lung LT, Barantalab F, Liew YK, Alreshidi MA, Abduljaleel SA, and Hamat RA. 2015. Comparative proteomic analysis of extracellular proteins expressed by various clonal types of Staphylococcus aureus and during planktonic growth and biofilm development. *Front Microbiol* 6:524. 10.3389/fmicb.2015.00524

Bhaigyabati T, Kirithika T, Ramya J, and Usha K. 2011. Phytochemical constituents and antioxidant activity of various extracts of corn silk (Zea mays L). *Research Journal of Pharmaceutical Biological & Chemical Sciences* 2:986-993.

Chambers HF. 2001. The changing epidemiology of Staphylococcus aureus? *Emerging Infectious Diseases* 7:178.

Chen X, Shang F, Meng Y, Li L, Cui Y, Zhang M, Qi K, and Xue T. 2015. Ethanol extract of Sanguisorba officinalis L. inhibits biofilm formation of methicillin-resistant Staphylococcus aureus in an ica-dependent manner. *J Dairy Sci* 98:8486-8491. 10.3168/jds.2015-9899

Chen YW, Zhao P, Borup R, and Hoffman EP. 2000. Expression profiling in the muscular dystrophies: identification of novel aspects of molecular pathophysiology. *J Cell Biol* 151:1321-1336.

Cramton SE, Gerke C, Schnell NF, Nichols WW, and Götz F. 1999. The intercellular adhesion (ica) locus is present in Staphylococcus aureus and is required for biofilm formation. *Infection & Immunity* 67:5427.

Fedtke I, Götz F, and Peschel A. 2004. Bacterial evasion of innate host defenses – the Staphylococcus aureus lesson. *International Journal of Medical Microbiology* 294:189.

Habtemariam S. 1998. Extract of corn silk (stigma of Zea mays) inhibits the tumour necrosis factor-alpha- and bacterial lipopolysaccharide-induced cell adhesion and ICAM-1 expression. *Planta Medica* 64:314-318.

Hasanudin K, Hashim P, and Mustafa S. 2012. Corn silk (Stigma maydis) in healthcare: a phytochemical and pharmacological review. *Molecules* 17:9697-9715.

Health UDo, Control HSCfD, and Prevention. 1999. Four pediatric deaths from community-acquired methicillin-resistant Staphylococcus aureus — Minnesota and North Dakota, 1997-1999. *Morbidity & Mortality Weekly Report* 48:707.

Hillerton JE, and Berry EA. 2005. Treating mastitis in the cow – a tradition or an archaism. *Journal of Applied Microbiology* 98:1250-1255.

Hiramatsu K, Cui L, Kuroda M, and Ito T. 2001. The emergence and evolution of methicillin-resistant Staphylococcus aureus. *Trends in Microbiology* 9:486-493.

Huijps K, Lam TJ, and Hogeveen H. 2008. Costs of mastitis: facts and perception. *Journal of Dairy Research* 75:113-120.

Jefferson KK, Goldmann DA, and Pier GB. 2005. Use of confocal microscopy to analyze the rate of vancomycin penetration through Staphylococcus aureus biofilms. *Antimicrob Agents Chemother* 49:2467-2473. 10.1128/AAC.49.6.2467-2473.2005

Joh D, Wann ER, Kreikemeyer B, Speziale P, and Höök M. 1999. Role of fibronectin-binding MSCRAMMs in bacterial adherence and entry into mammalian cells. *Matrix Biology* 18:211-223.

Joshi S, Mumtaz S, Singh J, Pasha S, and Mukhopadhyay K. 2018. Novel Miniature Membrane Active Lipopeptidomimetics against Planktonic and Biofilm Embedded Methicillin-Resistant Staphylococcus aureus. *Sci Rep* 8:1021. 10.1038/s41598-017-17234-z

Jr ZH. 2000. Identification and effects of maize silk volatiles on cultures of Aspergillus flavus. *J Agric Food Chem* 48:921-925.

Kiedrowski MR, and Horswill AR. 2011. New approaches for treating staphylococcal biofilm infections. *Annals of the New York Academy of Sciences* 1241:104.

Kozytska S, Stauss D, Pawlik MC, Hensen S, Eckart M, Ziebuhr W, Witte W, and Ohlsen K. 2010. Identification of specific genes in Staphylococcus aureus strains associated with bovine mastitis. *Veterinary Microbiology* 145:360-365.

Lowy FD. 2003. Antimicrobial resistance: the example of Staphylococcus aureus. *J Clin Invest* 111:1265-1273. 10.1172/JCI18535

Malinowski E, and Kłossowska A. 2010. Mastitis caused by coagulase-negative staphylococci in cows. *Medycyna Weterynaryjna*.

Naimi TS, Ledell KH, Boxrud DJ, Groom AV, Steward CD, Johnson SK, Besser JM, O'Boyle C, Danila RN, and Cheek JE. 2001. Epidemiology and Clonality of Community-Acquired Methicillin-Resistant Staphylococcus aureus in Minnesota, 1996–1998. *Clinical Infectious Diseases* 33:990.

O'Gara JP. 2007. ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. *Fems Microbiology Letters* 270:179.

Petrelli D, Repetto A, D'Ercole S, Rombini S, Ripa S, Prenna M, and Vitali LA. 2008. Analysis of meticillin-susceptible and meticillin-resistant biofilm-forming Staphylococcus aureus from catheter infections isolated in a large Italian hospital. *Journal of Medical Microbiology* 57:364.

Piepers S, De ML, De KA, Opsomer G, Barkema HW, and De VS. 2007. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. *Journal of Dairy Research* 74:478.

Pozo JLD, and Patel R. 2007. The Challenge of Treating Biofilm-associated Bacterial Infections. *Clinical*

Pharmacology & Therapeutics 82:204-209.

Pozzi C, Waters EM, Rudkin JK, Schaeffer CR, Lohan AJ, Tong P, Loftus BJ, Pier GB, Fey PD, Massey RC, and O'Gara JP. 2012. Methicillin resistance alters the biofilm phenotype and attenuates virulence in Staphylococcus aureus device-associated infections. *PLoS Pathog* 8:e1002626. 10.1371/journal.ppat.1002626

Queck SY, Khan BA, Wang R, Bach TH, Kretschmer D, Chen L, Kreiswirth BN, Peschel A, Deleo FR, and Otto M. 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. *PLoS Pathog* 5:e1000533. 10.1371/journal.ppat.1000533

Richmond MH. 1979. Beta-lactam antibiotics and beta-lactamases: two sides of a continuing story. *Reviews of Infectious Diseases* 1:30.

Szweda P, Schielmann M, Frankowska A, Kot B, and Zalewska M. 2014. Antibiotic resistance in Staphylococcus aureus strains isolated from cows with mastitis in eastern Poland and analysis of susceptibility of resistant strains to alternative nonantibiotic agents: lysostaphin, nisin and polymyxin B. *Journal of Veterinary Medical Science* 76:355.

Watts JL. 1988. Etiological agents of bovine mastitis. *Veterinary Microbiology* 16:41-66.

Xue T, Chen X, and Shang F. 2014. Short communication: Effects of lactose and milk on the expression of biofilm-associated genes in Staphylococcus aureus strains isolated from a dairy cow with mastitis. *J Dairy Sci* 97:6129-6134. 10.3168/jds.2014-8344

Ziebuhr W, Heilmann C, Gotz F, Meyer P, Wilms K, Straube E, and Hacker J. 1997. Detection of the intercellular adhesion gene cluster (ica) and phase variation in Staphylococcus epidermidis blood culture strains and mucosal isolates. *Infect Immun* 65:890-896.

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 $P < 0.05$).

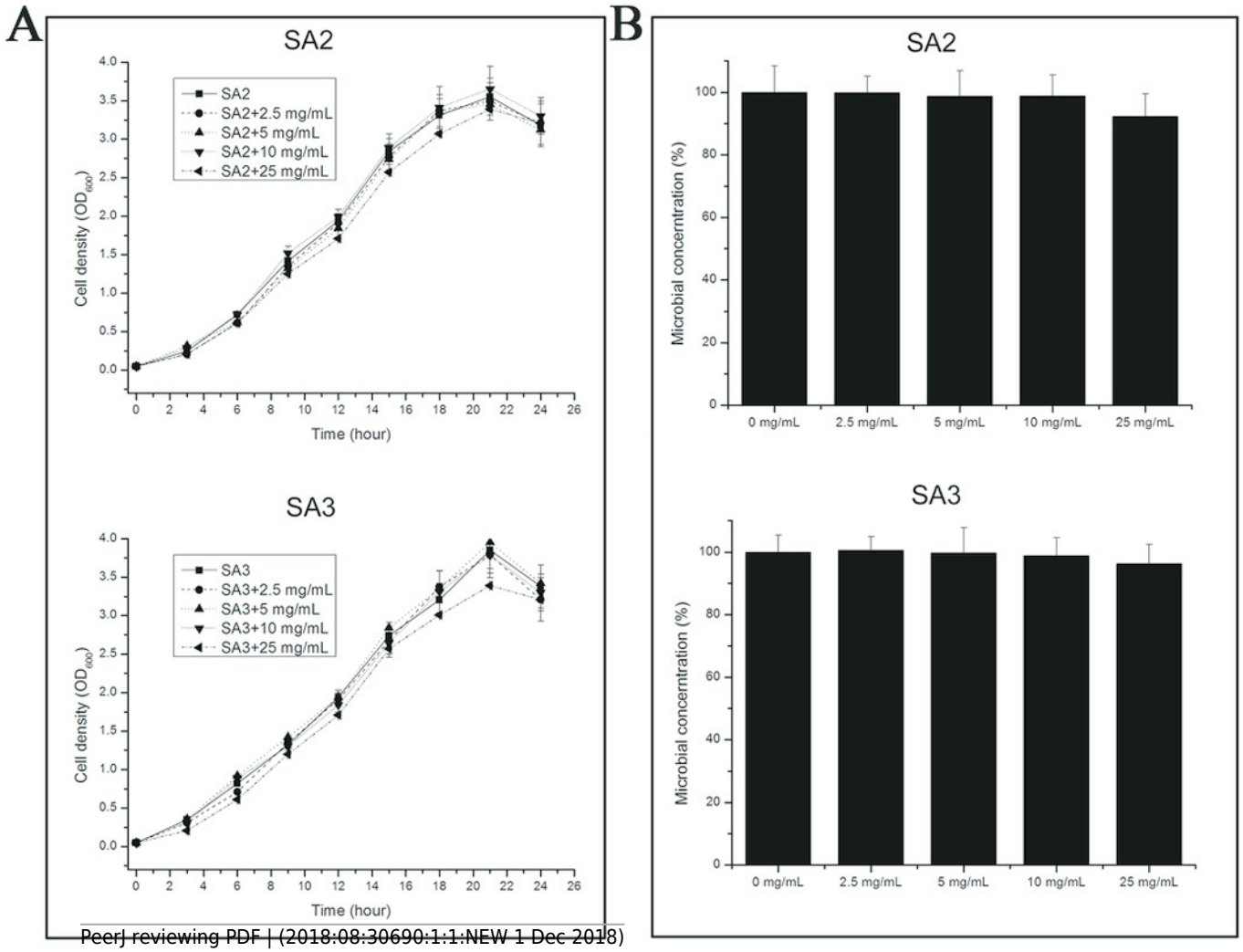


Figure 2

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 $P < 0.01$).

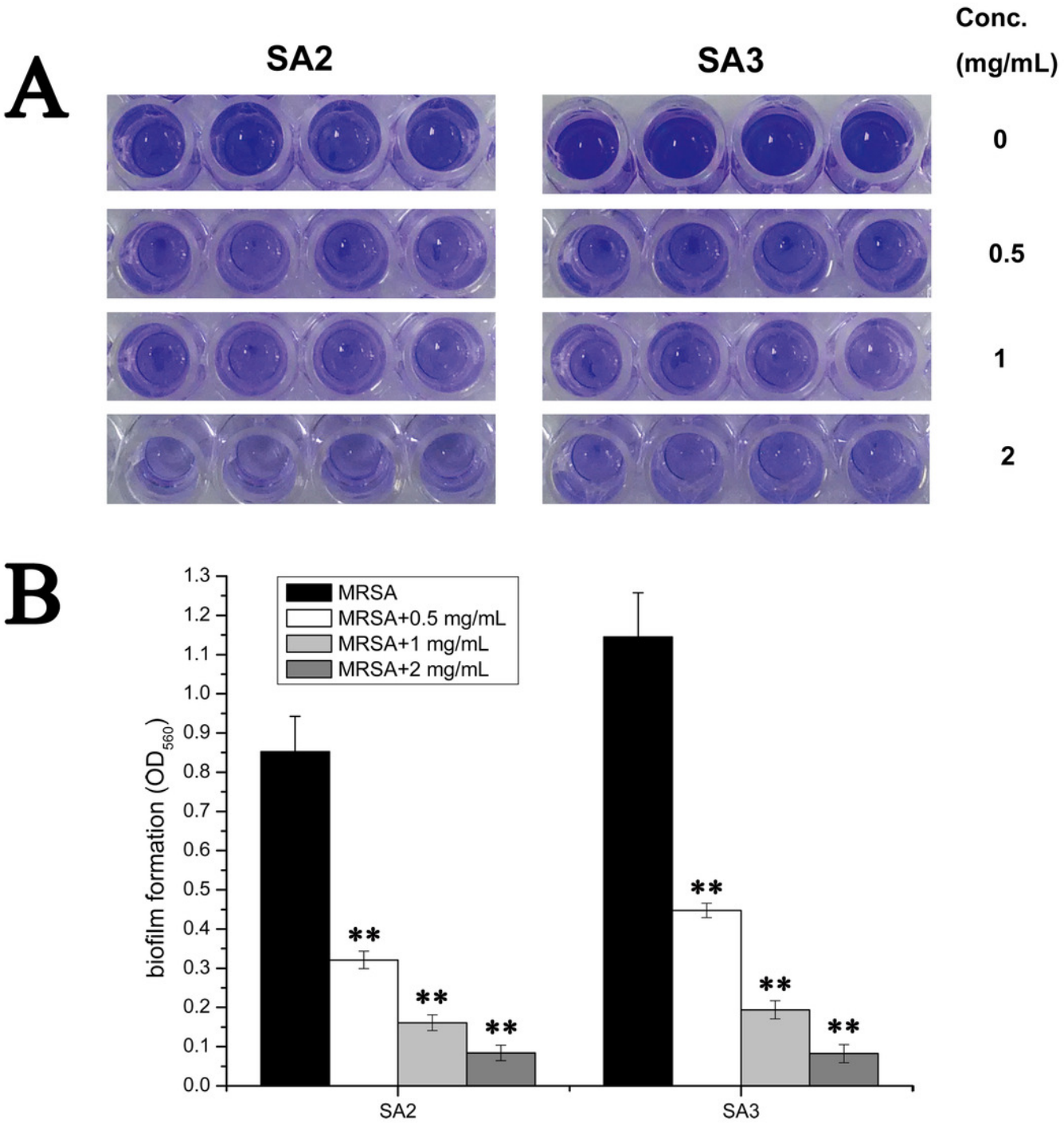
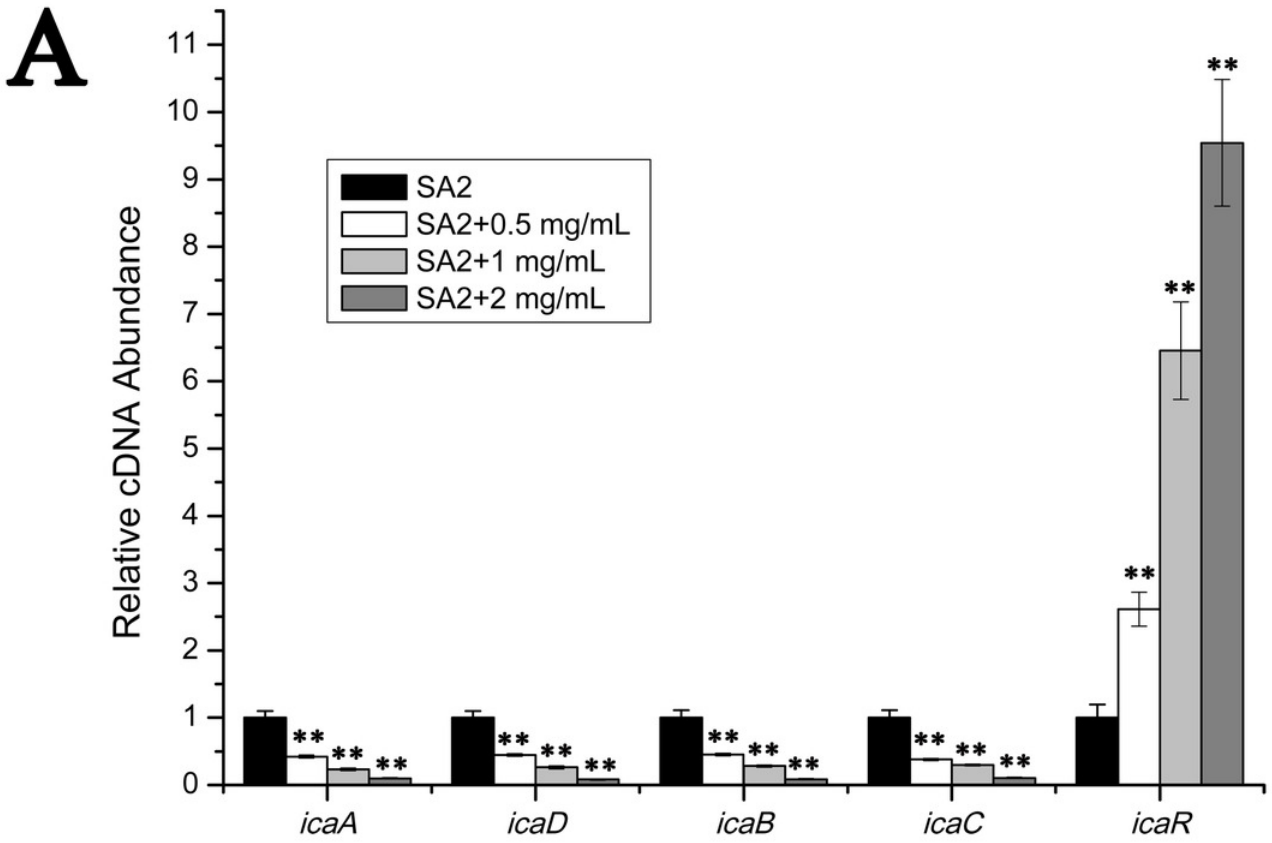

A
SA2
SA3
Conc. (mg/mL)
0
0.5
1
2
B
MRSA
MRSA+0.5 mg/mL
MRSA+1 mg/mL
MRSA+2 mg/mL
biofilm formation (OD560)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
SA2
SA3

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

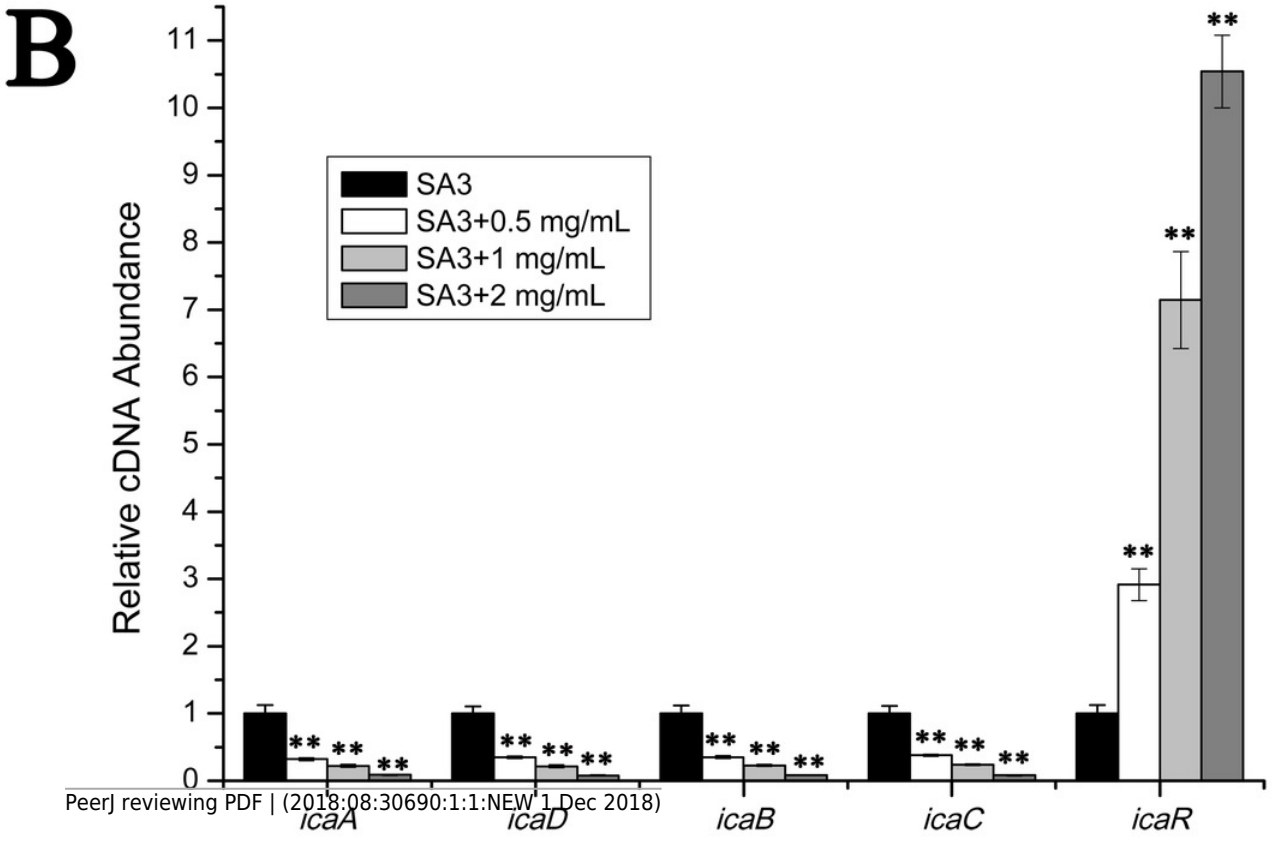
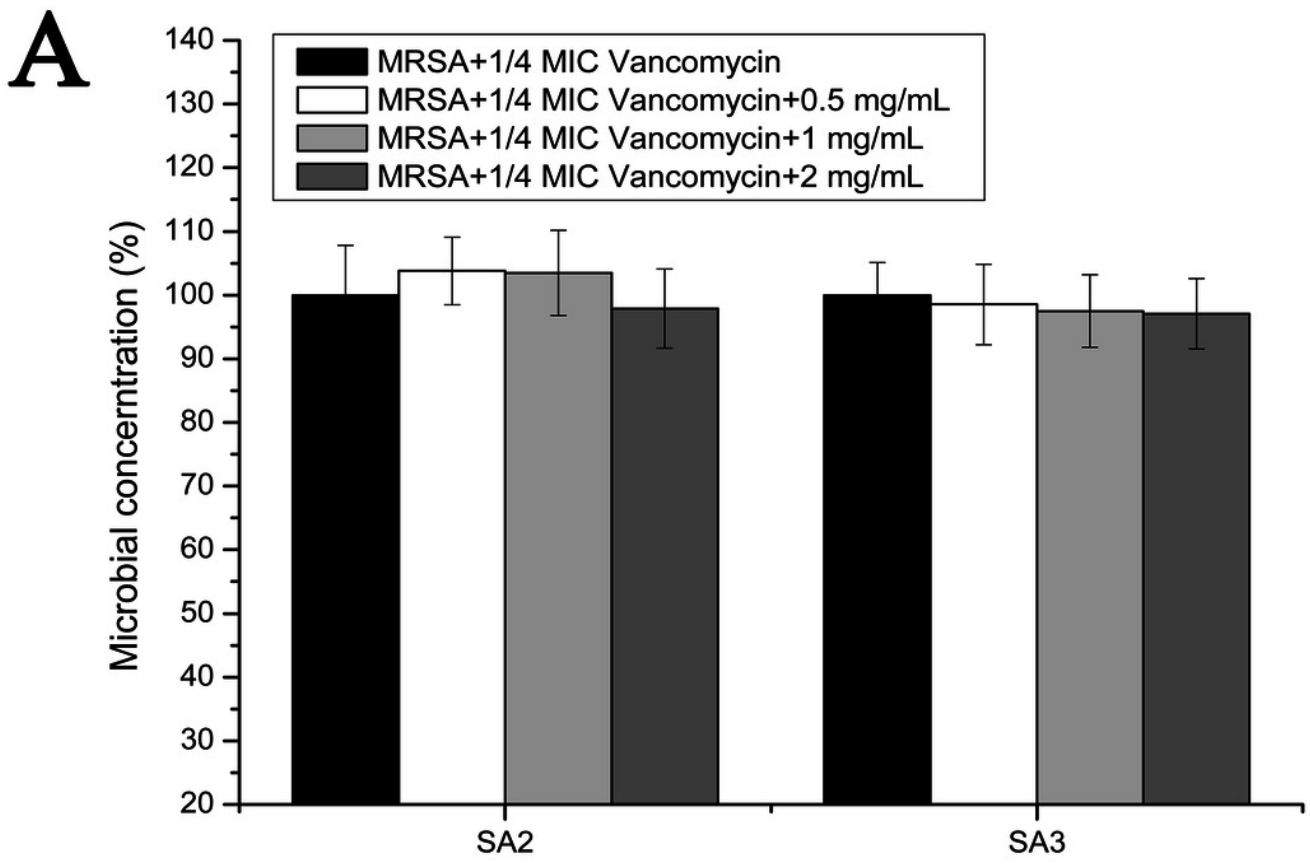

B
Relative cDNA Abundance
SA3
SA3+0.5 mg/mL
SA3+1 mg/mL
SA3+2 mg/mL
icaA
icaD
icaB
icaC
icaR

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

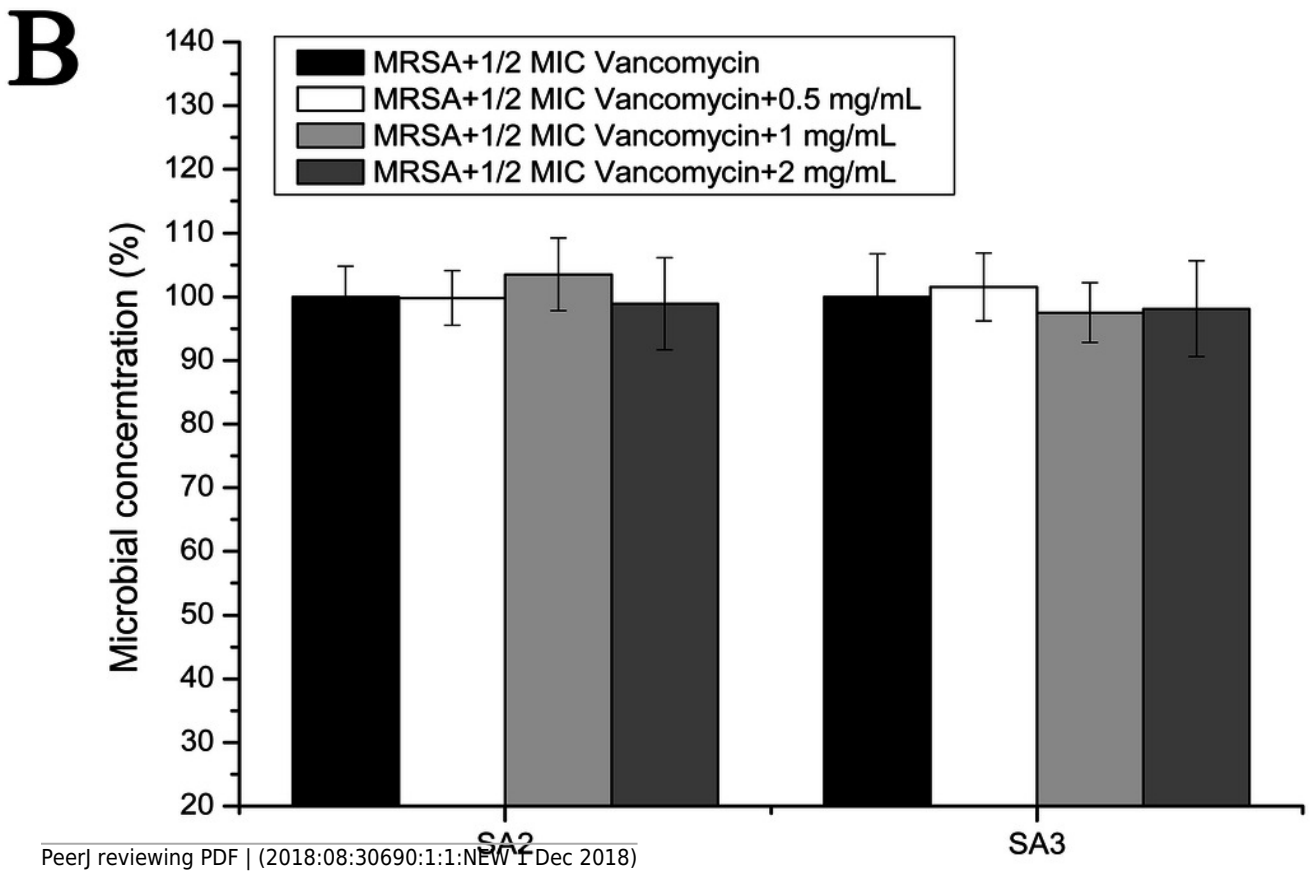
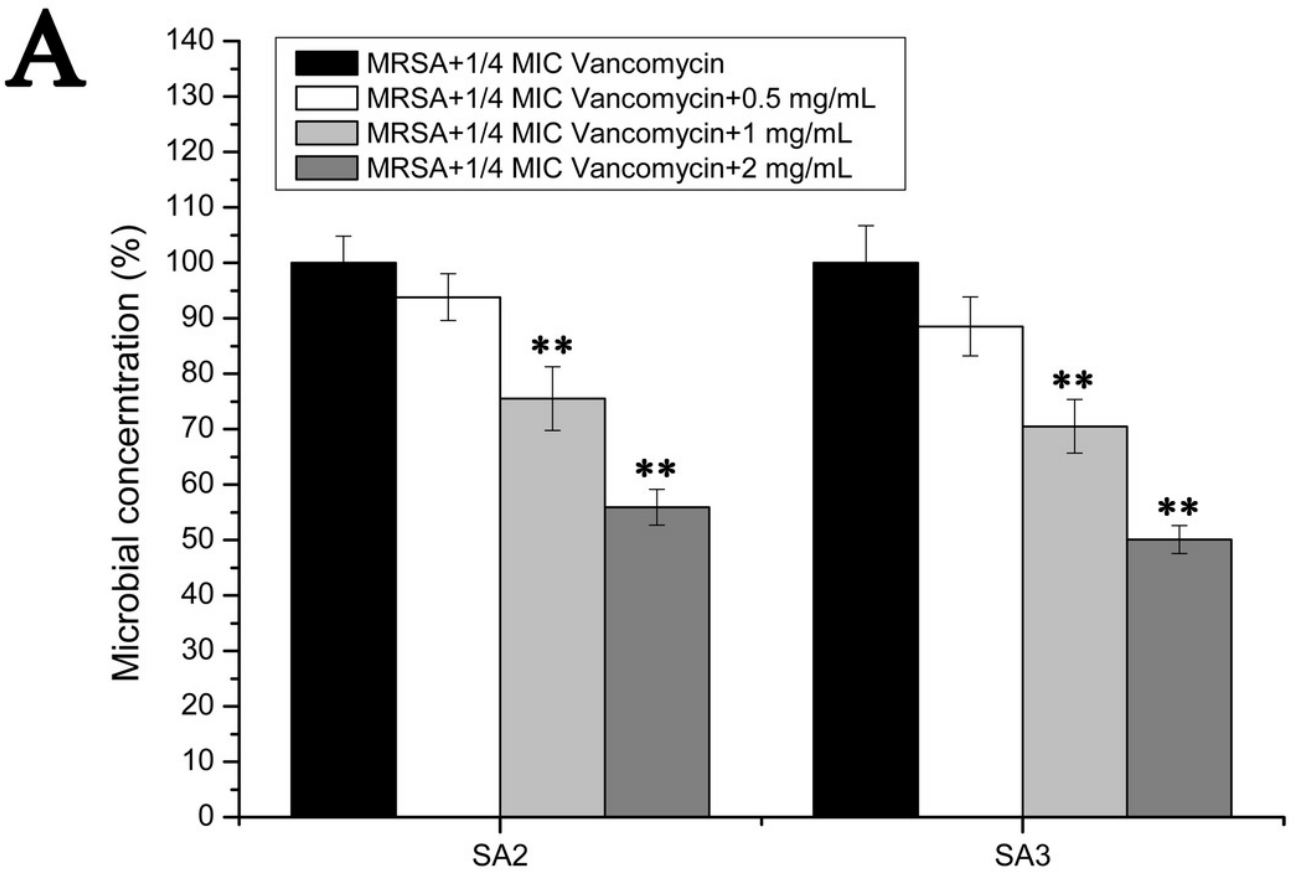

B
MRSA+1/2 MIC Vancomycin
MRSA+1/2 MIC Vancomycin+0.5 mg/mL
MRSA+1/2 MIC Vancomycin+1 mg/mL
MRSA+1/2 MIC Vancomycin+2 mg/mL
Microbial concerntration (%)
140
130
120
110
100
90
80
70
60
50
40
30
20
SA2
SA3

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

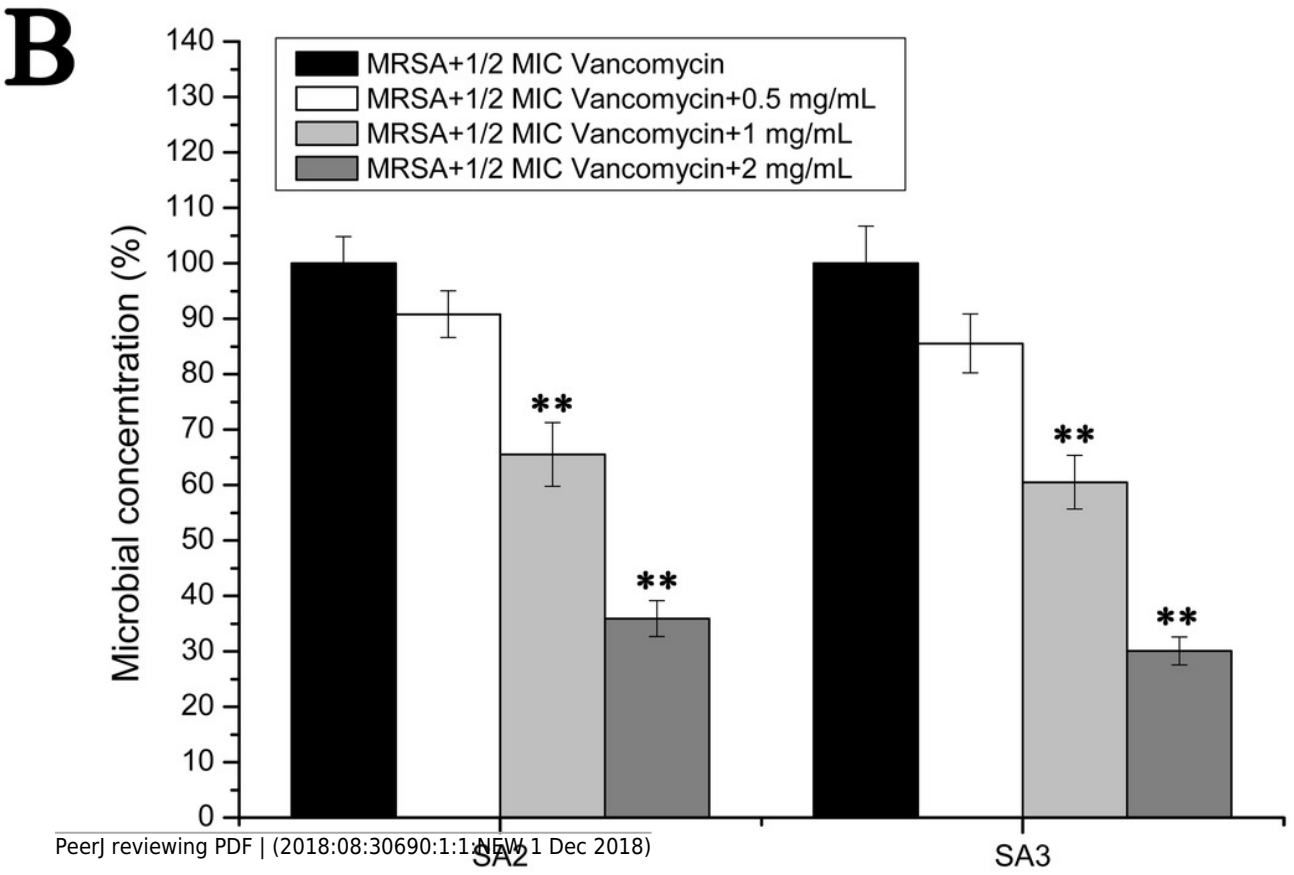

B
MRSA+1/2 MIC Vancomycin
MRSA+1/2 MIC Vancomycin+0.5 mg/mL
MRSA+1/2 MIC Vancomycin+1 mg/mL
MRSA+1/2 MIC Vancomycin+2 mg/mL
Microbial concerntration (%)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
**
**
**
**
SA2
SA3

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