Eugenol inhibits quorum sensing and biofilm of toxigenic MRSA strains isolated from food handlers employed in Saudi Arabia

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ABSTRACT
Food handlers are important component in assessment and maintenance of food quality as they are carriers of food pathogens causing spoilage. Food spoilage is attributed to quorum sensing (QS) controlled development of biofilms. Therefore, there is an urgent need to develop novel QS and biofilm inhibitors to prevent spoilage of food products. In the present study, toxin producing biofilm forming methicillin-resistant *Staphylococcus aureus* (MRSA) were isolated from food handlers. Further, eugenol was screened for its QS and anti-biofilm properties. Analysis of nasal and hand swabs revealed the presence of seven toxigenic and biofilm forming MRSA strains. Eugenol demonstrated significant anti-QS activity in CVO26 and also reduced the QS-regulated production of elastase, protease, chitinase, pyocyanin and exopolysaccharide (EPS) in PAO1 considerably. Eugenol demonstrated 17%–86%, 24%–69%, 30%–91%, 9%–94% and 4%–89% reduction in biofilm biomass of *S. aureus* ATCC 25923 and MRSA strains FSA3, FSA11, FSA13 and FSA32, respectively. Sub-inhibitory concentrations of eugenol also decreased the metabolic activity in biofilm cells. Molecular docking analysis showed high binding affinity of eugenol that represents its biofilm inhibitory activity. This is the first report on the carriage of toxigenic drug-resistant biofilm forming *S. aureus* by food handlers and inhibition of their biofilms in the Kingdom of Saudi Arabia. The findings give a clear insight into the food safety hazards associated with the carriage of *S. aureus* and present eugenol as a broad-spectrum anti-QS and anti-biofilm agent.

Introduction
Foodborne illness is one of the major health concerns worldwide and according to the estimates of World Health Organization (WHO) around 30% population in the developed countries suffers from food-related health hazards annually, whereas in the third world countries mortality of two million is estimated per year [1]. Food handlers are a common and persistent cause of the spread of foodborne diseases [2] and *Staphylococcus aureus* is one of the important pathogens often transmitted via food contaminated by an infected food handler.

Staphylococci are ubiquitously found in nature and are frequently isolated from food and environmental sources. *Staphylococcus aureus* is a foodborne pathogen that can cause localized and invasive infections in humans due to consumption of contaminated food [3]. The infection causing ability of *S. aureus* is attributed primarily to the production of staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin (TSST) [4]. Although toxin production is the major cause of the foodborne illness caused by the *S. aureus*, other factors like biofilm formation and antibiotic resistance also contribute to its pathogenicity. Drug-resistant staphylococci are a major public health concern as the bacteria can circulate easily in the environment through the food chain [5]. In the past two decades, infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) has increased considerably across the globe. Multi-drug resistant strains of *S. aureus* have been frequently recovered from food stuff and food handlers [6]. Staphylococci can also form biofilms on various surfaces and impart resistance to the pathogen against antibiotics and sanitizers [7]. Therefore, biofilm formation is a marker of pathogenicity for drug-resistant *S. aureus* [8]. Biofilm formation has been extensively studied in clinical settings, but limited information is available regarding staphylococcal biofilm in the food industry.

Food industry is currently facing severe problem of food spoilage and biofilm formation by foodborne pathogens and even with the application of modern
preservation techniques, losses due to microbial spoilage of food are still huge [9]. Foodborne pathogens like Pseudomonas spp., E. coli, Staphylococcus spp., Salmonella spp., Campylobacter jejuni and Yersinia enterocolitica have been identified to form biofilms [10]. Attachment of these foodborne pathogens to the food product or the processing surfaces leads to mass food spoilage, severe public health risk and economic losses [10]. Recently, bacterial quorum sensing (cell-to-cell communication) has received huge attention due to its role in the attachment, growth and survival of pathogenic bacteria in biofilm mode in foods [11]. Since quorum sensing (QS) in bacteria is mediated by the production of small signal molecules called autoinducers, therefore, any interference in the QS signalling system can effectively alter the microbial gene expression related to food spoilage and biofilm formation.

Since the discovery of marine alga (Delisea pulchra) as QS inhibitor, many synthetic and natural compounds have been investigated for their QS and biofilm inhibitory activity. However, the stability and toxicity associated with these compounds limit their use in food industries [12]. This has led to search for a novel stable and non-toxic QS inhibitor for their potential use in the prevention of food spoilage. Eugenol is a major constituent of clove oil and is known to possess several biological properties, including antimicrobial, antioxidant, anti-inflammatory, anticaimetic and antispasmodic activities. It is also important industrially and has been used as a flavouring agent in food and cosmetic products [13]. Recently, biofilm inhibition and eradication properties of eugenol against MRSA and methicillin-sensitive S. aureus (MSSA) clinical strains have been reported [14]. However, the effect of eugenol on QS and biofilm of MRSA strains isolated from food handler’s is not studied yet.

Since human carriers are the most important cause of transmission of foodborne illness, there is a need to investigate the association between food handlers and transmission of food-related illness. Therefore, the present study was aimed at assessing the prevalence of toxigenic, biofilm forming MRSA isolated from the nose and hands of food handlers employed in the restaurants of King Saud University, Riyadh. Additionally, the goal was also to investigate the anti-QS and biofilm inhibitory activity of eugenol against isolated MRSA strains. Bacterial characteristics like antibiotic resistance and biofilm formation were also studied as limited data is available on these aspects, especially in the Arab world.

Materials and methods

Sample collection

Samples were collected from the hands (interdigital region, index fingers, thumbs and palms of both right and left hands) and anterior nares of 100 male food handlers employed in the King Saud University, Riyadh, restaurants and cafeterias during meal preparation. One swab was used for each region.

Swabs were streaked on Baird–Parker plates (Oxoid, UK) immediately (within 1 h) after collection and the plates were incubated at 35 °C for 48 h in our laboratory.

Staphylococcal isolation and identification

After incubation, five black colonies from each plate (presence and absence of halo) were identified. Gram staining, production of catalase and coagulase were used as screening tests. The coagulase-positive strains were submitted to the kit ‘Staphytest Test Dry Spot’ (Oxoid, UK). The two positive clumping species were subjected to the Voges–Proskauer test (S. aureus positive).

Detection of toxins

Only one identified isolate, per positive individual, was investigated using an immunological technique to verify its ability to synthesize SE(s). The strains were grown in 10 mL of Tryptone Soya Broth (Oxoid, UK) by shaking aerobically for 16–18 h at 37 °C. After centrifugation at 9000 × g for 20 min at 4 °C, production of SEs A, B, C, D and TSST-1 was determined by reversed passive latex agglutination using two commercial kits, SET-RPLA and TST-RPLA (Oxoid, UK), according to the manufacturer’s protocols. Negative controls were used with all the tested samples.

Screening for methicillin resistance

Resistance to oxacillin was determined in all S. aureus isolates using an oxacillin broth screening test recommended by the British Society for Antimicrobial Chemotherapy (BSAC).

Microtitre plate assay

The ability of the strains to form a biofilm was investigated in flat-bottom 96-well polystyrene microtitre plates according to the protocol of Stepanovic’ et al. [15]. For each strain, four wells of a microtitre plate were filled with 200 µL of bacterial suspension in Tryptone Soya Broth (Oxoid, UK) supplemented with 0.5% glucose, and the plates were incubated aerobically at 37 °C for 24 h under static conditions. Then, the content of each well was aspirated, and each well was washed three times with 250 µL of sterile physiological saline. The attached bacteria were fixed with methanol for 15 min, and then the plates were emptied and left to dry. The plates were stained for 5 min with 200 µL of crystal violet per well. Excess stain was rinsed-off with tap water, and after the
plates were air-dried, the dye bound to biofilm was resolubilized with 160 μL of ethanol. The OD of each well was measured at 570 nm by using a Multiskan Microplate Reader (Thermo Scientific).

**Congo red agar method**

Congo red agar (CRA) method was adopted for screening of biofilm formation by MRSA isolates as described by Freeman et al. [16]. Briefly, plates were inoculated and incubated aerobically for 24–48 h at 37 °C. Positive result was indicated by black colonies with a dry crystalline consistency.

**Determination of minimum inhibitory concentration (MIC)**

MIC of eugenol was determined against Chromobacterium violaceum CVO26, Pseudomonas aeruginosa PAO1, S. aureus ATCC 25923 and four MRSA strains by broth macrodilution method (CLSI, 2007). Sub-MICs were selected for the assessment of anti-virulence and anti-biofilm activity in the above test strains. Eugenol used in the present study was purchased from Hi-Media Laboratories, India.

**Quantitative estimation of violacein**

Extent of violacein production by C. violaceum (CVO26) in the presence of sub-MICs of eugenol was studied by extracting violacein and quantifying photometrically using the method of Husain et al. [17].

**Effect on virulence factor production**

Effect of sub-MICs of eugenol on virulence factors of P. aeruginosa PAO1 such as lasB elastase, protease, pyocyanin, chitinase, EPS extraction and quantification was assessed as described previously [18].

**Analysis of lasB transcriptional activity in E. coli**

Measurements of β-galactosidase luminescence in E. coli MG4/pKDT17 were done by adopting the method described by Pearson et al. [19].

**Assay for biofilm inhibition**

Wells of a microtitre plate were filled with 200 μL of bacterial suspension in Tryptone Soya Broth (Oxoid, UK) supplemented with 0.5% glucose, sub-MICs of eugenol and the plates were incubated aerobically at 37 °C for 24 h under static conditions. Further, the protocol of Stepanović et al. [15] was followed as described above.

**Light microscopy analysis of biofilm inhibition**

Glass cover slips (18 mm diameter) were placed in six well tissue culture plates. Wells of the plates were dispensed with 1 mL of tryptic soy broth (TSB) media containing sub-MICs of eugenol. Further, 1 mL of standardized cell suspension was added to each well and incubated at 37 °C for 48 h. Well without test agents served as positive control. After the formation of biofilm, medium was discarded, and discs were washed three times with sterile phosphate-buffered saline (PBS) and stained with 0.1% crystal violet and incubated at 37 °C for 10 min. The discs were visualized under a bright field light microscope (Olympus, Japan) at 40 X.

**Biofilm metabolic activity measurement**

The metabolic (respiratory) activity of biofilm was determined by using (XTT) reduction assay. Biofilm formation was done as described above. Following incubation (24 h), the contents of each well were removed, and wells were washed three times with PBS to remove loosely attached cells. The sodium salt of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) was dissolved in PBS to 1 mg/mL concentration, filter sterilized and stored at −80 °C. Menadione was dissolved in acetone to 1 mmol/L and sterilized immediately before each measurement. Working solution of XTT/menadione reagent was freshly prepared before each assay in the ratio of 12.5:1. Following washing, 100 mL PBS was added to each well of a 96-well microtitre plate. 13.5 mL of XTT/menadione mixture was then added to each well; the plate was gently shaken, then covered (in darkness) and incubated at 37 °C for 3 h. Following incubation, the absorbance was measured at 490 nm. Blank well, negative and positive controls were performed as described above. Each assay was performed in triplicate [20].

**In silico analysis**

**Preparation of enzyme and molecular docking**

The three-dimensional (3D) crystal structure of sarA protein structure (PDB ID code 2FNP) was extracted from Research Collaboratory for Structural Bioinformatics (RCSB) protein databank. All the hetero atoms as well as solvent were removed. The 3D structure of eugenol and c-di-GMP were retrieved from pubchem-compound database (CID3314 and CID6323195). AutoDock tools 4.0 [21] was used to carry out docking of eugenol and c-di-GMP within the binding site of sarA. Lamarckian genetic algorithm, a combination between the genetic algorithm and the local search Pseudo-Solis and Wets algorithm, was used as a
parameter for the molecular docking. The grid box dimension was set to 60 x 60 x 60 Å around active site of sarA making sure that eugenol and c-di-GMP can freely rotate inside the grid. The number of docking runs was set to 15. The final poses were selected on the basis of their binding energies. The final complexes were subsequently visualized using PyMol [22].

Results and discussion

Prevalence of toxigenic MRSA

Due to inadequate personal hygiene and cross contamination, food handlers are vector of foodborne disease. Therefore, in this study we have collected samples from hands and nasal cavity of 100 male food handlers employed in different restaurants and cafeterias of King Saud University, Riyadh. Of the total collected samples, 43% of nose samples and 15% of hand samples were found to be carrying S. aureus (Table 1). Our results on nasal and skin carriage of S. aureus by food handler’s (43% and 15%, respectively) were in agreement to the previous report by Loeto et al. [23] and El-Shenawy et al. [24] demonstrating the prevalence of S. aureus among food handlers of Botswana and Egypt, respectively. Carriage rates of S. aureus reported by several investigators differ and these variations may be due to the ecological differences of the studied population.

Staphylococcal food poisoning is an intoxication caused by the ingestion of food containing pre-formed SE. Among different types of SEs, Enterotoxin A is most commonly associated with staphylococcal food poisoning and to a lesser extent to Enterotoxins D and B which are also known to cause poisoning [25]. Food handlers carrying toxigenic S. aureus in their noses or hands are causes for food contamination via direct contact or through respiratory secretions [26]. Results of the present investigation showed that among the 58 S. aureus isolates tested for SEs and TSST by reversed passive latex agglutination, 36 (62%), 2 (3.4%) and 1 (1.7%) were positive for SE, TSST-1 or both, respectively (Table 1). Our findings are in agreement with those of the Fueyo et al. [27] who reported 1.2%, 3.7% and 3.7% prevalence of SE, TSST-1 or both among S. aureus isolates from nasal swabs of human carriers.

Further, out of the 58 isolates screened, 7 (12%) were found to be MRSA. Results obtained from investigation of MRSA isolates were in accordance with the findings of Dagniew et al. [6] who reported 9.8% methicillin resistance among the isolated S. aureus from food handlers in Ethiopia. Detection of MRSA in the present study is vital because food handlers carrying MRSA have been identified as major cause of community-acquired foodborne outbreaks [28,29].

Table 1. Prevalence of nasal and hand carriage of coagulase positive Staphylococcus aureus among food handlers working in KSU restaurants.

| Source  | Total samples (n) | S. aureus +ve n (%) | Enterotoxigenic S. aureus +ve n (%) | Toxic shock syndrome toxicigenic S. aureus +ve n (%) |
|---------|------------------|---------------------|-------------------------------------|-----------------------------------------------|
| Nose    | 100              | 43 (43%)            | 26 (60%)                            | 3 (7%)                                         |
| Hand    | 100              | 15 (15%)            | 10 (66%)                            | ND*                                           |
| Total   | 200              | 58 (29%)            | 36 (62%)                            | 3 (5%)                                         |

*ND = Not detected.

Figure 1. Distribution of toxins in S. aureus isolated from food handlers.

(29%) and SED (14%) as depicted in Figure 1. TSST was detected in 5% of the isolated S. aureus strains. Our findings are in agreement with those of the Fueyo et al. [27] who reported 1.2%, 3.7% and 3.7% prevalence of SE, TSST-1 or both among S. aureus isolates from nasal swabs of human carriers.

Further, out of the 58 isolates screened, 7 (12%) were found to be MRSA. Results obtained from investigation of MRSA isolates were in accordance with the findings of Dagniew et al. [6] who reported 9.8% methicillin resistance among the isolated S. aureus from food handlers in Ethiopia. Detection of MRSA in the present study is vital because food handlers carrying MRSA have been identified as major cause of community-acquired foodborne outbreaks [28,29].

Screening for biofilm formation

Biofilm formation capacity of the MRSA strains was evaluated by CRA method and microtiter plate method (Figure 2(A,B)). In CRA method, six S. aureus isolates were positive for biofilm formation, showing black coloration of the colony. However, using microtiter plate (MTP) method, four MRSA strains formed strong biofilm, while one of the strains formed moderate biofilm and two test strains demonstrated weak biofilm forming capability as shown in Table 2. Staphylococcus aureus ATCC 25923 was used as positive control for biofilm formation. Since little information is available regarding staphylococcal biofilms in the food environment, isolated MRSA were screened for biofilm formation. Our findings find support from the observation made by Marino et al. [29] who showed moderate to high biofilm formation among staphylococci isolated from food samples and food handlers. The ability of food pathogens like S. aureus to adhere to an abiotic surface and form a biofilm in food environments may ultimately lead to food poisoning [30].
Determination of minimum inhibitory concentration (MIC)

MIC of eugenol was determined against *C. violaceum* CVO26, *P. aeruginosa* PAO1 and biofilm forming MRSA strains. Eugenol demonstrated a minimum inhibitory concentration of 160 µg/mL against CVO26 and PAO1. MIC of eugenol against *S. aureus* ATCC 25923 and four biofilm forming MRSA strains ranged from 42 to 665 µg/mL.

Assessment of QS and biofilm inhibitory activity of eugenol was done at sub-inhibitory concentrations, i.e. concentrations below the MICs.

Effect of eugenol on quorum sensing regulated traits

Significant problems in the food industry are due to the microbial spoilage of foods and biofilm formation by foodborne bacteria. Micro-organisms adhere to surfaces to survive the cleaning process [9]. Moreover, biofilms are more resistant to antimicrobials compared to planktonic cells, and this makes their elimination from food processing facilities a big challenge. Several studies have shown that bacterial spoilage of foods is regulated by cell-to-cell communication or QS mechanism that plays a major role in various stages of biofilm formation in bacteria [31]. Therefore, the present study was focused on the anti-QS and biofilm inhibitory effect of eugenol.

The QS inhibitory potential of eugenol was screened using the *C. violaceum* biosensor strain CVO26. The CviR-dependent QS system of *C. violaceum* coordinates the production of violacein pigment. CviR (QS receptor) in *C. violaceum* binds to the autoinducer (AHL) at high cell density. This CviR-AHL complex binds to DNA and activates expression of the *vio* genes required for the production of violacein. The compounds inhibiting the violacein pigment without significant reduction of the bacteria are termed as QS inhibitors. Eugenol exhibited a concentration-dependent decrease in QS-regulated violacein production, and statistically significant inhibition was recorded at all tested concentrations (Figure 3). Maximum reduction of 80% was recorded at 96 µg/mL concentration while lowest of 41% decrease over control was observed at 12 µg/mL eugenol concentration without any significant decrease in growth of the bacteria. Similar QS inhibitory properties of menthol and methyl eugenol based on violacein inhibition in CVO26 have been reported previously [17,32]. QS inhibitory activity of phytochemicals is attributed to the similarity in their chemical structure to those of AHL signal molecules and also because of their ability to degrade signal receptors (LuxR/LasR) [33,34]. Therefore, the reduction of QS-dependent violacein production in *C. violaceum* by the eugenol is plausibly due to inhibition of the CviR-dependent QS signalling either by prevention of AHL signal reception or decrease in the expression of the AHL family of synthases.

To ascertain the anti-QS property of eugenol, it was also tested against QS-regulated virulence factors of *P. aeruginosa* PAO1 at its respective sub-MICs. *Pseudomonas aeruginosa* has three QS systems, namely, *las, rhl* and *pqs*. The *las* and the *rhl* systems are LuxI/LuxR homologues of Las/LasR and of Rhl/RhlR, respectively [35]. Virulence factors such as elastase, protease, pyocyanin production, EPS

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### Table 2. Screening of MRSA strains for biofilm formation by Congo red agar and microtitre plate method.

| Strain designations | Biofilm assays | OD 570 nm | Inference |
|---------------------|----------------|-----------|-----------|
|                     | Congo red agar method* | Microtitre plate method** |
| FSA-1               | –              | 0.184 ± 0.001 | +         |
| FSA-2               | +              | 0.540 ± 0.059  | +++       |
| FSA-3               | +              | 0.897 ± 0.003  | +++       |
| FSA-11              | +              | 0.913 ± 0.014  | +++       |
| FSA-12              | –              | 0.261 ± 0.009  | +         |
| FSA-13              | +              | 0.955 ± 0.053  | +++       |
| FSA-32              | +              | 0.904 ± 0.033  | +++       |
| SA ATCC 25923       |                | 0.952 ± 0.021  | +++       |

*–* indicates no biofilm formation.  
+ indicates positive biofilm formation.  
*;+++*; strong biofilm; OD570 > 0.8.  
*;++*; moderate biofilm; OD570 > 0.4 to ≤ 0.8.  
+; weak biofilm; OD570 > 0.1 to ≤ 0.4

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*Figure 2. Screening of MRSA strains for biofilm formation: (A) Congo red agar (CRA) assay and (B) microtitre plate assay.*
production and biofilm formation in *P. aeruginosa* are regulated by three above-mentioned QS systems. Effect of eugenol on QS-regulated functions of *P. aeruginosa* PAO1 was determined as shown in Table 3. Significant reduction (p ≤ 0.001) in elastase (47%–82%), total protease activity (44%–87%) and pyocyanin production (45%–85%) of PAO1 was recorded at tested sub-MICs (21–84 μg/mL) of eugenol. Chitinase activity was lowered significantly (39.8%–63.2%) at sub-MICs ranging from 0.2 to 0.8% v/v eugenol. Similarly, decrease in EPS production was also dose-dependent but statistically significant reduction (49.1%) was observed at 84 μg/mL concentration. Considering las, rhl and pqs systems regulate the expression of numerous virulence-related genes, using eugenol to inhibit these QS systems would significantly decrease *P. aeruginosa* virulence.

**Effect of eugenol on β-galactosidase activity**

Effect of eugenol on AHL production was also assessed using β-galactosidase assay in *E. coli* MG4/pKDT17. Untreated PAO1 produced 590 miller units of AHL whereas at 10.5, 21, 42 and 84 μg/mL concentration of eugenol, quantity of AHL produced was 551, 474, 403 and 244 miller units, respectively. The results obtained demonstrated a significant decrease of 31.6% and 58.6% at 42 and 84 μg/mL eugenol concentrations (Figure 4). Reduced AHL levels of treated PAO1 demonstrates that eugenol decreases both elastase activity and the transcriptional activation of lasB in *E. coli*, which indicates that eugenol inhibits the las system as reported by Zhou et al. [36]

**Effect of eugenol on biofilm formation of MRSA**

Four MRSA strains that formed strong biofilms along with *S. aureus* ATCC 25923 were selected to study the biofilm inhibitory property of eugenol at sub-inhibitory concentrations (1/2 × MIC-1/8 × MIC). In the quantitative microtiter plate assay, eugenol exhibited a dose-dependent reduction in the biofilm biomass of MRSA strains. Eugenol demonstrated 17%–86%, 24%–69%, 30%–91%, 9%–94% and 4%–89% reduction in biofilm biomass of *S. aureus* ATCC 25923 and MRSA strains FSA3, FSA11, FSA13 and FSA32, respectively (Figure 5). The architecture of *S. aureus* (ATCC 25923) and MRSA biofilms grown with and without eugenol (0.5 × MIC) was analysed by light microscopy. The light microscopic images show significantly impaired biofilm growth in eugenol-supplemented media.

### Table 3. Effect of sub-MICs of eugenol on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAO1.

| Concentration (µg/mL) | Elastase activitya | Total proteaseb | Pyocyanin productionc | Chitinase activityd | EPS productione |
|----------------------|------------------|----------------|----------------------|-------------------|-----------------|
| Control              | 0.141 ± 0.024    | 1.010 ± 0.027  | 5.47 ± 0.1           | 0.128 ± 0.015     | 0.997 ± 0.032   |
| 21                   | 0.075 ± 0.028 (47)  | 0.571 ± 0.015 (44)  | 3.00 ± 0.5 (45)   | 0.091 ± 0.024 (28.9) | 0.901 ± 0.054 (9.6) |
| 42                   | 0.040 ± 0.015 (71)** | 0.177 ± 0.022 (82)** | 1.2 ± 0.05 (78)** | 0.065 ± 0.033 (49.2)** | 0.617 ± 0.045 (38.1)** |
| 84                   | 0.024 ± 0.019 (82)** | 0.124 ± 0.008 (87)** | 0.8 ± 0.045 (85)** | 0.047 ± 0.021 (63.2)** | 0.507 ± 0.029 (49.1)** |

Note: Values in the parentheses indicate percent reduction over control.
*aElastase activity is expressed as the absorbance at OD490.
*bTotal protease activity is expressed as the absorbance at OD600.
*cPyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
*dChitinase activity is expressed as the absorbance at OD570.
*eEPS production is expressed as absorbance at OD480.

The data represents mean values of three independent experiments. “significance at p ≤ 0.05; **significance at p ≤ 0.005; ***significance at p ≤ 0.001.
sample in comparison to the untreated samples (Figure 6). This is the first report on the biofilm inhibition of MRSA strains isolated from hands and nose of food handlers. In MRSA, biofilm formation is ica-independent and is mediated by proteins binding to proteins of the extracellular matrix [37]. As the biofilm experiments were carried-out in TSB medium supplied with 0.5% glucose, it has been reported previously that glucose can induce ica-independent biofilm formation in MRSA [37]. Therefore, it is envisaged that eugenol is effective against ica-independent biofilm formation. Similar findings were reported with eugenol in inhibition of biofilms of MRSA strains of clinical origin [14,38].

**Biofilm metabolic activity (XTT assay)**

XTT reduction assay was used to evaluate the metabolic activity of cells in biofilm mode after 24 h. Eugenol at sub-MICs demonstrated a decrease of 28%–72% in the respiratory activity of *S. aureus* 25923 cells growing in

![Figure 5. Effect of sub-MICs of eugenol on biofilm formation in MRSA strains.](image1)

![Figure 6. Inhibition of biofilm of MRSA isolate at sub-MICs of eugenol under light microscopy: (A) FSA13 untreated control, (B) 1/2 × MIC of eugenol, (C) FSA32 untreated control and (D) 1/2 × MIC of eugenol.](image2)
biofilm mode. Similar dose-dependent reduction in the metabolic activity of MRSA strains was recorded at respective sub-MICs as depicted in Figure 7. XTT reduction assay showed inhibition percentage range of 13%–64%, 24%–90%, 54%–88% and 45%–93% in biofilm cells of FSA3, FSA11, FSA13 and FSA32, respectively.

The results of inhibition of biofilm biomass and metabolic activity by sub-MICs of eugenol were almost similar. Moreover, the XTT reduction assay confirmed the inhibition of biofilm by sub-MICs of eugenol. However, results of XTT reduction assay could not be correlated with crystal violet assay as in strains FSA13 and FSA32 reduction in metabolic activity was significant as compared to the decrease in biofilm biomass of the two strains at tested sub-MICs. Reduced metabolic activity of biofilm cells is mainly attributed to the decreased nutrient and oxygen supply and such reduction as a physiological change can account for the resistance of biofilms to antimicrobial agents [39,40]. Also, many reports have indicated an inverse correlation or no correlation between biofilm biomass and metabolic activity for plant extract or essential oil [40–42]. However, our results showed that the eugenol had similar results in inhibition of biofilm formation and metabolic activity at higher sub-MICs tested.

In silico study

Molecular docking

The molecular docking was complementary applied to gain a better insight of the interaction of eugenol within the binding site of sarA. sarA has been reported to be an important regulator responsible for biofilm development in S. aureus. Previous findings have reported the upregulation of sarA transcript in biofilm [43]. It has also been reported earlier that targeting sarA inhibition would be an effective way of inhibiting the biofilm in S. aureus [44,45]. Therefore, sarA was taken as a molecular target for eugenol and structure–activity relationship of

Table 4. Binding efficacy of eugenol against sarA and the amino acid residues involved in their complex formation.

| Enzyme | Compounds | Autodock binding free energy (Kcal/mol) | Residues Involved | Hydrogen bond formation | Hydrophobic Interactions |
|--------|-----------|-----------------------------------------|------------------|------------------------|-------------------------|
| sarA   | c-di-GMP  | −5.88                                   | R184, E186, E189, T191 | N185, E186, H187, E189, T191 |
|        | Eugenol   | −5.13                                   | L153, K154, V192  | Y152, L153, K154, E189, R190, T191, L193 |

Figure 7. Percent reduction in metabolic activity of MRSA cells in biofilm mode at sub-MICs of eugenol.

Figure 8. Molecular docking analysis of sarA with (a) eugenol and (b) c-di-GMP.
eugenol was assessed against sarA binding site. As a reference model, the known inhibitor, c-di-GMP was docked in ligand binding domain of sarA. Earlier studies have demonstrated that c-di-GMP treatment can be used as a novel intervention strategy to control S. aureus biofilm formation and virulence [46]. Literature shows that c-di-GMP inhibits the biofilm formation in S. aureus clinical isolates, including MRSA strains [46]. The molecular docking scores of eugenol and c-di-GMP against sarA are depicted in Table 4 and Figure 8. It was revealed through molecular docking that eugenol was equally effective against sarA as compared to c-di-GMP. Eugenol was found to interact with the binding affinity of −5.09Kcal/mol against sarA. In this study, we also demonstrated that some key amino acid residues are involved in the positioning of eugenol within their respective binding site of sarA. It was found that the binding of eugenol within the active site of sarA was equally contributed by hydrogen bonding as well as hydrophobic interaction of the active site residues. Here, L153, K154, V192, Y152, E189, R190, T191 and L193 were the key residues involved in accommodating eugenol within the binding site of sarA. L153 and K154 were the common amino acids found to be involved in making hydrogen bonding as well as hydrophobic interaction with eugenol (Table 4 and Figure 8). The importance of these active site residues of sarA has already been discussed in previous studies [47]. The molecular docking scores of eugenol and c-di-GMP against sarA are shown in Table 4. Docking analysis further confirms that eugenol can interfere with QS and its regulated virulence and biofilm. Hence, it is envisioned that eugenol can be exploited as a broad-spectrum QS and biofilm inhibitor.

Conclusion
The study provides an insight on the carriage of S. aureus among food handlers. Findings of the current investigation give information regarding the prevalence of different types of toxins, prevalence of methicillin resistance and biofilm formation in the pathogen. The results of the present study demonstrate that eugenol has significant anti-QS and biofilm inhibitory activity. To the best of our knowledge, this is the first time that the biofilm inhibitory activity of eugenol has been reported against biofilm formed by MRSA isolated from food handlers. These results assume great importance as the above-studied traits influence bacterial survival and contamination of food and food processing environment. Specific QS and biofilm inhibitors can find application in food industry to enhance safety and quality of foods. Our observations should help in the better management of food products and food handlers to enhance food safety and safety of the consumers not only in KSU restaurants but also across the whole Kingdom of Saudi Arabia.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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