Contents lists available at ScienceDirect

Food Science and Human Wellness

journal homepage: http://www.keaipublishing.com/en/journals/food-science-and-human-wellness

Antibiofilm activity of 3,3′-diindolylmethane on *Staphylococcus aureus* and its disinfection on common food-contact surfaces

Hui Zhang[a], Xiaomei Guo[a], Lei Tian[b], Na Wang[c], Yuqing Li[d], Ariel Kushmaro[e,f], Robert Marks[e,f], Qun Sun[b,*]

[a] *College of Biomass Science and Engineering, Sichuan University, Chengdu 610064, China*
[b] *Key Laboratory of Bio-resources and Eco-environment of the Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610064, China*
[c] *Key Laboratory of Green Chemistry and Technology, Ministry of Education, College of Chemistry, Sichuan University, Chengdu 610064, China*
[d] *State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases & Department of Cariology and Endodontics, West China Hospital of Stomatology, Sichuan University, Chengdu 610065, China*
[e] *Avram and Stella Goldstein-Goren Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel*
[f] *The Ilse Katz Center for Meso and Nanoscale Science and Technology, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel*

ARTICLE INFO

Article history:
Received 3 March 2021
Received in revised form 30 March 2021
Accepted 14 April 2021
Available Online 10 May 2022

Keywords:
Biofilm inhibition
3,3′-Diindolylmethane
Extracellular DNA
Extracellular polysaccharide
Food-contact surfaces

ABSTRACT

This study explored the antibiofilm efficacy of 3,3′-diindolylmethane (DIM) on *Staphylococcus aureus* and its disinfection on common food-contact surfaces. The minimum biofilm inhibitory concentration (MBIC) of DIM on *S. aureus* was 62.5 μmol/L, while it did not impede the bacterial growth evaluated by growth curve and XTT reduction assay. DIM in the concentration range of 31.2−62.5 μmol/L demonstrated a dose-dependent antibiofilm activity to *S. aureus*, as confirmed by light microscopic (LM), confocal laser scanning microscopic (CLSM), and scanning electron microscopic (SEM) analyses. At DIM of 62.5 μmol/L, the biomass of *S. aureus* biofilm was significantly reduced by 97% and its average thickness by 58% ($P < 0.05$). DIM of 62.5 μmol/L inhibited the bacterial initial adhesion and proliferation, as well as cell motility; the release of extracellular DNA (eDNA) and extracellular polysaccharide (EPS) were reduced by 75% and 69%, respectively. DIM exhibited a strong inhibition to *S. aureus* biofilm formation on common food-contact surfaces, including 304 stainless steel, glass, and polyvinyl chloride (PVC) but not disperse the mature biofilm. Overall, our investigation identified DIM as a promising antibiofilm agent and its suitability to prevent the biofilm formation of *S. aureus* on common food-contact surfaces utilized during food processing.

1. Introduction

Microbial biofilms are defined as multicellular communities with complex architectures that enable microorganisms to grow and adhere to abiotic or living surfaces [1]. Most of the pathogens that cause foodborne diseases have capacity to form biofilms on most utensil surfaces and under almost all environmental conditions encountered in food production plants [2]. Bacteria are more likely to form biofilms on food-contact surface due to the residue of food ingredients in the food industry [3]. Compared with planktonic bacteria, biofilm-forming cells are more resistant to antimicrobial agents as they have a barrier composed of extracellular polysaccharide (EPS), extracellular DNA (eDNA) and proteins which prevent or reduce the contact with antimicrobial agents [4,5]. When biofilms are formed on food-contact surfaces, they are difficult to remove due to the insoluble extracellular matrix (ECM) in which the bacterial cells develop [6,7]. Therefore, biofilms forming on food-contact surfaces made of various materials, such as glass, stainless steel [8] and plastic [9], are often responsible for food contamination and foodborne bacterial outbreaks.

* Corresponding author at: College of Life Sciences, Sichuan University, Chengdu 610064, China.
E-mail address: qunsun@scu.edu.cn (Q. Sun)
Peer review under responsibility of KeAi Communications Co., Ltd.

Publishing services by Elsevier

http://doi.org/10.1016/j.fshw.2022.04.017

Hence, how to prevent the formation of biofilms is a challenge for the food industry.

Staphylococcus aureus is a commensal and opportunistic pathogen that can form biofilm on food and food-contact surfaces, causing food contamination [10]. It was reported that approximately 20%–25% of foodborne bacterial outbreaks were caused by *S. aureus* in China [11]. Biofilm formation of *S. aureus* is the main cause of *Staphylococcal* foodborne outbreaks. In the food industry, a variety of sanitizers, including benzalkonium chloride, quaternary ammonium compounds, and hydrogen peroxides, have been used to control biofilm formation and prevent food contamination [12]. However, the extensive use of sanitizers in the food industry not only failed to completely remove or inactivate pathogens, but has also led to the emergence of resistant bacteria [13-15]. In recent years, resistant bacteria have been found in a variety of foods, such as raw and processed meat [16,17] and dairy products [18]. In order to solve the problems of food contamination caused by biofilm formation and bacterial resistance caused by the wide application of sanitizers, it is urgent to find novel antibiofilm agents that may help to reduce the emergence of bacterial resistance.

Indole (Fig. S1A), a metabolite of tryptophan degradation has been shown to regulate bacterial behaviors such as biofilm formation [19], motility [20], virulence [21] and antibiotic resistance [22]. In addition to indole itself, many indole derivatives, including naturally occurring and synthetic ones, have been shown to affect bacterial behaviors. Compared with indole, most of indole derivatives (7-hydroxyindole [23], indole-3-acetaldehyde [24], 3-indolylacetonitrile and indole-3-carboxyaldehyde [25]) have even shown stronger antibiofilm activity. 3,3′-diindolylmethane (DIM) (Fig. S1B) is the derivative of indol-3-carbinol abundant in *Brassica* vegetables, which is considered a potent anticancer agent with a variety of biological activities, including anti-inflammatory [26], antiproliferative [27] effects *in vitro* and in animal models. Kushmaro et al. [28] reported the antibiofilm activity of DIM against common gram-negative bacteria of medical purpose, however, the effect of DIM on biofilm formation, extracellular matrix closely related to biofilm of gram-positive foodborne bacteria *S. aureus*, and its potential application prospect in food industry are still unknown.

Hence, in order to investigate the possible antibiofilm effect of DIM, the present study established the experimental model of the *S. aureus* biofilms, an important gram-positive foodborne pathogen. This allowed the evaluation of DIM effect on biofilm biomass, morphology, and the growth cycle of biofilm formation. The effect of DIM on cell motility and extracellular matrix of *S. aureus*, as well as the biofilm formed on common food-contact surfaces was also determined.

2. Materials and methods

2.1 Chemicals, bacteria strain, and culture condition

DIM (≥ 98%; CAS: 1968-05-4) was purchased from the Aladdin Industrial Corporation (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO) (Macklin Biochemical Company, Shanghai, China) to a storage concentration of 2×10^{-2} μmol/L. All other chemicals are analytical grade. The strain of *S. aureus* used in this study was previously isolated and stored at −80 °C with 25% glycerol in our laboratory. The bacteria were inoculated in tryptone soy broth (TSB) at 37 °C for overnight. Then, overnight cultures of *S. aureus* with optical density value of 0.5 at 600 nm (around 1×10^8 CFU/mL) were considered as standard cell suspension used for subsequent experiments.

2.2 Assessment of biofilm biomass

The effect of DIM on *S. aureus* biofilm formation was measured in 96-well polystyrene microplates (Corning 3599, USA) as described according to the previously reported method with minor modifications [29-31]. The lowest concentration of DIM, which did not significantly ($P > 0.05$) inhibit the biofilm formation of the bacteria, was considered as MBIC [32]. In other words, when the concentration reached MBIC, the inhibition effect of biofilm could not be improved even if the concentration was increased. Briefly, 5 μL DIM was added individually to each well containing 175 μL brain heart infusion (BHI) broth that was supplemented with 2% glucose, 2% sucrose and 2% NaCl in a sterile 96-well polystyrene microplate to reach final concentrations of 7.8, 15.6, 31.2, 62.5 and 100 μmol/L. 5 μL of DIM was replaced with 5 μL of DMSO solution as the control. One percent inoculum from standard cell suspension (20 μL diluted standard bacterial suspension in a 1:10 ratio) of *S. aureus* were gently added to each well and incubated at 37 °C for 24 h to form biofilm. After incubation, the planktonic cells were discarded and the wells were washed with 200 μL sterile PBS (Solarbio Science & Technology Company, Beijing, China) for 3 times in order to remove unattached cells. Subsequently, the remaining attached bacteria were fixed with 200 μL of 95% (*V/V*) methanol solution. After 15 min, the fixatives were discarded and the dried wells were stained with 200 μL of 0.1% crystal violet solution (Solarbio Science & Technology Company, Beijing, China) for 20 min. Then, the staining solutions were discarded and the wells were washed twice with distilled water. After drying, 95% (*V/V*) ethanol solutions were used to dissolve the stained biofilm for 15 min and the biofilm biomass (optical density value) was quantified using a microplate reader (BIORAD680, USA) at 570 nm. The percentage of inhibition was calculated according to the following formula [33].

$$\text{Biofilm inhibition } (\%) = \frac{\text{Control OD}_{570\text{ nm}} - \text{Treated OD}_{570\text{ nm}}}{\text{Control OD}_{570\text{ nm}}} \times 100$$

2.3 Growth curve analysis

The effect of DIM on *S. aureus* growth was tested as described by Packiavathy et al. [34]. To obtain growth curve, one percent inoculum from standard cell suspension (around 1×10^8 CFU/mL) of *S. aureus* were added to 50 mL sterile BHI broth with DIM at 31.2 and 62.5 μmol/L and incubated at 37 °C with 130 r/min for 24 h. The optical density value at 600 nm was measured using UV-visible spectrophotometer (Shimadzu UV-2450, Japan) at every 2 h interval up to 24 h.

2.4 XTT reduction assay

The effect of DIM on the metabolic activity of *S. aureus* was examined using the XTT reduction assay according to the

previous method with minor modifications [33]. The sodium salt of XTT and phenazine methosulfate (PMS) was dissolved in PBS at a concentration of 20 and 300 μg/mL, respectively. The biofilm was incubated as described above (method section 2.2). After incubation, the planktonic cells were collected from the 96-well polystyrene microplates. The biofilm cells were washed with sterile PBS for 3 times; the planktonic cells were washed with sterile PBS through repeated centrifugation at 850 × *g* for 5 min and the precipitates were collected. Then, 200 μL of combined solution of XTT and PMS were added to the 96-well polystyrene microplates with washed biofilm and planktonic cells, separately. Following that, 96-well polystyrene microplates were incubated at 37 °C with 130 r/min for 3 h in the dark. Metabolic activity was evaluated by measuring the optical density value at 490 nm using the above microplate reader.

2.5 In situ visualization of S. aureus biofilm

2.5.1 Light microscopic (LM) analysis

In order to verify the impact of DIM on the *S. aureus* biofilm, the biofilm was investigated microscopically in the presence and absence of DIM as in previous reports [35,36]. Briefly, the sterile coverslips (*φ* 14 mm) were placed in each well of 24-well polystyrene microplate. 25 μL DIM was added to each well containing 875 μL BHI broth that was supplemented with 2% glucose, 2% sucrose and 2% NaCl in a sterile 24-well polystyrene microplate to reach final concentrations of 31.2 and 62.5 μmol/L. One percent inoculum from standard cell suspension (100 μL diluted standard bacterial suspension in a 1:10 ratio) of *S. aureus* were gently added to each well and incubated at 37 °C for 24 h to allow the formation of biofilm. After incubation, the coverslips were washed with sterile PBS for 3 times and stained with 0.1% crystal violet solution for 20 min. Following staining the coverslips were washed with sterile water to remove the excess stain and air dried. Stained coverslips were placed on glass slide and images were captured by a light microscope (Olympus BX53F, Japan) at magnifications of 400×.

2.5.2 Confocal laser scanning microscopic (CLSM) analysis

The effect of DIM on structure of *S. aureus* biofilm was further analyzed with CLSM (Leica TCS SP5II, Germany) [33,37]. The biofilm was grown on the sterile coverslips (*φ* 14 mm) as described the above method 2.5.1. After removal of planktonic cells, biofilm cells were washed with 0.85% NaCl solution and were stained with 3.34 μmol/L SYTO 9 from LIVE/DEAD BacLight bacterial viability kit L7012 (Thermo Fisher Scientific, USA) at 25 °C for 15 min in the dark. The stained biofilm was observed with CLSM. Images were captured using LAS-X software (version 3.3.0; Leica Application Suite X, Leica Microsystems CMS GmbH), and the three-dimensional structure of biofilm was reconstructed using the ImageJ software (version 1.48; National Institutes of Health). In addition, quantitative parameters of the three-dimensional structure were calculated using COMSTAT 2.1 software provided by Dr. Claus Sternberg, DTU Systems Biology, Technical University of Denmark, Denmark.

2.5.3 Scanning electron microscopic (SEM) analysis

To validate the observation obtained with the LM and CLSM, the effect of DIM on *S. aureus* biofilm was further examined with SEM (JSM-7500F, Japan) according to the previous method with slightly modifications [38,39]. Briefly, the sterile coverslips (*φ* 8 mm) were placed in each well of 48-well polystyrene microplate. Then, the biofilm was grown on the sterile coverslips (*φ* 8 mm) with DIM at 31.2 and 62.5 μmol/L as described the above section 2.2. Afterwards, the coverslips were washed with PBS for 3 times and immediately fixed with 2.5% glutaraldehyde solution (Leagene Biotechnology Company, Beijing, China) at 4 °C for 3 h. The coverslips were dehydrated in a graded ethanol series of 30%, 50%, 60% 70%, 90%, 95% and 100% for 10 min. The samples were then critical-point dried and immediately sputter coated with gold. The biofilm was visualized under SEM at magnifications of 2 000× and 5 000×, respectively.

2.6 Growth cycle of S. aureus biofilm formation assay

To obtain growth cycle of *S. aureus* biofilm, one percent inoculum from standard cell suspension of *S. aureus* were added to 96-well polystyrene microplates with 200 μL sterile BHI broth that was supplemented with 2% glucose, 2% sucrose and 2% NaCl. After inoculation at 37 °C for different times, the biofilm was quantified with crystal violet staining as described previously at various times up to 120 h. To obtain the effect of DIM on the growth cycle of *S. aureus* biofilm formation, DIM of 62.5 μmol/L was added at different time points of the growth cycle. Then, the biofilm was quantified after 24 h [40].

2.7 Cell motility assay

The motility assays were performed according to the previous reports with minor modifications. Briefly, the plates containing 1% tryptone, 0.25% NaCl and 0.3% agar were prepared for sliding motility [41]. Then, 15 μL of standard cell suspension was inoculated on the center of the plates in the absence or presence of DIM (31.2, 62.5 μmol/L) [42]. After incubation at 37 °C for 24 h, the cell motility of *S. aureus* was observed.

2.8 Extracellular matrix measurement

2.8.1 eDNA measurement

The biofilm was grown as described in section 2.5.1 without sterile coverslips, in which eDNA was measured as described by Rice et al. [43]. After discarding planktonic cells and washing the wells, 5 μL EDTA (0.5 mol/L) was added into each well at 4 °C. After 1 h, 700 μL TEN buffer (Leagene Biotechnology Company, Beijing, China) was added into each well to resuspend biofilm cells. After centrifugation for 5 min at 4 °C and 18 000 × *g*, 100 μL of each supernatant was transferred to a tube containing 300 μL of TE buffer (Solarbio Science & Technology Company, Beijing, China). Then, the mixture and equal volume of binding buffer (Gel extraction kit, OMEGA) was added to adsorption columns. After 2 min at room temperature, eDNA was collected by centrifugation at 4 °C and 18 000 × *g* for 60 s. The adsorption columns were washed with wash

buffer (Gel extraction kit, OMEGA) through repeated centrifugation at 1 800 × *g* for 30 s. Finally, after adding sterile water, eDNA was collected by centrifugation. The concentration of eDNA was measured by spectrophotometer (BioDrop BD1110, England).

2.8.2 EPS measurement

EPS in biofilm were determined by colorimetric method of anthrone sulfuric acid [44]. DIM was added to round cell culture dish (φ 60 mm) containing 5 mL BHI that was supplemented with 2% glucose, 2% sucrose and 2% NaCl to reach final concentrations of 31.2 and 62.5 μmol/L. Then, 1% inoculum from standard cell suspension of *S. aureus* were added to each dish and incubated at 37 °C for 24 h to allow the formation of biofilm. After discarding planktonic cells, the biofilm was gently washed 3 times with sterile PBS to remove unattached cells before suspended in 2 mL of PBS. The biofilm cell suspensions were centrifuged at 9 500 × *g* for 10 min. The resulting supernatant and 6 mL anthrone sulfuric acid solution (0.100 g anthrone reagent are soluble in 100 mL 80% sulfuric acid) were mixed thoroughly, then heat mixture in boiling water at 95 °C for 15 min, and cool mixture in ice water for 15 min. The optical density value at 625 nm were recorded, and the concentration of EPS was calculated according to the standard glucose curve.

2.9 Antibiofilm activity of DIM on common food-contact surfaces

Biofilm was grown on the sterile 304 stainless steel, glass and PVC pieces (φ 8 mm) placed in 48-well polystyrene microplates without and with DIM (15.6, 31.2 and 62.5 μmol/L) as described the above section 2.2. After incubation, the 304 stainless steel, glass and PVC pieces were washed with sterile PBS for 3 times and stained with crystal violet as described in section 2.2. The biofilm biomass (optical density value) was quantified using the above microplate reader at 570 nm. In addition, the number of active cells in biofilm was determined by plate counting method. Briefly, after washing the pieces, the biofilm was resuspended with 1 mL PBS. The suspensions that were diluted with 1:10, 1:100 and 1:1 000 was spread TSB solid plate and the count was recorded after incubating at 37 °C for 24 h.

2.10 Mature Biofilm disruption assay on common food-contact surfaces

Mature biofilm was grown on the sterile 304 stainless steel, glass and PVC pieces (φ 8 mm) placed in 48-well polystyrene microplates without DIM. After incubation, the 304 stainless steel glass and PVC pieces were washed with sterile PBS for 3 times, then mature biofilm were treated with DIM (15.6, 31.2 and 62.5 μmol/L) and positive control. NaOH (10 g/L) and HNO_3 (10 g/L) solution were adopted as positive control. After 15, 30 and 45 min of treatment, biofilm was stained as described the above section 2.2. The biofilm biomass (optical density value) was quantified using microplate reader at 570 nm.

2.11 Statistical analysis

All experiments were performed in triplicate at least three times. All data were expressed as arithmetic mean ± standard deviation and analyzed with Dunnett-ANOVA test using the SPSS software (version 20.0; SPSS, I., USA). A probability value at $P < 0.05$ was considered significant.

3. Results

3.1 Dose-dependent inhibition of biofilm formation in S. aureus by DIM

The antibiofilm activity of DIM was assessed by measuring the binding of crystal violet to biofilm cells of *S. aureus* on 96-well polystyrene microplate. A concentration dependent increase in antibiofilm activity was observed (Fig. 1). DIM at 31.2 and 62.5 μmol/L showed 50% and 90% antibiofilm activity, respectively. No significant increase in the antibiofilm activity was observed at higher concentrations than 62.5 μmol/L ($P > 0.05$). Thus 62.5 μmol/L was considered as MBIC.

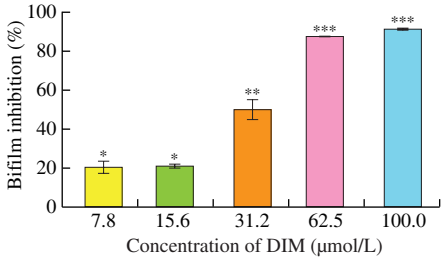

Fig. 1 The effect of DIM on biofilm formation of *S. aureus*. Data were presented as the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.2 Non-fatal effect of DIM on S. aureus growth

The effects on bacterial growth and biofilm structure are the two important criteria to determine the efficacy of any antibiofilm

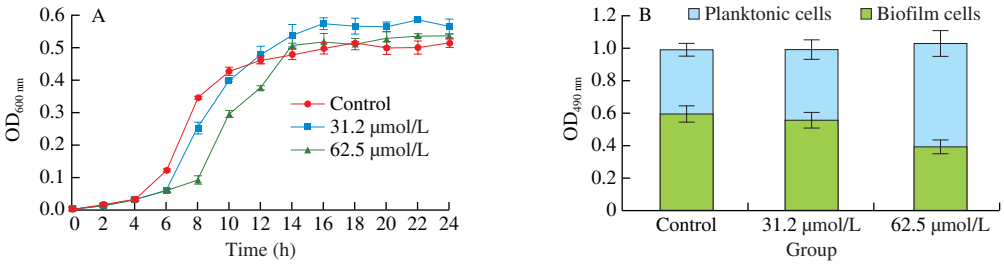

Fig. 2 The effect of DIM on growth of *S. aureus*. (A) A graph depicting the $OD_{600\,nm}$ of *S. aureus* grown in the presence and absence of DIM. (B) The viability of planktonic and biofilm cells of *S. aureus* were determined separately by XTT assay. Control: dimethyl sulfoxide. Data were presented as the mean ± S.D.

agent [32]. The promising antibiofilm agents have the characteristics of having little or no effect on bacterial growth under conditions of high antibiofilm activity [45]. In order to explore whether the high inhibition effect of DIM on biofilm was realized by inhibiting the growth, the concentrations with at least 50% antibiofilm activity (MBIC and 1/2 MBIC) were selected to study the effect on *S. aureus* growth. After 24 h of incubation, no significant differences were observed in the cell densities between control and DIM (31.2 and 62.5 μmol/L) treated (Fig. 2A). Further, DIM have no antibacterial activity as confirmed by quantifying and comparing the total number of metabolically active cells in the control and treated wells using XTT reduction assay. The number of metabolically active cells in the biofilm cells was reduced in a dose-dependent manner and the number of metabolically active cells in planktonic cells was found to increase in a dose-dependent manner when treated with DIM from 31.2 μmol/L to 62.5 μmol/L (Fig. 2B). On the other hand, no significant difference was detected among the total metabolically active cells in control and DIM-treated-samples ($P > 0.05$). This confirmed that DIM has no antibacterial activity against *S. aureus* at tested concentrations.

3.3 *In situ visualization of S. aureus biofilm*

3.3.1 *LM and CLSM analysis*

The effect on bacterial growth and biofilm architecture are the two important criteria to determine the efficacy of antibiofilm agents. The LM and CLSM images clearly revealed the morphology of *S. aureus* biofilm on the coverslips after DIM treatment. The untreated coverslips showed a clumping of complex biofilm, whereas a remarkable reduction in biofilm formation was observed on the DIM-treated-coverslips (Fig. 3). Moreover, the biofilm covered surface area and biofilm thickness in the presence of DIM were reduced in a dose-dependent manner (Fig. 3B). Further, the three-dimensional images analysis using COMSTAT 2.1 software showed a reduction in the thickness of *S. aureus* biofilm on the coverslips in the presence of DIM. The biomass and thickness of the biofilm was reduced in a dose-dependent manner when treated with DIM from 31.2 μmol/L to 62.5 μmol/L. The biomass of *S. aureus* biofilm was significantly reduced by 30% when exposed to 31.2 μmol/L DIM but by 97%, when exposed to 62.5 μmol/L ($P < 0.05$). The thickness of *S. aureus* biofilm was significantly reduced by 34%, and by 58% when exposed to 31.2, 62.5 μmol/L of DIM, respectively ($P < 0.05$) (Table 1). These images and quantitative analysis suggested that DIM had the ability to inhibit the formation of *S. aureus* biofilm.

Table 1
Inhibitory effect of DIM on the formation of *S. aureus* biofilm.

Parameters	Control[a]	31.2 μmol/L	62.5 μmol/L
Biomass ($\mu m^3/\mu m^2$)	9.329 ± 0.212^A	6.508 ± 0.201^B	0.301 ± 0.039^C
Average thickness (μm)	34.762 ± 0.729^A	22.821 ± 1.114^B	14.683 ± 0.569^C
Maximum thickness (μm)	41.869 ± 1.291^A	37.861 ± 1.386^B	23.627 ± 0.963^C
Surface to volume ratio ($\mu m^2/\mu m^3$)	3.378 ± 0.124^A	3.901 ± 0.172^B	4.734 ± 0.143^C

Data were presented as the mean ± S.D. [a]Control: dimethyl sulfoxide. [A–C] Different capital letters in the same line indicate significant differences ($P < 0.05$).

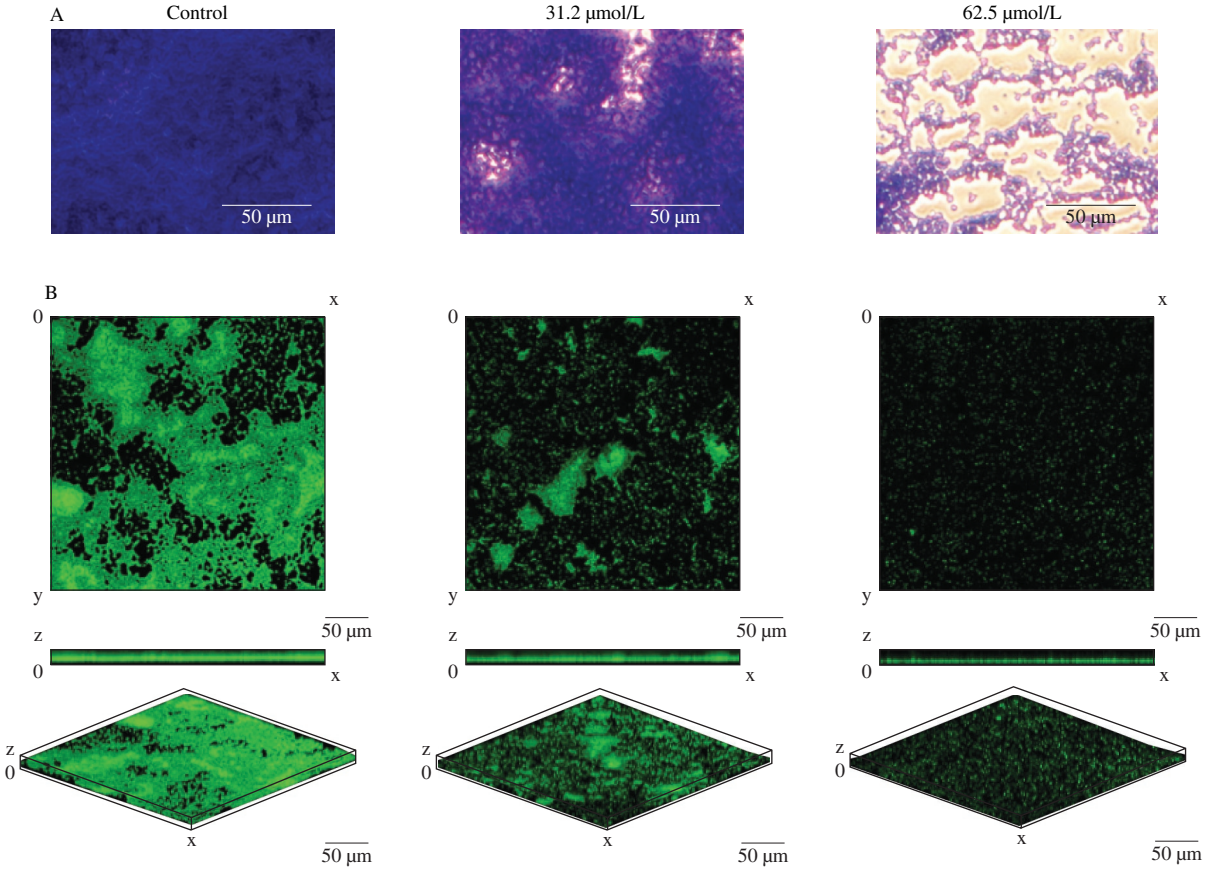

Fig. 3 Microscopic visualization of antibiofilm efficacy of DIM on *S. aureus*. (A) Light microscopic images (400×). (B) Confocal laser scanning microscope images (400×) in the presence and absence of DIM.

3.3.2 SEM analysis

SEM analysis was also carried out to confirm the antibiofilm potential of DIM on formation of *S. aureus* biofilm. The biofilm of untreated control was observed to adhere on the surface of coverslips and form thick aggregates, while the biofilm exposed to DIM gradually decreased. As shown in Fig. 4, the biofilm treated with 31.2 μmol/L DIM showed slackened and small cell clusters. And the biofilm in the presence of higher concentration of DIM (62.5 μmol/L) were of dispersed colonies of fewer cells and attached loosely on the glass coverslips. Moreover, extracellular matrix (the red arrow) was clearly visible in the untreated control group, but not in the treatment group.

3.4 Effect of DIM on the growth cycle of S. aureus biofilm

Biofilm formation generally divided into several steps: initial attachment, bacterial aggregation, maturation and dispersion [46]. The effect of DIM on the growth cycle of *S. aureus* biofilm is shown in Fig. 5. The growth cycle of *S. aureus* biofilm was measured and the results showed that between 0 and 3 h there was the bacterial initial adhesion stage, between 3 and 8 h was the bacterial aggregation stage occurred, 8–48 h was the biofilm maturation stage and the biofilm diffusion stage occurred after 48 h (Fig. 5A). To explore which phase of the growth cycle of *S. aureus* biofilm DIM influenced, DIM of 62.5 μmol/L was added to the cultures at different time points during the growth cycle of *S. aureus* biofilm. Addition of DIM immediately (0 h) after inoculation resulted in 90% inhibition of biofilm formation (Fig. 5B). DIM inhibited the biofilm obviously when added at 2, 4 and 6 h after inoculation during the adhesion and proliferation stages. Though, 8 h following inoculation during the growth phase, the biofilm cultures were resistant to DIM (Fig. 5B). These results demonstrated that DIM interfered with the formation of *S. aureus* biofilm when added during the bacterial initial adhesion and proliferation stage.

3.5 Effect of DIM on the cell motility of S. aureus

During the initial bacterial adhesion stage, motility plays a crucial role in adhesion to surface and to the subsequent biofilm formation [41]. *S. aureus* is a non-flagellated bacterium with a motility defined as sliding or colony spreading. Hence, the ability to interfere with the sliding of *S. aureus* of DIM was tested. Fig. 6 showed a dose-dependent attenuation of sliding with DIM treated in the concentration range from 31.2 μmol/L to 62.5 μmol/L. The maximum inhibition of sliding was observed at 62.5 μmol/L.

3.6 Effect of DIM on the release of eDNA and EPS of S. aureus

ECM is a key factor in the formation of the three-dimensional structure of biofilm [47]. eDNA and EPS play important roles in the initial adhesion and the proliferation stage of bacteria,

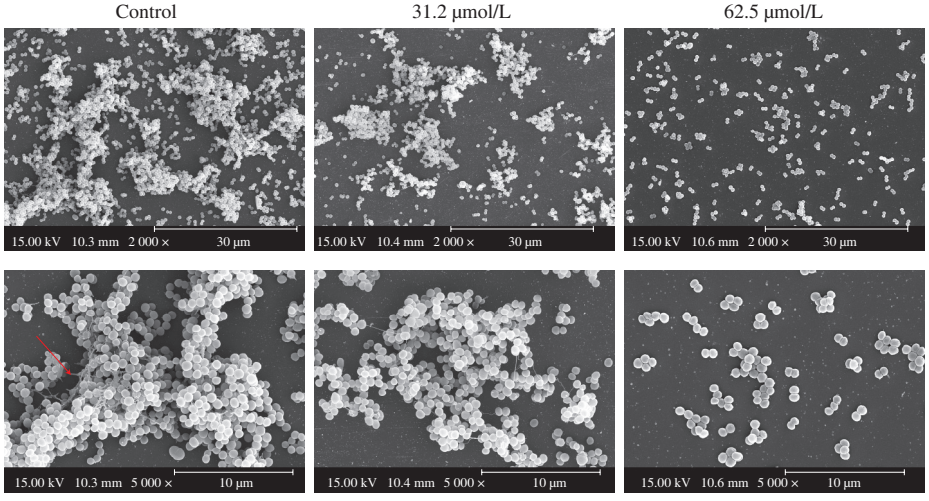

Fig. 4 Scanning electron micrographs of *S. aureus* biofilm formed on the coverslips in the presence and absence of DIM. Images were taken at 2 000× and 5 000× magnification. Red arrow, extracellular matrix.

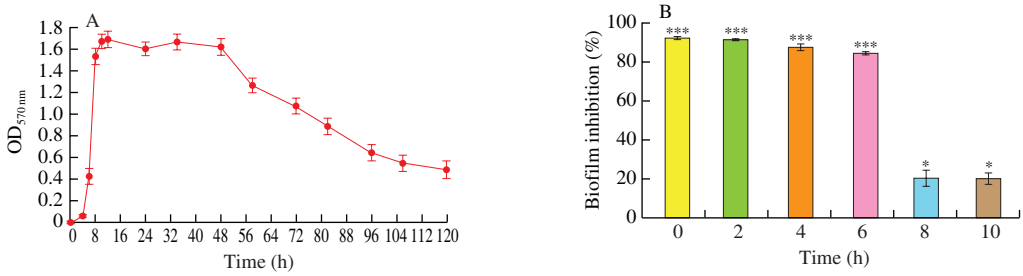

Fig. 5 (A) The growth cycle of *S. aureus* biofilm. (B) The effect of DIM on the *S. aureus* biofilm after adding at different time points in growth cycle of *S. aureus* biofilm. Data were presented as the mean ± S.D. $^{*}P < 0.05$, $^{***}P < 0.001$.

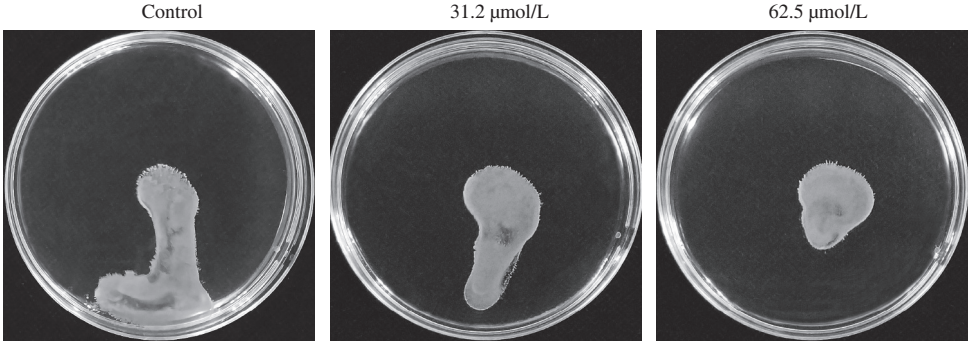

Fig. 6 The effect of DIM on sliding of *S. aureus*. Control: dimethyl sulfoxide.

respectively [48,49]. Therefore, we examined the inhibitory ability of DIM on the release of eDNA and EPS. The concentration of eDNA and EPS in *S. aureus* biofilm are given in Fig. 7. A significantly decrease of eDNA and EPS was observed in a dose-dependent manner with DIM treated in the concentration range from 31.2μmol/L to 62.5 μmol/L ($P < 0.05$). At the concentration of 62.5 μmol/L, the eDNA and EPS content in the extracellular matrix was 1.7 and 11.6 μg/mL. They were inhibited by 75% and 69%, respectively.

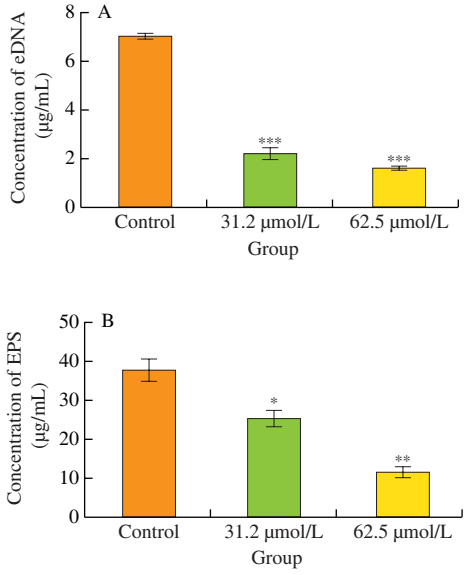

Fig. 7 (A) The effect of DIM on the release of eDNA. (B) The effect of DIM on the release of EPS. Control: dimethyl sulfoxide. Data were presented as the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.7 Effect of DIM on biofilm formation of S. aureus on common food-contact surfaces

The surface property of the carrier is also a crucial factor affecting the attachment and biofilm formation [50,51]. Hence, we investigated whether DIM inhibit the biofilm formation of *S. aureus* on common food-contact surfaces. Compared with PVC and glass, *S. aureus* is more likely to form biofilm on 304 stainless steel surfaces (Fig. 8A). The ability of DIM to inhibit the formation of *S. aureus* biofilm on 304 stainless steel, glass and PVC was determined. Fig. S2 shows a dose-dependent increase of biofilm inhibition rate with DIM treated in the concentration range from 15.6 μmol/L to 62.5 μmol/L. DIM at 62.5 μmol/L had better antibiofilm activity on 304 stainless steel and glass surface, and the biofilm inhibition rate was nearly 80%. As can be seen from Fig. 8B, the number of viable cells in the biofilm formed on 304 stainless steel was the highest, confirming that *S. aureus* was more likely to form biofilm on 304 stainless steel. A significantly decrease of viable cells was observed in a dose-dependent manner with DIM treated in the concentration range from 15.6 μmol/L to 62.5 μmol/L ($P < 0.05$) (Fig. 8B). Approximately 4.4×10^6 CFU/coupon of *S. aureus* in the biofilm on 304 stainless steel was detected in the absence of DIM, whereas 3.0×10^5 CFU/coupon of *S. aureus* was observed in the presence of DIM (62.5 μmol/L) (Fig. 8B). The results showed that DIM was able to inhibit the formation of *S. aureus* biofilm on common food-contact surfaces such as glass, PVC, and especially 304 stainless steel that was the most commonly utilized in food industry.

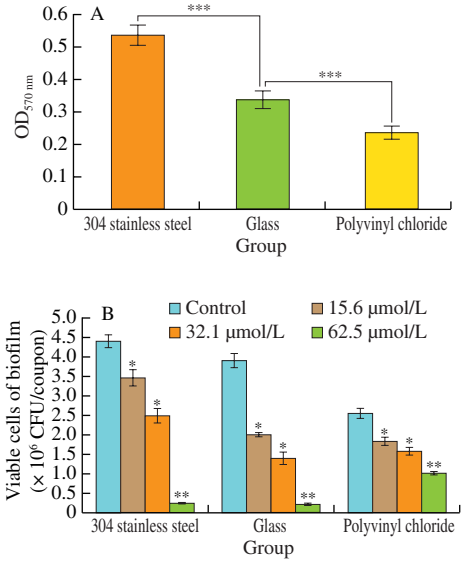

Fig. 8 (A) The ability of biofilm formation by *S. aureus* on common food-contact surfaces. (B) The effect of DIM on the viable cells in the *S. aureus* biofilm. Control: dimethyl sulfoxide. Data were presented as the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.8 Effect of DIM on mature biofilms of S. aureus on common food-contact surfaces

When evaluating the mature biofilm disruption ability of DIM on *S. aureus* on common food-contact surfaces, the crystal violet

quantification showed that DIM did not exhibit any significant disruption on mature biofilms of *S. aureus* (Table S1). DIM failed to demolish mature biofilm of *S. aureus* at all tested concentrations.

4. Discussion

S. aureus is one of the leading foodborne pathogens that cause foodborne outbreaks worldwide [52]. *S. aureus* can form biofilm on food and food-contact surfaces by adherence, colonization, and development of extracellular matrix, thereby affecting the quality of food. Importantly, biofilm is difficult to completely remove once formed, and resistant bacteria are increasing due to the wide application of sanitizers in the food industry. Hence, the current study focused on an antibiofilm agent that could effectively inhibit the biofilm formation of *S. aureus* without exerting any selection pressure over the growth. Here we reported the antibiofilm activity of DIM on the foodborne pathogen *S. aureus*. The antibiofilm activity of DIM at MBIC was up to 90% in 96-well polystyrene microplates by crystal violet staining assay, which was higher than those by most small molecule compounds reported so far [53-55]. In addition, the results of growth curve and XTT reduction assay revealed its inefficiency in killing *S. aureus* cells. Thus, it is appropriate to state that the antibiofilm potential of DIM at MBIC is not due to its inhibitory effect on bacterial growth.

The most prominent feature of a biofilm is the three-dimensional structure formed by colony aggregation and stacking [56]. The micrographs of CLSM demonstrated that DIM imposed a collapse of the biofilm architecture of *S. aureus* by loosening its microcolonies. Furthermore, the substantial reduction in biomass and thickness of biofilm treatment with DIM was demonstrated through COMSTAT 2.1 software analysis. Likewise, SEM analysis confirmed the remarkable antibiofilm efficiency of DIM. These results suggested that DIM prevented the attachment of the bacterial cells and the formation of three-dimensional architecture resulting in a failure in the formation of a biofilm. This is in accordance with the previous reports that many antibiofilm agents were able to disrupt the complex biofilm architecture and slacken microcolonies [57-59].

The planktonic cells aggregated to form a complex three-dimensional structure of biofilm through a variety of physiological factors and exhibited different physiological activities such as cell motility, initial attachment, cell proliferation and production of ECM at different stages [49]. We found that DIM terminated biofilm growth of *S. aureus* when it was added at the initial adhesion and proliferation stage. This may have been due to its effect on bacterial motility as it was reported that motility was related to the adhesion of cells to abiotic surfaces and that played an important role in biofilm formation [60]. Indeed, our study revealed that DIM could inhibit the sliding of *S. aureus*. Kaito et al. [61] and Fuente-Núñez et al. [62] reported the factors influenced in motility could inhibit the efficiency of biofilm formation.

S. aureus biofilm is often encased in a self-produced extracellular matrix which is the major reason for the three-dimensional structure of biofilm [63]. eDNA is an essential matrix molecule, and previous reports suggested the presence of eDNA on bacterial cell surface enhances adhesion and surface aggregation due to the involvement of acid-base interactions [48,64,65]. We measured the content of eDNA in the biofilm and the results demonstrated DIM also inhibited the eDNA release. As previously reported, antibiofilm agents may impede the formation of *S. aureus* biofilm by reducing the expression of *cidA*, a murein hydrolase regulator, to inhibit eDNA release [66]. EPS, a major contributor to the structural integrity of biofilm, is produced mainly during the proliferation stage [48]. Previous reports showed the ability of potent antibiofilm to reduce the EPS content in *S. aureus* biofilm [67,68]. A similar phenomenon was observed in our study demonstrating that DIM could prevent the production of EPS by *S. aureus*. These results suggested that DIM might inhibit the formation of *S. aureus* biofilm by inhibiting the release of eDNA and EPS, thus damaging the integrity of three-dimensional structure. This is supported by the SEM images showing that the ECM around the biofilm cells decreased with increasing DIM concentration.

Although the majority of planktonic cells are susceptible to common antimicrobials, mature biofilms with ECM are usually resistant to antimicrobials [69], thus, it is important to remove previously established biofilms. Similar to multiple previous studies of testing small molecule compounds, DIM failed to disrupt mature biofilm of *S. aureus* at all concentrations tested [33,35]. Because ECM is formed by the aggregation of proteins, eDNA, polysaccharides and other macromolecules, the breaking down of a mature biofilm may be largely depending on the chemical processes including acid/base (e.g., HNO_3, NaOH, etc.) effects, or enzymatic processes, such as proteases, nucleases, lysostaphin and glycogen hydrolases, etc. [70].

In the food industry, biofilm formed on food-contact surfaces has shown to be a major cause of food contamination by a variety of investigations [71,72]. We found that *S. aureus* had the greatest biofilm formation on 304 stainless steel among the three-common food-contact surfaces tested. Interestingly, DIM was shown to reduce the biofilm formation of *S. aureus* on 304 stainless steel by nearly 80% and the viable cells in biofilm by 90% in our investigation. Indeed, the inhibitory effect of antibiofilm agents on the biofilm formation of *S. aureus* has been reported in multiple previous studies [35,54,73], but most of them were based on 96-well polystyrene microplates analysis and few had evaluated their effects on food-contact surface commonly used in food industry like 304 stainless steel. Rodriguesde et al. [74] did report the effect of carvacrol on the biofilm formation of *S. aureus* on both 96-well polystyrene microplates and 304 stainless steel surfaces, however, carvacrol could only effectively inhibit the biofilm on 96-well polystyrene microplates but not on stainless steel surfaces, neither to the viable cells (reduced by around 15% after 72 h). These results suggested that DIM exert strong inhibition activity against the formation of *S. aureus* biofilm on 304 stainless steel, the representing food-contact surface in food industry.

Additionally, animal and pharmacokinetic experiments showed that DIM was low in toxicity—it was well tolerated in healthy subjects at single doses of up to 200 mg (0.8 mmol) [75,76], which is 12 times higher than the highest concentration used in the present sutdy, and DIM had high stability under both acidic and high temperature conditions, which is common during food processing [77,78]. Thus, DIM has a good application prospect in the food industry. Since foodborne pathogens may still be remained on food-contact surfaces at small quantity thus to form colony and even biofilms, especially in hard-to-clean areas, such as cracks, gaskets, and pipe joints, despite the routine cleaning, sanitation and disinfection applied during food processing. DIM mainly hindered the bacterial adhesion and colony formation to prevent the biofilm formation, thus it is suitable to

be used to wash and temporarily stay on the food-contact surface after conventional cleaning and disinfection to prevent the biofilm formation. Of course, at present the inhibition effect of DIM on biofilm formation is preliminarily investigated in the laboratory, and the process parameters of its application in the food industry need to be further determined.

5. Conclusion

The antibiofilm activity of DIM on *S. aureus* was evaluated for the first time and our investigation demonstrated that DIM could be applied to inhibit the formation of *S. aureus* biofilm on common food-contact surfaces. Since DIM had little effect on bacterial growth even at the concentration of effective biofilm inhibition, it has the potential to be developed into a novel antibiofilm agent to prevent the biofilm formation of common foodborne pathogens like *S. aureus* on food-contact surfaces thus ensuring food safety.

Declaration of competing interest

The authors declared that there was no conflict of interest.

Acknowledgments

This work was supported by the National Key Research and Development Projects (2019YFE0103800); Sichuan Science and Technology Program (2019YFH0113, 2021YFH0060, 2021YFH0072, 2021ZHFP0045, 2021YFN0092); and the Fundamental Research Funds for the Central Universities (2018CDLZ-07, 2018CDPZH-9, 2019CDPZH-23, 2020CDLZ-17).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.1016/j.fshw.2022.04.017.

References

[1] A. Lianou, G.J.E. Nychas, K.P. Koutsoumanis, Strain variability in biofilm formation: a food safety and quality perspective, Food Res. Int. 137 (2020) 109424. http://dx.doi.org/10.1016/j.foodres.2020.109424.

[2] A. Bridier, P. Sanchez-Vizuete, M. Guilbaud, et al., Biofilm-associated persistence of food-borne pathogens, Food Microbiol. 45 (2015) 167-178. http://dx.doi.org/10.1016/j.fm.2014.04.015.

[3] M. Zou, D. Liu, Effects of carbon sources and temperature on the formation and structural characteristics of food-related *Staphylococcus epidermidis* biofilms, Food Sci. Hum. Well. 9 (2020) 370-376. http://dx.doi.org/10.1016/j.fshw.2020.05.007.

[4] H. Kim, M.J. Moon, C.Y. Kim, et al., Efficacy of chemical sanitizers against *Bacillus cereus* on food contact surfaces with scratch and biofilm, Food. Sci. Biotechnol. 28 (2019) 581-590. http://dx.doi.org/10.1007/s10068-018-0482-2.

[5] A.K. Mangalappalli-Illathu, S. Vidovic, D.R. Korber, Differential adaptive response and survival of *Salmonella enterica* serovar enteritidis planktonic and biofilm cells exposed to benzalkonium chloride, Antimicrob. Agents Chemother. 52 (2008) 3669-3680. http://dx.doi.org/10.1128/AAC.00073-08.

[6] M. Sharma, S.K. Anand, Biofilms evaluation as an essential component of HACCP for food/dairy processing industry – a case, Food Control 13 (2002) 469-477. http://dx.doi.org/10.1016/S0956-7135(01)00068-8.

[7] C.K. Bower, J. Mcguire, M.A. Daeschel, The adhesion and detachment of bacteria and spores on food-contact surfaces, Trends Food Sci. Technol. 7 (1996) 152-157. http://dx.doi.org/10.1016/0924-2244(96)81255-6.

[8] S.C. Marques, J.D.G.O.S. Rezende, L.A.D.F. Alves, et al., Formation of biofilms by *Staphylococcus aureus* on stainless steel and glass surfaces and its resistance to some selected chemical sanitizers, Braz. J. Microbiol. 38 (2007) 538-543. http://dx.doi.org/10.1590/S1517-83822007000300029.

[9] A.G. Ríos-Castillo, C. Ripolles-Avila, J.J. Rodríguez-Jerez, Evaluation of bacterial population using multiple sampling methods and the identification of bacteria detected on supermarket food contact surfaces, Food Control 119 (2020) 107471. http://dx.doi.org/10.1016/j.foodcont.2020.107471.

[10] D. Grace, A. Fetsch, *Staphylococcus aureus*—a foodborne pathogen: epidemiology, detection, characterization, prevention, and control: anoverview, *Staphylococcus aureus*, (2018) 3-10. http://dx.doi.org/10.1016/B978-0-12-809671-0.00001-2.

[11] W. Wei, B. Zulqarnain, J. Tao, et al., Enterotoxigenicity and antimicrobial resistance of *Staphylococcus aureus* isolated from retail food in China, Front. Microbiol. 8 (2017) 2256. http://dx.doi.org/10.3389/fmicb.2017.02256.

[12] Y. Pan, F. Breidt, S. Kathariou, Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment, Appl. Environ. Microbiol. 72 (2006) 7711-7717, http://dx.doi.org/10.1128/AEM.01065-06.

[13] D.A.L. Silva, A.C. Camargo, S.D. Todorov, et al., *Listeria* spp. contamination in a butcher shop environment and *Listeria monocytogenes* adhesion ability and sensitivity to food-contact surface sanitizers, J. Food Saf. 37 (2016) e12313. http://dx.doi.org/10.1111/jfs.12313.

[14] M.S. Sidhu, S. Langsrud, A. Holck, Disinfectant and antibiotic resistance of lactic acid bacteria isolated from the food industry, Microb. Drug Resist. 7 (2001) 73-83. http://dx.doi.org/10.1089/107662901750152846.

[15] L. Haubert, M.L. Zehetmeyr, Y.M.N. Pereira, et al., Tolerance to benzalkonium chloride and antimicrobial activity of *Butia odorata* Barb. Rodr. extract in *Salmonella* spp. isolates from food and food environments, Food Res. Int. 116 (2019) 652-659. http://dx.doi.org/10.1016/j.foodres.2018.08.092.

[16] Q.C. Li, Y. Li, Y.Y. Tang, et al., Prevalence and characterization of *Staphylococcus aureus* and *Staphylococcus argenteus* in chicken from retail markets in China, Food Control, 96 (2019) 158-164. http://dx.doi.org/10.1016/j.foodcont.2018.08.030.

[17] X.J. Yang, J.M. Zhang, S.B. Yu, et al., Prevalence of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* in retail ready-to-eat foods in China, Front. Microbiol. 7 (2016) 816. http://dx.doi.org/10.3389/fmicb.2016.00816.

[18] J.S. Dai, S. Wu, J.H. Huang, et al., Prevalence and characterization of *Staphylococcus aureus* isolated from pasteurized milk in China, Front. Microbiol. 10 (2019) 641. http://dx.doi.org/10.3389/fmicb.2019.00641.

[19] J. Lee, A. Jayaraman, T.K. Wood, Indole is an inter-species biofilm signal mediated by SdiA, BMC Microbiol. 7 (2007) 42-56. http://dx.doi.org/10.1186/1471-2180-7-42.

[20] J.T. Lee, S. Bhuta, T.C. Calcaterra, et al., Acellular dermal matrix grafts for prevention of microarterial thrombophlebitis, Arch. Otolaryngol. Head. Neck. Surg. 133 (2007) 42-45. http://dx.doi.org/10.1001/archotol.133.1.42.

[21] B. Bettina, A. Akwasi, I. Yevgeniy, et al., A family of indoles regulate virulence and shiga toxin production in pathogenic *E. coli*, PLoS One 8 (2013) e54456. http://dx.doi.org/10.1371/journal.pone.0054456.

[22] N.M. Vega, K.R. Allison, A.S. Khalil, et al., Signaling-mediated bacterial persister formation, Nat. Chem. Biol. 8 (2011) 431-433. http://dx.doi.org/10.1038/nchembio.915.

[23] J. Lee, T. Bansal, A. Jayaraman, et al., Enterohemorrhagic *Escherichia coli* biofilms are inhibited by 7-hydroxyindole and stimulated by isatin, Appl. Environ. Microbiol. 73 (2007) 4100-4109. http://dx.doi.org/10.1128/AEM.00360-07.

[24] J.H. Lee, Y.G. Kim, C.J. Kim, et al., Indole-3-acetaldehyde from *Rhodococcus* sp. BFI 332 inhibits *Escherichia coli* O157:H7 biofilm formation, Appl. Microbiol. Biotechnol. 96 (2012) 1071-1078. http://dx.doi.org/10.1007/s00253-012-3881-y.

[25] J.H. Lee, M.H. Cho, J. Lee, 3-Indolylacetonitrile decreases *Escherichia coli* O157:H7 biofilm formation and *Pseudomonas aeruginosa* virulence, Environ. Microbiol. 13 (2011) 62-73. http://dx.doi.org/10.1111/j.1462-2920.2010.02308.x.

[26] Y. Ye, S.H. Miao, Y. Wang, et al., 3,3′-Diindolylmethane potentiates tumor necrosis factorrelated apoptosisinducing ligand-induced apoptosis of gastric

cancer cells, Oncol. Lett. (2015) 2393-2397. http://dx.doi.org/10.3892/ol.2015.3008.

[27] B. Prabhu, D. Balakrishnan, S. Sundaresan, Antiproliferative and anti-inflammatory properties of diindolylmethane and lupeol against *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine induced bladder carcinogenesis in experimental rats, Hum. Exp. Toxicol. 35 (2016) 685-692. http://dx.doi.org/10.1177/0960327115597985.

[28] A. Kushmaro, R. Marks, K. Golberg, China, Patent No. CN107835685A, 2018.

[29] S. Stepanović, D. Vuković, V. Hola, et al., Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci, APMIS 115 (2010) 891-899. http://dx.doi.org/10.1111/j.1600-0463.2007.apm_630.x.

[30] G.A. O′Toole, Microtiter dish biofilm formation assay, J. Vis. Exp. 47 (2011) e2437. http://dx.doi.org/10.3791/2437.

[31] S. Stepanović, D. Vuković, I. Dakić, et al., A modified microtiter-plate test for quantification of *Staphylococcal* biofilm formation, J. Microbiol. Methods 40 (2000) 175-179. http://dx.doi.org/10.1016/S0167-7012(00)00122-6.

[32] G.A. Subramenium, D. Viszwapriya, P.M. Iyer, et al., covR mediated antibiofilm activity of 3-furancarboxaldehyde increases the virulence of group a *Streptococcus*, PLoS One 10 (2015) e0127210. http://dx.doi.org/10.1371/journal.pone.0127210.

[33] M. Sivaranjani, S. Gowrishankar, A. Kamaladevi, et al., Morin inhibits biofilm production and reduces the virulence of *Listeria monocytogenes* — an *in vitro* and *in vivo* approach, Int. J. Food Microbiol. 237 (2016) 73-82. http://dx.doi.org/10.1016/j.ijfoodmicro.2016.08.021.

[34] I.A.S.V. Packiavathy, P. Sasikumar, S.K. Pandian, et al., Prevention of quorum-sensing-mediated biofilm development and virulence factors production in *Vibrio* spp. by curcumin, Appl. Microbiol. Biotechnol. 97 (2013) 10177-10187. http://dx.doi.org/10.1007/s00253-013-4704-5.

[35] J.R. Bai, K. Zhong, Y.P. Wu, et al., Antibiofilm activity of shikimic acid against *Staphylococcus aureus*, Food Control 95 (2019) 327-333. http://dx.doi.org/10.1016/j.foodcont.2018.08.020.

[36] S. Gowrishankar, N. Duncun Mosioma, S. Karutha Pandian, Coral-associated bacteria as a promising antibiofilm agent against methicillin-resistant and -susceptible *Staphylococcus aureus* biofilms, J. Evidence-Based Complementary Altern. Med. 2012 (2012) 1-17. http://dx.doi.org/10.1155/2012/862374.

[37] A. Bridier, F. Dubois-Brissonnet, A. Boubetra, et al., The biofilm architecture of sixty opportunistic pathogens deciphered using a high throughput CLSM method, J. Microbiol. Methods 82 (2010) 64-70. http://dx.doi.org/10.1016/j.mimet.2010.04.006.

[38] S. Gowrishankar, A. Kamaladevi, K.S. Ayyanar, et al., *Bacillus* amyloliquefaciens-secreted cyclic dipeptide-cyclo(*L*-leucyl-*L*-prolyl) inhibits biofilm and virulence in methicillin-resistant *Staphylococcus aureus*, RSC. Adv. 5 (2015) 95788-95804. http://dx.doi.org/10.1039/C5RA11641D.

[39] Â. Luís, F. Silva, S. Sousa, et al., Antistaphylococcal and biofilm inhibitory activities of gallic, caffeic, and chlorogenic acids, Biofouling 30 (2013) 69-79. http://dx.doi.org/10.1080/08927014.2013.845878.

[40] H. Xiang, F. Cao, D. Ming, et al., Aloe-emodin inhibits *Staphylococcus aureus* biofilms and extracellular protein production at the initial adhesion stage of biofilm development, Appl. Microbiol. Biotechnol. 101 (2017) 6671-6681. http://dx.doi.org/10.1007/s00253-017-8403-5.

[41] M.T. Butler, Q. Wang, R.M. Harshey, Cell density and mobility protect swarming bacteria against antibiotics, Proc. Natl. Acad. Sci. USA 107 (2010) 3776-3781. http://dx.doi.org/10.1073/pnas.0910934107.

[42] J. Monte, A. Abreu, A. Borges, et al., Antimicrobial activity of selected phytochemicals against *Escherichia coli* and *Staphylococcus aureus* and their biofilms, Pathogens 3 (2014) 473-498. http://dx.doi.org/10.3390/pathogens3020473.

[43] K.C. Rice, E.E. Mann, J.L. Endres, et al., The cidA murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*, Proc. Natl. Acad. Sci. USA 104 (2007) 8113-8118. http://dx.doi.org/10.1073/pnas.0610226104.

[44] Z. Ren, T. Cui, J. Zeng, et al., Molecule targeting glucosyltransferase inhibits *Streptococcus mutans* biofilm formation and virulence, Antimicrob. Agents Chemother. 60 (2015) 126-135. http://dx.doi.org/10.1128/AAC.00919-15.

[45] X. Liu, X. Xiang, T. Hui, et al., Chemical agents modulating bacterial biofilm formation and development, Chin. J. Biotechnol. 33 (2017) 1433-1465. http://dx.doi.org/10.13345/j.cjb.170133.

[46] S. Lin, L. Yang, G. Chen, et al., Pathogenic features and characteristics of food borne pathogens biofilm: biomass, viability and matrix, Microb. Pathog. 111 (2017) 285-291. http://dx.doi.org/https://doi.org/10.1016/j.micpath.2017.08.005.

[47] H. Koo, K.M. Yamada, Dynamic cell–matrix interactions modulate microbial biofilm and tissue 3D microenvironments, Curr. Opin. Cell Biol. 42 (2016) 102-112. http://dx.doi.org/https://doi.org/10.1016/j.ceb.2016.05.005.

[48] T. Das, P.K. Sharma, H.J. Busscher, et al., Role of extracellular DNA in initial bacterial adhesion and surface aggregation, Appl. Environ. Microbiol. 76 (2010) 3405-3408. http://dx.doi.org/10.1128/AEM.03119-09.

[49] L. Lu, W. Hu, Z. Tian, et al., Developing natural products as potential anti-biofilm agents, Chin. Med. 14 (2019) 1-17. http://dx.doi.org/10.1186/s13020-019-0232-2.

[50] K. Arunasri, S.V. Mohan, Chapter 2.3-biofilms: microbial life on the electrode surface, in: S.V. Mohan, S. Varjani, A. Pandey (Eds.), Microbial Electrochemical Technology, Elsevier, 2019, pp. 295-313. http://dx.doi.org/https://doi.org/10.1016/B978-0-444-64052-9.00011-X.

[51] M. Krsmanovic, D. Biswas, H. Ali, et al., Hydrodynamics and surface properties influence biofilm proliferation, Adv. Colloid Interface Sci. 288 (2021) 102336. http://dx.doi.org/https://doi.org/10.1016/j.cis.2020.102336.

[52] B. Umarani, K. Richa, S. Paresh, et al., Antimicrobial and anti-biofilm activity of hexadentated macrocyclic complex of copper (II) derived from thiosemicarbazide against *Staphylococcus aureus*, Sci. Rep. 8 (2018) 8050. http://dx.doi.org/10.1038/s41598-018-26483-5.

[53] A. Valliammai, A. Selvaraj, P. Mathumitha, et al., Polymeric antibiofilm coating comprising synergistic combination of citral and thymol prevents methicillin-resistant *Staphylococcus aureus* biofilm formation on titanium, Mater. Sci. Eng. C 121 (2021) 111863. http://dx.doi.org/10.1016/j.msec.2021.111863.

[54] W. Qian, M. Liu, Y. Fu, et al., Antimicrobial mechanism of luteolin against *Staphylococcus aureus* and *Listeria monocytogenes* and its antibiofilm properties, Microb. Pathog. 142 (2020) 104056. http://dx.doi.org/10.1016/j.micpath.2020.104056.

[55] A.K. Khan, A. Ahmed, M. Hussain, et al., Antibiofilm potential of 16-oxo-cleroda-3, 13(14) *E*-diene-15 oic acid and its five new *γ*-amino *γ*-lactone derivatives against methicillin resistant *Staphylococcus aureus* and *Streptococcus mutans*, Eur. J. Med. Chem. 138 (2017) 480-490. http://dx.doi.org/10.1016/j.ejmech.2017.06.065.

[56] J.L. You, X.L. Xue, L.X. Cao, et al., Inhibition of *Vibrio* biofilm formation by a marine actinomycete strain A66, Appl. Microbiol. Biotechnol. 76 (2007) 1137-1144. http://dx.doi.org/10.1007/s00253-007-1074-x.

[57] S.N. Khan, K. Shakir, I. Jawed, et al., Enhanced killing and antibiofilm activity of encapsulated cinnamaldehyde against *Candida albicans*, Front. Microbiol. 8 (2017) 1641. http://dx.doi.org/10.3389/fmicb.2017.01641.

[58] S.R. Martínez, D.M. Rocca, V. Aiassa, et al., Linezolid as an eradication agent against assembled methicillin-resistant *Staphylococcus aureus* biofilms, RSC. Adv. 6 (2016) 101023-101028. http://dx.doi.org/10.1039/c6ra19670e.

[59] D. Ming, D.C. Wang, F.J. Cao, et al., Kaempferol inhibits the primary attachment phase of biofilm formation in *Staphylococcus aureus*, Front. Microbiol. 8 (2017) 2263. http://dx.doi.org/10.3389/fmicb.2017.02263.

[60] A. Borges, M.J. Saavedra, M. Simoes, The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria, Biofouling 28 (2012) 755-767. http://dx.doi.org/10.1080/08927014.2012.706751.

[61] C. Kaito, K. Sekimizu, Colony spreading in *Staphylococcus aureus*, J. Bacteriol. 189 (2007) 2553-2557. http://dx.doi.org/10.1128/JB.01635-06.

[62] C.d.l. Fuente-Núñez, V. Korolik, M. Bains, et al., Inhibition of bacterial biofilm formation and swarming motility by a small synthetic cationic peptide, Antimicrob. Agents Chemother. 56 (2012) 2696-2704. http://dx.doi.org/10.1128/AAC.00064-12

[63] C.R. Arciola, D. Campoccia, P. Speziale, et al., Biofilm formation in *Staphylococcus* implant infections. a review of molecular mechanisms and implications for biofilm-resistant materials, Biomaterials 33 (2012) 5967-5982. http://dx.doi.org/10.1016/j.biomaterials.2012.05.031.

[64] T. Das, B.P. Krom, H.C.V.D. Mei, et al., DNA-mediated bacterial aggregation is dictated by acid–base interactions, Soft. Matter. 7 (2011) 2927-2935. http://dx.doi.org/10.1039/c0sm01142h.

[65] T. Das, S. Sehar, L. Koop, et al., Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation, PLoS One 9 (2014) e91935. http://dx.doi.org/10.1371/journal.pone.0091935.

[66] D.C. Wang, Q. Jin, H. Xiang, et al., Transcriptional and functional analysis of the effects of magnolol: inhibition of autolysis and biofilms in *Staphylococcus aureus*, PLoS One 6 (2011) e26833. http://dx.doi.org/10.1371/journal.pone.0026833.

[67] N.A. Al-Shabib, F.M. Husain, I. Ahmad, et al., Rutin inhibits mono and multi-species biofilm formation by foodborne drug resistant *Escherichia coli* and *Staphylococcus aureus*, Food Control 79 (2017) 325-332. http://dx.doi.org/10.1016/j.foodcont.2017.03.004.

[68] A. Farha, Q. Yang, G. Kim, et al., Inhibition of multidrug-resistant foodborne *Staphylococcus aureus* biofilms by a natural terpenoid (+)-nootkatone and related molecular mechanism, Food Control 112 (2020) 107154. http://dx.doi.org/10.1016/j.foodcont.2020.107154.

[69] J. Parra-Ruiz, C. Vidaillac, W.E. Rose, et al., Activities of high-dose daptomycin, vancomycin, and moxifloxacin alone or in combination with clarithromycin or rifampin in a novel *in vitro* model of *Staphylococcus aureus* biofilm, Antimicrob. Agents Chemother. 54 (2010) 4329-4334. http://dx.doi.org/10.1128/AAC.00455-10.

[70] M.K. Suresh, R. Biswas, L. Biswas, An update on recent developments in the prevention and treatment of *Staphylococcus aureus* biofilms, Int. J. Med. Microbiol. 309 (2019) 1-12. http://dx.doi.org/10.1016/j.ijmm.2018.11.002.

[71] X.M. Shi, X.N. Zhu, Biofilm formation and food safety in food industries, Trends Food Sci. Technol. 20 (2009) 407-413. http://dx.doi.org/10.1016/j.tifs.2009.01.054.

[72] B. Speranza, M.R. Corbo, The impact of biofilms on food spoilage, in: A. Bevilacqua, M.R. Corbo, M. Sinigaglia (Eds.), The Microbiological Quality of Food, Woodhead Publishing, 2017, pp. 259-282. http://dx.doi.org/10.1016/B978-0-08-100502-6.00014-5.

[73] A. Nostro, A.S. Roccaro, G. Bisignano, et al., Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms, J. Med. Microbiol. 56 (2007) 519-523. http://dx.doi.org/10.1099/jmm.0.46804-0.

[74] J.B.d.S. Rodriguesde, R.J. Carvalho, N.T. Souza, et al., Effects of oregano essential oil and carvacrol on biofilms of *Staphylococcus aureus* from food-contact surfaces, Food Control 73 (2017) 1237-1246. http://dx.doi.org/10.1016/j.foodcont.2016.10.043.

[75] J.L. Xiu, E.S. Park, H.P. Man, et al., 3,3′-Diindolylmethane suppresses the growth of gastric cancer cells via activation of the Hippo signaling pathway, Oncol. Rep. 30 (2013) 2419-2426. http://dx.doi.org/10.3892/or.2013.2717.

[76] G.A. Reed, J.M. Sunega, D.K. Sullivan, et al., Single-dose pharmacokinetics and tolerability of absorption-enhanced 3,3′-diindolylmethane in healthy subjects, Cancer Epidemiol. Biomarkers Prev. 17 (2008) 2619-2624. http://dx.doi.org/10.1158/1055-9965.EPI-08-0520.

[77] H.G. Shertzer, A.P. Senft, The micronutrient indole-3-carbinol: implications for disease and chemoprevention, Drug Metab. Drug Interact. 17 (2000) 159-88. http://dx.doi.org/10.1515/dmdi.2000.17.1-4.159.

[78] E. Ciska, J. Honke, Effect of boiling on the content of ascorbigen, indole-3-carbinol, indole-3-acetonitrile, and 3,3′-diindolylmethane in fermented cabbage, J. Agric. Food Chem. 60 (2012) 3645-3649. http://dx.doi.org/10.1021/jf300133k.