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Research Article

Evaluation of Antibacterial and Antibiofilm Properties of Kojic Acid against *Aeromonas sobria* and *Staphylococcus saprophyticus*

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Biofilms composed of microbes and extracellular polymeric substances (EPSs) pose a significant risk to human health and lead to economic loss in the food industry. In this study, the antimicrobial and antibiofilm properties of kojic acid (KA) against *Aeromonas sobria* (*A. sobria*) and *Staphylococcus saprophyticus* (*S. saprophyticus*) were investigated by determining the leakage of DNA and protein, cell morphology, biofilm formation, the metabolic activity of biofilms, excretion of EPS, and biofilm architecture. The results indicated that the values of minimum inhibitory concentration (MIC) of *A. sobria* and *S. saprophyticus* after KA treatment were 0.4 mg/mL and 1.6 mg/mL, respectively. 1 × MIC KA showed unignorable antimicrobial activity against the two bacteria, leading to alterations in the bacterial physicochemical characteristics and cell death. Sub-MICs of KA can inhibit biofilm formation and decrease the metabolic activity and excretion of EPS, and these inhibition effects were in a dose-dependent manner. These results were further confirmed by the visual images obtained from scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Moreover, *S. saprophyticus* is more susceptible to KA in inhibiting biofilm formation, and for *A. sobria*, changes in the cell structure and the permeability of the cell membrane were more obvious. This research highlighted the antibacterial and antibiofilm activity of KA against *A. sobria* and *S. saprophyticus*.

1. Introduction

Fish is a good source of food. It can be farmed or wild caught which contains macronutrients such as protein, lipids, ash, and carbohydrates [1, 2]. Fish consumption in daily basis can prevent various disease [3]. However, the quality of fish after being slaughtered is prone to deterioration due to digestive enzymes and lipases and the activity of various microbes [4]. Despite the initial microbiota being made up of a large amount of microorganisms, only few species overmaster at the end of storage, known as specific spoilage organisms (SSOs) [5]. It was found that *Aeromonas sobria* and *Staphylococcus saprophyticus* are considered to be SSOs, dominating in fish stored aerobically during refrigeration. *A. sobria* is Gram-negative, rod-shaped, flagellated, highly active, and facultative anaerobic bacteria in turbot [6]. Previous studies have reported that *A. sobria*, a zoonotic pathogen, can not only cause furunculosis and septicemia in fish but also lead to gastroenteritis and wound infection in humans [7]. *S. saprophyticus* is Gram-positive bacteria and also known as major spoilage bacteria in pomfrets [8]. As an opportunistic pathogen, *S. saprophyticus* has various clinical manifestations, including cutaneous abscesses, cellulitis, and invasive disease with bacteremia, pneumonia, and musculoskeletal infections. Besides, *A. sobria* and *S. saprophyticus* have a strong ability to form a multilayered biofilm. Biofilms

are clusters of bacteria formed on biotic or abiotic surfaces, which include extracellular polymeric substances (EPSs), membrane proteins, fimbriae, and flagella [9–13]. Biofilms pose a risk to food safety because they enhance bacteria's pathogenicity and spoilage ability, which are very difficult to be eliminated by disinfectants, antibiotics, and preservatives [14]. Food and processing equipment are contaminated by food-borne pathogens. Bacteria form biofilms on the surface, and they mainly survive in biofilms rather than in planktonic forms in the food industry [15–17]. The virulence and biofilm formation of pathogens cause food safety problems and economic loss in the food industry [18, 19]. Therefore, there is considerable research interest in controlling the growth and biofilm formation of food-borne pathogens. Recently, antibiotics are commonly used to inhibit bacteria, but they can also induce antibiotic resistance. Thus, it is essential to find an antibacterial agent that has a broad spectrum of activity, and the antimicrobial effect is complex.

Kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran4-one, KA) is a natural organic acid produced by fungi such as *Aspergillus oryzae* (*A. oryzae*) and *Aspergillus flavus* (*A. flavus*) [20, 21]. KA and its derivatives are widely used as food additives, presenting interesting bioactivities for preventing enzymatic browning of shrimp and antimicrobial and antiviral activities [22, 23]. Studies have shown that KA could prolong the shelf life of pork and duck breast meat by inhibiting *Acinetobacter*, *Photobacterium*, *Myroides*, and *Pseudomonas* spp. [24]. It was also found that KA exhibits a potent antimicrobial effect on *Pseudomonas* and *Shewanella* on the preservation of refrigerated sea bass fillets [22, 25]. However, previous studies only presented a key role in antibacterial activity of KA. Until now, we have a lack of understanding about the mechanisms of KA on biofilm formation of *A. sobria* and *S. saprophyticus*.

The aim of the present study was to provide a quantitative understanding of the antibacterial and antibiofilm properties against *A. sobria* and *S. saprophyticus* of KA. The antibacterial activity of KA was assessed by measuring minimum inhibitory concentrations (MICs), the changes in the cell microstructure, and the leakage of protein and nucleic acids in the cytoplasm. The antibiofilm activity of KA against *A. sobria* and *S. saprophyticus* focused on extracellular polymeric substances and metabolic activity of the biofilm and biofilm architecture. The findings might provide the further application of KA in food-borne pathogens and spoilage bacteria in seafood.

2. Materials and Methods

2.1. Bacterial Strains and Chemicals. Strains of *Aeromonas sobria* QY32 and *Staphylococcus saprophyticus* SY24 were originally obtained from spoiled Pacific white shrimp and identified by using 16s rRNA and a VITEK®2 CompactA system (BIOMÉRIEUX, France) and then stored in sterilized tryptone soy broth (TSB) with 25% glycerin at −80°C. Before use, the strains were individually incubated at 30°C for 18 h in brain heart infusion broth (BHI) to reach the stationary phase. Then, the bacterial suspension was grown in TSB at 30°C to 10^9 CFU/mL. All the culture media were purchased from Qingdao Hope Bio-Technology Co., Ltd. (Qingdao, China). The natural preservative of KA used in this study was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China).

2.2. Determination of Minimum Inhibitory Concentrations (MICs). The MICs of KA for two bacteria were determined using a microdilution method according to the method proposed by Wang et al. [25]. Briefly, the test bacterial suspension was mixed with serially 2-fold diluted KA solutions prepared in TSB at 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg/mL to achieve the initial inoculum of tested bacteria at approximately 10^6 CFU/mL. Then, the mixed solutions were incubated in 96-well microplates at 30°C for 24 h. Finally, the MIC of the sample was the lowest concentration at which bacterial growth was completely inhibited by recorded OD_{600nm} with a microplate reader (BioTek Synergy 2, Winooski, VT, United States).

2.3. Challenge Tests. To evaluate the effect of KA against *A. sobria* and *S. saprophyticus*, overnight cultured bacterial suspensions (10^6 CFU/mL) were exposed to broth dilution with various concentrations of KA (0.1, 0.2, and 0.4 mg/mL for *A. sobria* and 0.4, 0.8, and 1.6 mg/mL for *S. saprophyticus*). After treatment, the bacterial suspensions were cultured at 30°C for 24 h under static conditions and inoculated in sterile TSB without KA served as the control [26]. Then, the suspensions were collected to evaluate the antibacterial and antibiofilm activities of KA.

2.4. Evaluation of the Antibacterial Activity

2.4.1. Determination of the Leakage of DNA and Protein. Cellular material leakage was measured according to the method proposed by Liu et al. [27]. Briefly, 1 mL of two bacterial suspensions was mixed with 4 mL of neutralization buffer (0.85% NaCl solution and 0.5% sodium thiosulfate). The OD values at 260 nm and 280 nm were determined in triplicates.

2.4.2. Electron Microscopy Observation. Bacteria were cultured in 12-well plates to evaluate the disruption of KA on morphological changes [28]. Overnight cultures of *A. sobria* and *S. saprophyticus* were inoculated with a sterile coverslip. Briefly, after removing planktonic cells, each well was washed three times with sterile 0.1 M PBS (pH 7.0, 0.14 M NaCl). Samples were fixed overnight at 4°C with 2.5% glutaraldehyde (Solarbio, China). Then, each sample was dehydrated by a series of ethanol (50%, 70%, 80%, 90%, and 100%) for 10 min each time. The coverslips with bacteria were splattered with gold and observed by SEM (SU5000; Hitachi, Tokyo, Japan). The accelerating voltage was 5 KV.

2.5. Evaluation of the Antibiofilm Activity

2.5.1. Biofilm Inhibition Assay. Biofilm-forming capacity of the two strains with KA at different concentrations was analyzed in 96-well plates by using the crystal violet assay as previously described [26]. Bacteria were added to TSB containing KA at different concentrations at 30°C for 24 h. After incubation, the suspension of the biofilms was washed three times with sterile PBS (0.1 M, pH 7.2) to remove planktonic cells. Subsequently, the plate was stained with 200 μL of 0.2% (w/v) crystal violet for 5 min and then washed carefully to remove the redundant dye with sterile water. Finally, 200 μL of 33% acetic acid (v/v) was added to each well, and OD_{600nm} was recorded.

To collect pellets, the bacterial suspension was centrifuged at 5, 000g at 4°C. Then, the pellets were resuspended in 0.9% NaCl and serially diluted to count planktonic bacteria.

2.5.2. Measurement of the Metabolic Activity of Biofilms. The method to analyze the metabolic activity of biofilms was performed using a 2, 3-bis (2-methoxy-4-nitro-5-sulfo-pheny)-2H-tetrazolium-5 carboxanilide (XTT) reduction assay [29]. Biofilms formed in 96-well plates were washed with PBS three times. The plates were incubated with 150 μL XTT (Sigma Aldrich, UK) for 2 h at 37°C in the dark place. The absorbance results were measured at 490 nm.

2.5.3. Quantification of Extracellular Polymeric Substances (EPSs). The method to investigate extracellular polymeric substances in biofilms was conducted according to the literature [30, 31]. The biofilms were established in 6-well plates to quantity EPS. The bacterial suspension was centrifuged at 5, 000g for 15 min to remove bacterial cells. After that, 300 μL of the supernatant was mixed with 1, 200 μL Alcian blue stain and incubated at room temperature for 1 h. The mixture was centrifuged at 7, 000g for 10 min to remove the suspension. After that, samples were mixed with 1, 200 μL ethanol and then centrifuged at 7, 000g for 10 min to obtain the precipitate. Two milliliters of SDS was added to suspend the obtained precipitate. The OD_{620nm} of the supernatants was measured.

2.5.4. Visualization of the Biofilms Using Confocal Laser Scanning Microscopy (CLSM). After 24 h incubation of a sterile coverslip in 12-well plates, the suspension was removed. The samples were fixed with 4% glutaraldehyde (Sangon Biotech, Co., Ltd., Shanghai, China) for 30 min at 4°C. Then, the coverslips were rinsed with 0.1 M PBS and stained with SYBR Green I (Sangon Biotech, Co., Ltd., Shanghai, China) in the dark for 30 min at room temperature. Finally, the excess strain was washed off using 0.1 M PBS and then dried in the air. The samples were observed by CLSM (TSC SP8, Leica, Germany) with a 10 × objective microscope at 488 nm (excitation wavelength) and 525 ± 25 nm (emission wavelength) [32].

Table 1: The MICs of *A. sobria* and *S. saprophyticus* after KA treatment.

	A. sobria	*S. saprophyticus*
MIC (mg/mL)	0.4	1.6

2.6. Statistical Analysis. All experiments were carried out in triplicates. For each data, means ± standard deviations (SDs) were calculated, and statistical significances were analyzed with ANOVA and Duncan's test and conducted with $P < 0.05$.

3. Results and Discussion

3.1. MICs. The MICs of KA against *A. sobria* and *S. saprophyticus* were determined for evaluating the potential inhibitory effect of KA on the two bacteria. The values of MICs of *A. sobria* and *S. saprophyticus* after KA treatment were 0.4 mg/mL and 1.6 mg/mL, respectively (Table 1). The results were consistent with those of previous studies that showed that KA was more efficient against Gram-negative bacteria than against Gram-positive ones [21].

3.2. Analysis of the Antibacterial Activity

3.2.1. Effect of KA on the Leakage of DNA and Protein. Cell membrane damage was validated by the leakage of cellular materials such as DNA and protein, which reflected the bactericidal effect of bacteriostatic agents [33, 34]. It was observed that the contents of DNA and protein in the two bacteria had a significant dose-dependent promotion after KA treatment at different densities due to the increase in permeability ($P < 0.05$) [35]. As shown in Figure 1, the increase rate of *A. sobria* was higher than that of *S. saprophyticus*. It was evident that the bactericidal effect of KA against *A. sobria* was stronger than that of *S. saprophyticus*, indicating that the extent of damage in cell integrity of Gram-negative was higher than that of Gram-positive. It can also be observed in SEM analysis (Figures 2(a) and 2(b)).

3.2.2. Effect of KA on Morphological Changes. The morphological changes in *A. sobria* (Figure 2(a)) and *S. saprophyticus* (Figure 2(b)) after KA treatment at different densities were observed by scanning electron microscopy. After treatment with 1/4 × MIC KA for 24 h, there was little damage found in cells. During treatment with 1/2 and 1 × MIC KA, the structure of cells was destroyed, and only small cell clusters of bacteria remained attached. It is worth noting that bacterial cells of *A. sobria* were shriveled, deformed, and ruptured (Figure 2(a)). However, the morphology of *S. saprophyticus* was only shriveled and had a coarse outer surface. The destruction of cell membranes was not catastrophic (Figure 2(b)). The group of 1 × MIC KA was more efficient than that of 1/2 × MIC treatment, especially in the group of *A. sobria*. These changes in cell morphology were consistent with the above results, showing that KA played a key role in altering the membrane integrity

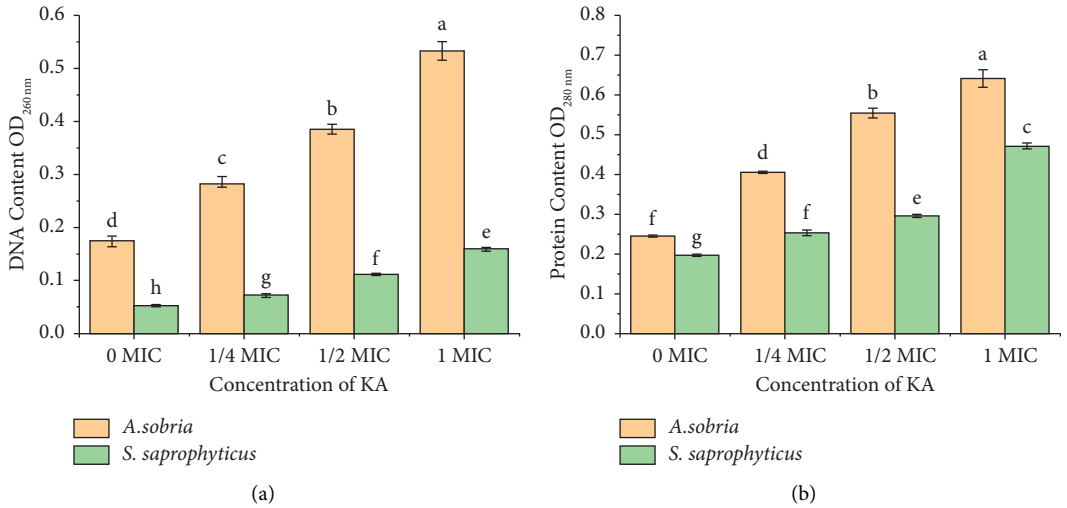


Figure 1: The leakage of DNA (a) and protein (b) of *A. sobria* and *S. saprophyticus* after KA treatment at different densities at 30°C for 24 h. The different lowercase letters represent significant differences ($P < 0.05$).

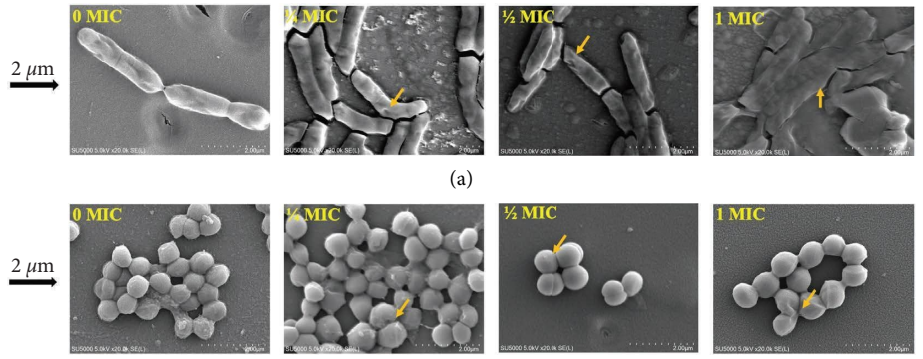


Figure 2: Scanning electron micrograph images of *A. sobria* (a) and *S. saprophyticus* (b) after KA treatment at different densities at 30°C for 24 h. The scale bar represents 2 μm.

of *A. sobria*. Similar findings also showed that KA destroyed the structure of Gram-negative bacteria (*Escherichia coli* and *Salmonella typhimurium*) and caused little damage to Gram-positive ones (*Listeria monocytogenes and Bacillus subtilis*) [21].

3.3. Analysis of the Antibiofilm Activity

3.3.1. Effect of KA on Biofilm Formation. The inhibition effects of different density of KA treatment of *A. sobria* and *S. saprophyticus* on biofilm formation are observed in Figure 3. The experiments in which planktonic bacteria were cultured with 0, 1/4, 1/2, and 1 × KA allowed the recovery to form a biofilm. In Figure 3(a), compared with 0 MIC, 1/4 × MIC could not cause a significant reduction in the viability of planktonic bacteria. Meanwhile, at 1/2 × MIC, planktonic cells were reduced by only 7% for *A. sobria* and 6% for *S. saprophyticus*. It was indicated that sublethal conditions have little effect on the activity of planktonic bacteria. However, it was noted that biofilm biomass of the two bacteria had a significant dose-dependent reduction after KA treatment at different densities. It also reported that there was no or minor effect on the activity of *Pseudomonas fluorescens* (*P. fluorescens*) under sub-lethal conditions with treatment (octyl gallate). But these treatments could significantly inhibit biofilm formation of *P. fluorescens* [36]. The amounts of biomass in the biofilm of *A. sobria* and *S. saprophyticus* were 0.21 and 0.25, measured by OD at 600 nm, in the control groups without KA, respectively. Figure 3(b) shows the inhibition rates of KA of biofilms at 1/4 and 1/2 × MICs were 11% and 61% for *A. sobria*, while those for *S. saprophyticus* were 8% and 70%, respectively. It was indicated that sub-MIC of KA prevented the formation of biofilms ($P < 0.05$) and that the group with 1 × MIC KA treatment significantly increased the removal of biofilms compared with a concentration of KA 0.4 mg/mL (*A. sobria*) and 1.6 mg/mL (*S. saprophyticus*) ($P < 0.01$). Therefore, the results indicated that KA had the ability to prevent biofilm formation of *A. sobria* and *S. saprophyticus*. It is reported that KA could play an important role in antibiofilm activity

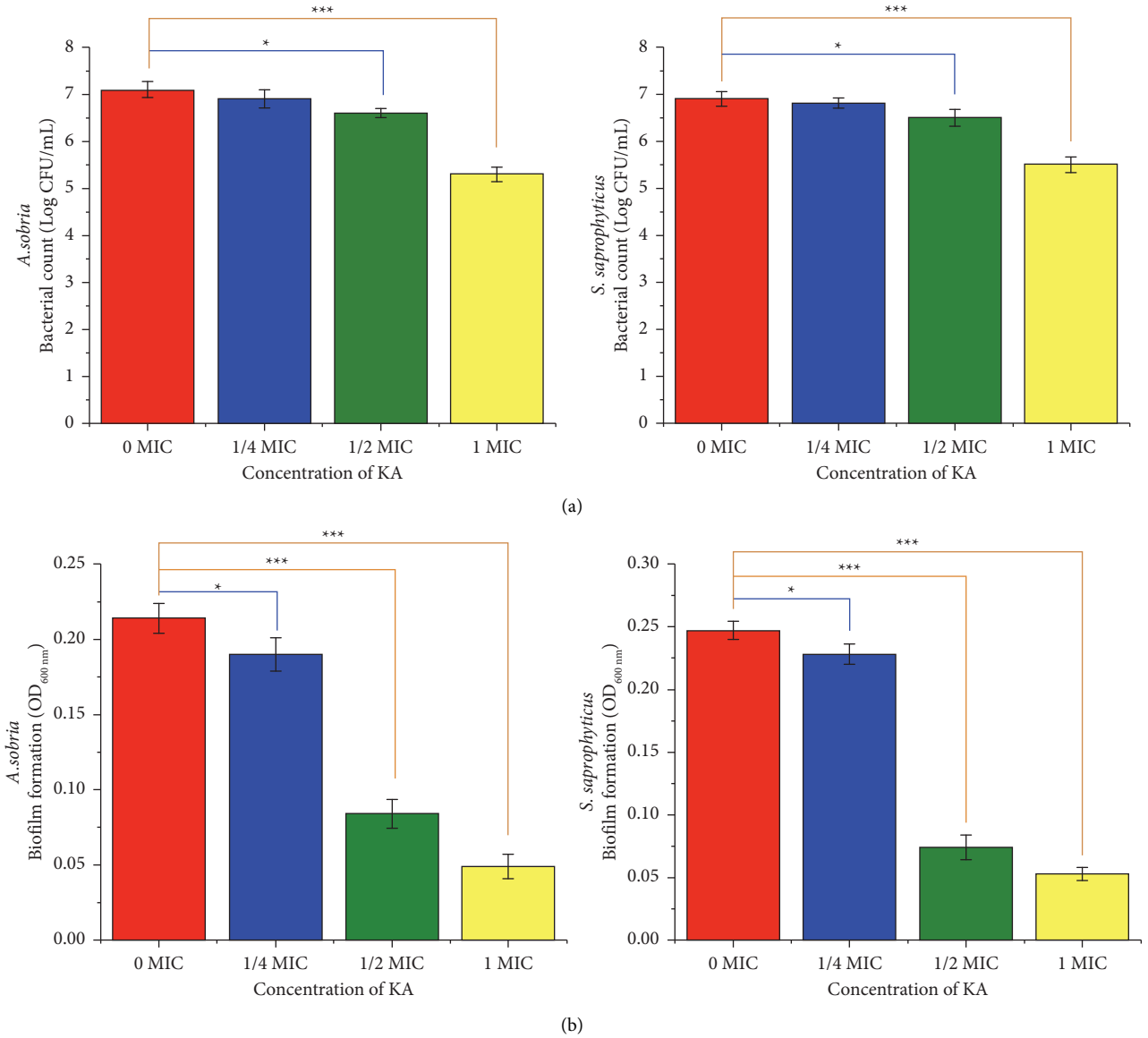


FIGURE 3: Effect on biofilm formation of *A. sobria* and *S. saprophyticus* after KA treatment at different densities at 30°C for 24 h. (a) Surviving planktonic cells in the culture supernatant treated with KA. (b) Biofilm formation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

against food-related bacteria including Gram-positive bacteria and Gram-negative ones [21].

3.3.2. Effect of KA on the Metabolic Activity of Bacterial Biofilm. As shown in Figure 4, it was observed that the inhibition rate of the metabolic activity of biofilms increased significantly after KA treatment at different densities. The metabolic activity of biofilms of *A. sobria* and *S. saprophyticus* was inhibited by 30% and 32% with 1/2 × MIC KA, respectively. At 1 × MIC, the metabolic activity of biofilms was reduced by 46% and 50%, respectively. The inhibition rates of the groups treated with 1 × MIC were significantly higher than those of others in both bacteria ($P < 0.05$). It was observed that the KA was able to decrease the metabolic activity and showed a stronger inhibition effect on S. *saprophyticus* Hou et al. found that KA showed unignorable antibiofilm activity to *Pseudomonas* species, which was consistent with our findings [22].

3.3.3. Effect of KA on the Inhibition Rate of Extracellular Polymeric Substances (EPSs). The aim of the assay was to research whether KA could inhibit or reduce the quantity of EPS formed by *A. sobria* and *S. saprophyticus*, due to EPS being the basis of the biofilm structure and its contribution to the function of biofilms [14]. With an increase in KA concentration, the amount of EPS was significantly decreased compared to that of the control treatment (Figure 5). From the results obtained, the inhibition rates of EPS in *A. sobria* and *S. saprophyticus* were 19% and 21% at 1/4 × MIC, respectively. There were no significant differences ($P > 0.05$). However, it was obvious that the inhibition rates at 1/2 × MIC were almost close to 1 × MIC, indicating

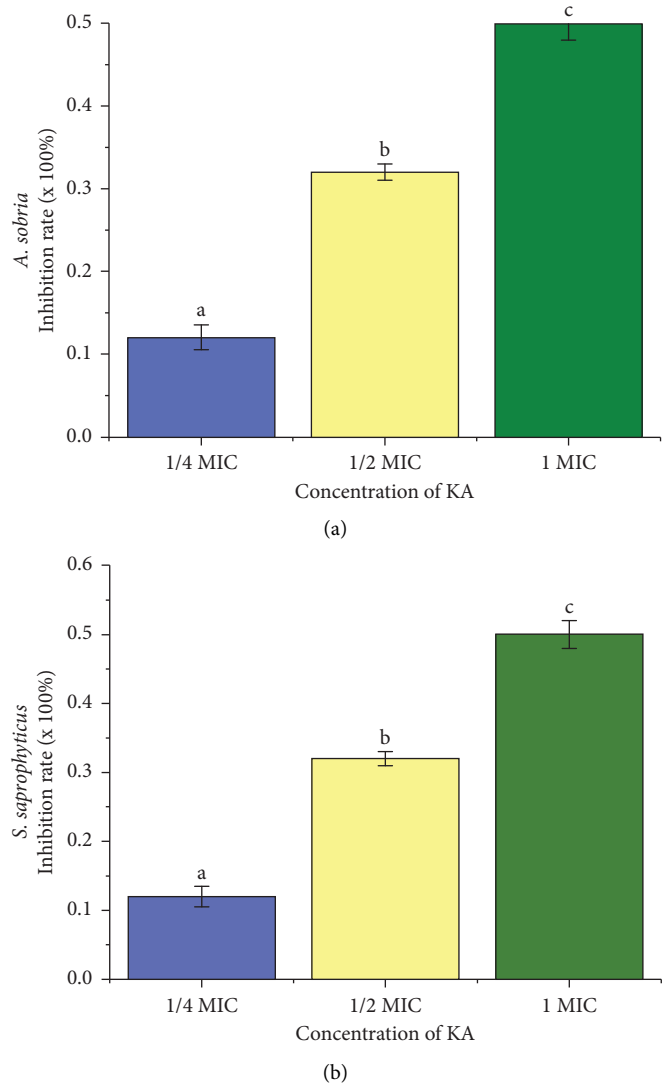


Figure 4: Inhibition effect on the metabolic activity of biofilms of *A. sobria* (a) and *S. saprophyticus* (b) after KA treatment at different densities at 30°C for 24 h. Values are exhibited as the percentage of the metabolic activity of biofilms. The different lowercase letters represent significant differences ($P < 0.05$).

there was a good performance at 1/2 × MIC against the two bacteria. Moreover, the reduction rate of KA at 1/2 × MIC reached up to 70% (*A. sobria*) and 75% (*S. saprophyticus*), exhibiting a significant difference between each other ($P < 0.05$). The results also displayed that the inhibition effect on excretion of EPS of *S. saprophyticus* was higher than that of *A. sobria,* consistent with the results of biofilm formation.

3.3.4. Characterization of Antibiofilm Activity of KA by CLSM. CLSM was used to visualize the changes in mature biofilms of *A. sobria* and *S. saprophyticus* after treatment with KA (Figure 6). No significant inhibitory effect was observed by 1/4 × MIC KA treatment compared with the control group. It was observed that 1/2 × MIC KA significantly inhibited the growth of biofilms. Furthermore, the cell cluster was rarely observed in the samples of the two bacteria treated with 1 × MIC KA. Therefore, KA inhibited the growth of mature biofilms both in *A. sobria* and *S. saprophyticus*. It is also reported that the effect of KA on the biofilms of *Acinetobacter baumannii* might be ascribed to the downregulation of *bfmR* via binding and blocking [37]. Visual results obtained by CLSM were in agreement with the above data, showing that KA could inhibit biofilm formation, excretion of EPS, and the metabolic activity of bacterial biofilms and that KA could significantly inhibit and eradicate the biofilm of *A. sobria* and *S. saprophyticus* at sub-MICs.

3.4. Proposed Antimicrobial and Antibiofilm Mechanisms of KA against A. sobria and S. saprophyticus. The structure of KA is shown in Scheme 1. In general, KA had different pathways to kill bacteria. Scheme 2 has shown the underlying mechanism. Biofilms are clusters of bacteria, including EPS, membrane proteins, fimbriae, and flagella.

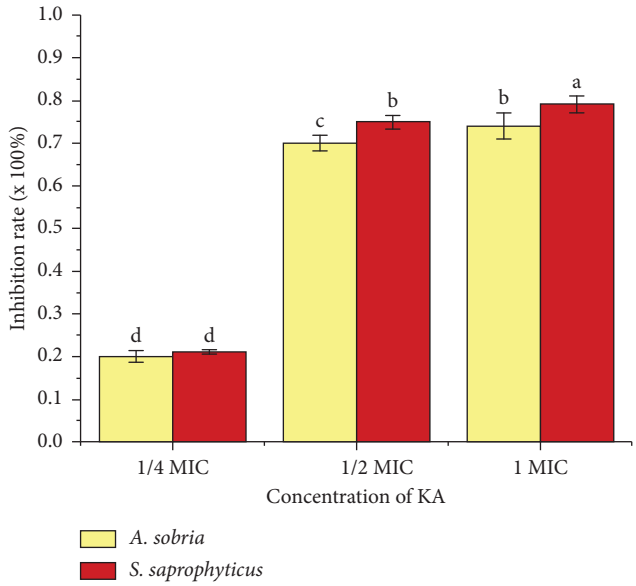


Figure 5: Inhibition effect on EPS of *A. sobria* and *S. saprophyticus* after KA treatment at different densities at 30°C for 24 h. Values are exhibited as the percentage of excretion of EPS. The different lowercase letters represent significant differences ($P < 0.05$).

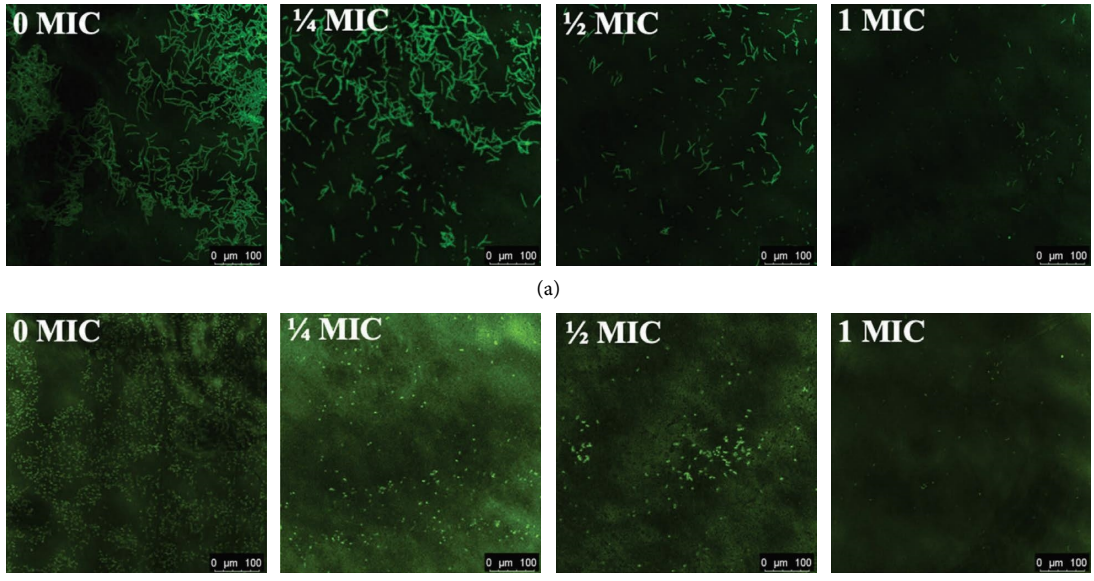
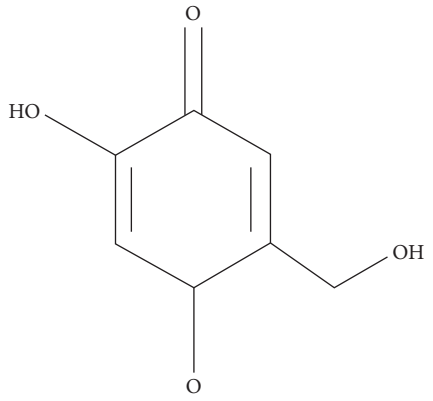
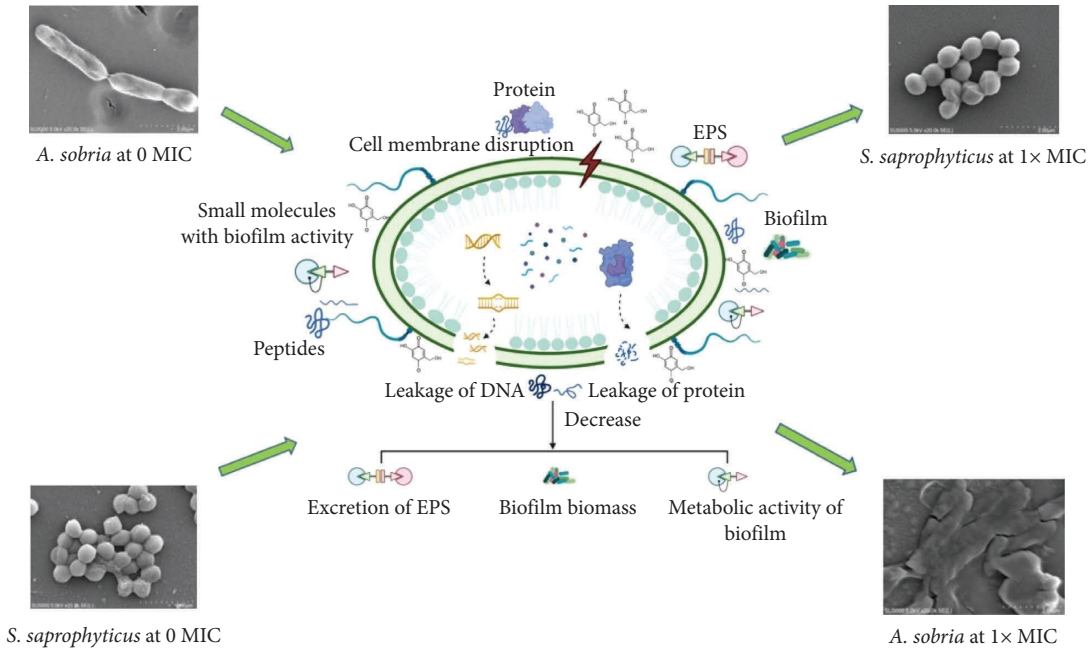


Figure 6: CLSM images of biofilms by *A. sobria* (a) and *S. saprophyticus* (b) after KA treatment at different densities at 30°C for 24 h.

For the antibiofilm mechanism of KA, KA destroyed EPS and the molecules with biofilm activity and biofilm formation, leading to decrease the activity of bacteria's pathogenicity and spoilage ability. For the antimicrobial mechanism of KA, the surface of cells treated with KA was shriveled, deformed, and ruptured, resulting in the leakage of DNA and protein, due to the increase in membrane permeability. It was hypothesized that KA promoted the membrane permeability by disrupting the cell membrane and interacting with the phospholipids of cells in the catechol group [38]. Moreover, as a chelator, KA could form a complex with metaions and destroy the balance of ions, causing the leakage of cellular materials, leading to shrinkage and even death.



Scheme 1: Molecular structure of KA.



Scheme 2: Proposed model for the mechanism of antimicrobial and antibiofilm properties of KA against *A. sobria* and *S. saprophyticus*.

4. Conclusions

In this study, the antimicrobial and antibiofilm properties of KA against *A. sobria* and *S. saprophyticus*, which can form a multilayered biofilm, were observed. The values of MICs of *A. sobria* and *S. saprophyticus* after KA treatment were 0.4 mg/mL and 1.6 mg/mL, respectively. 1 × MIC KA showed unignorable antimicrobial activity against the two bacteria by destroying the cell structure, leading to the leakage of DNA and protein and cell death. Although there were few changes in cell morphology at 1/4 × MIC and 1/2 × MIC, especially for *S. saprophyticus*, KA inhibited biofilm formation, which could decrease their pathogenicity and spoilage ability. Moreover, *S. saprophyticus* might be more susceptible to KA in inhibiting biofilm formation, whereas the cell membrane of *A. sobria* was more vulnerable. Therefore, further research needs to be carried out to understand the action mechanism of KA on spoilage bacteria.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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