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Research article

Antagonism and Antibiofilm Activity of Sterile Microbiota Growth Medium against *Klebsiella pneumoniae In Vitro*

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ABSTRACT

The metabolic bacteria extraction, Sterile Microbiota Growth Medium (SMGM), may play a role as an antibacterial and antibiofilm agent, reducing the virulence of pathogenic bacteria in vitro and in vivo. These extracts can be used to treat infections caused by antibiotic-resistant bacterial isolates. The present study aims to investigate the antibacterial and antibiofilm effects of SMGM extracted from non-*Staphylococcus aureus* growth media on the virulence of *Klebsiella pneumoniae*, as assessed by biofilm formation and bacterial growth rate. In the current study, 25 skin swabs were collected from healthy volunteers to isolate non-*S. aureus* isolates (*S. epidermidis*). From these isolates, SMGMs were prepared aseptically from 10 isolates of S. epidermidis by collecting the overnight growth (Nutrient broth) and preparing cell-free growth media after centrifugation and passing through Millipore filters. The microdilution method on a microtiter plate was used to evaluate the antibacterial effect of SMGM. Moreover, the microtiter plate and crystal violate method was used to assess the antibiofilm effect of different dilution of SMGM on the ability of *K. pneumoniea* to form biofilm *in vitro*. The results showed that all dilutions of SMGM ½, ¼, $^1/_8$, $^1/_{16}$, $^1/_{32}$, and $^1/_{64}$ reduced biofilm formation of *K. pneumoniae* ($P<0.05$). The study also demonstrated that SMGM reduced the growth rate of *K. pneumoniae* ($P<0.05$) at different time intervals (up to 48 h). It can be concluded from the current study that SMGM reduces the ability of *K. pneumoniae* to form biofilms and decreases the growth rate of planktonic cells.

Keywords: Antibiofilm, Antagonism, Biofilm, *Klebsiella pneumoniae*, Microbiome.

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1. INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen. It is a Gram-negative bacterium that plays an important role in hospital-acquired infections, particularly in immunosuppressed patients [1]. This bacterial species causes different infectious diseases, including pneumonia, urinary tract infections (UTIs), bloodstream infections, and device-related complications [2]. Biofilm formation by *K. pneumoniae* is an important virulence factor, comprising a structured microbial community encased in extracellular polymeric substances that enable the pathogen to adhere to both living and nonliving surfaces [3].

Biofilm formation not only improves bacterial persistence but also provides antimicrobial tolerance and reduces immune defenses against pathogens. Thus, it complicates treatment outcomes and contributes to the development of multidrug resistance (MDR) [4]. The appearance of resistance to carbapenems, especially among antibiotic-resistant *K. pneumoniae* isolates, creates additional treatment challenges, underscoring the urgent need for different strategies to prevent or disrupt biofilm formation and growth [5]. The concept of bacterial interference is a promising approach in scientific research, where the secretions or metabolites of comme-

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nsal or non-pathogenic bacteria stimulate the growth of pathogenic bacterial species and reduce their virulence [6]. *Staphylococcus epidermidis* is a Gram-positive bacterial species.

It is present as a normal flora of human skin and mucous membranes. This bacterial species is known for its role as a harmless commensal and an opportunistic pathogen, depending on the host's health status. Recent investigations have shown that *S. epidermidis* produces bioactive molecules that influence the behavior of other bacteria, including the disruption of quorum sensing and biofilm formation. An earlier study showed its possible as a source of novel antimicrobial or anti-biofilm agents [6].

Conditioned culture media from commensal organisms, such as many bacteria, serve as a significant source of secreted metabolites, enzymes, and signaling molecules that can inhibit the growth of pathogenic species [7]. Using these naturally occurring substances provides a potentially safe, cost-effective [7], and sustainable method for managing biofilm-related infections, avoiding the selective pressures associated with conventional antibiotics [8].

This study aimed to evaluate the effectiveness of *S. epidermidis* - derived culture media in declining biofilm formation of *K. pneumoniae in vitro*. The results highlight the role of growth culture products from normal flora in decreasing the virulence of multidrug-resistant *K. pneumoniae* by reducing biofilm formation. This involves developing innovative anti-biofilm strategies based on microbial ecological interactions.

2. MATERIALS and METHODS

2.1. Bacterial strains and culture conditions

The isolated *K. pneumoniae* strains were obtained from the Medical Microbiology Laboratory, Department of Biology, College of Science, University of Baghdad. The isolate was isolated previously from urine samples [9]. The isolate was previously characterized as a strong biofilm producer. The isolate was cultured onto nutrient agar and stored at 4°C for a short time. The bacterial isolate was stored (for a long time) at -20°C in 20% glycerol in nutrient broth (Himedia, India).

2.2. Non-*S. aureus* isolates (Microbiome isolations)

Twenty-five skin swabs were collected from healthy volunteers. Sterile swabs impregnated with normal saline were used to collect samples from uncovered skin (avoiding regions near the mucosa, the palms, and the soles). The swabs were inoculated onto either nutrient or blood agar plates. The plates were incubated at 37°C for one to two days. Two to three bacterial colonies per volunteer were randomly selected based on colony morphology. Common biochemical tests (oxidase, catalase, coagulase, and protein A) and culture on selective and differential media (Staph 110, milk agar, and mannitol salt agar) were used to identify the bacterial isolate as *S. epidermidis*.

2.3. Preparation of SMGM

Ten isolates of *S. epidermidis* were selected to check their SMGM effectivity against biofilm formation of *K. pneumoniea*. The SMGM preparation method was previously described [6]. A single bacterial colony of *S. epidermidis* was inoculated in 100 mL of nutrient broth and incubated at 37°C for 24 h. The growth cultured media were centrifuged at 5000 × *g* for 15 min, and the supernatant was filtered (0.22 µm PES filter). The sterility of the SMGM was evaluated by streaking onto nutrient agar plates. The control SMGM was prepared from non-inoculated nutrient broth.

2.4. Anti-biofilm effect of SMGM

The anti-biofilm effect of SMGM of each skin isolate of *Staphylococcus* (non-*S. aureus*) against the biofilm formation of *K. pneumoniae* using the microtiter plate test, the method described previously [6, 10]. Briefly, the *K. pneumoniae* inoculum was prepared by washing the overnight growth culture of bacteria (bacteria inoculated in tryptic soy broth (TSB) with sterile normal saline. Finally, the absorbance of the bacterial suspension (*K. pneumoniae*) was adjusted to 0.1 at 600 nm (optical density, OD^{600}) using sterile normal saline. Hundred microliters of TSB supplemented with 1% glucose (Hi-media), 15 µL of SMGM, and 10 µL of the *K. pneumoniae* inoculum was added to each well. Furthermore, *K. pneumoniae* biofilm production was examined without any additions (control). After incubation for 24 h at 37 °C, the wells were washed three times with 200 µL normal saline. The plates were dried by incubating for 30 min at 60 °C and stained with 125 µL 0.1% crystal violet for 15 min at 21 °C. Then, the plates were washed three times with 200 µL of distilled water. After drying the wells, 150 µL of 99% ethanol was added. The plates were left at 21 °C for 30 min prior to measuring the optical density at 570 nm. The results were taken in terms of the mean ± standard deviation (SD). The experiment was repeated three times.

2.5. Bacterial planktonic growth

Figure 2 shows the growth curves of *K. pneumoniae* in the presence or absence of $^1/_{16}$ SMGM. A 96-well microtiter plate was used in this experiment. Overnight growth of TSB was washed three times, diluted with sterile TSB, and adjusted to 0.1 at an OD^{600}. In the microtiter plate wells, 100 µL of TSB was added, with (test) or without a $^1/_{16}$ SMGM dilution (control). The absorbance at 600 nm was measured after incubation of microtiter plates for different time intervals at 37°C. The absorbance was measured at 4-h intervals using a microplate reader (BioTek 800, USA). Experiments were performed in triplicate. The effect of SMGM of each *Staphylococcus* (non-*S. aureus*) on biofilm formation of *K. pneumoniae* was evaluated.

2.6. Statistical analysis

Unpaired one-way ANOVA (Origin 8.6 Software, USA). The results were expressed in mean ± standard deviation (SD). Differences were considered statistically significant when values of $P < 0.05$ were obtained.

3. RESULTS

3.1. Non-*S. aureus* isolates (Microbiome isolations)

In the present study, 10 isolates of Gram-positive cocci were isolated and classified as Staphylococci but not S. aureus.

3.2. Effect of SMGM on biofilm formation

The results of the effect of different dilutions of SMGM on the biofilm formation of *K. pneumoniae* are illustrated in Figure 1. All dilutions negatively affected the biofilm formation. The significant reduction of biofilm formation was seen when the *K. pneumoniae* was exposed to the dilutions ½, ¼, $^1/_8$, $^1/_{16}$, $^1/_{32}$, and $^1/_{64}$ SMGM. No significant decrease in the biofilm formation of *K. pneumoniae* exposed to 1/128 of SMGM.

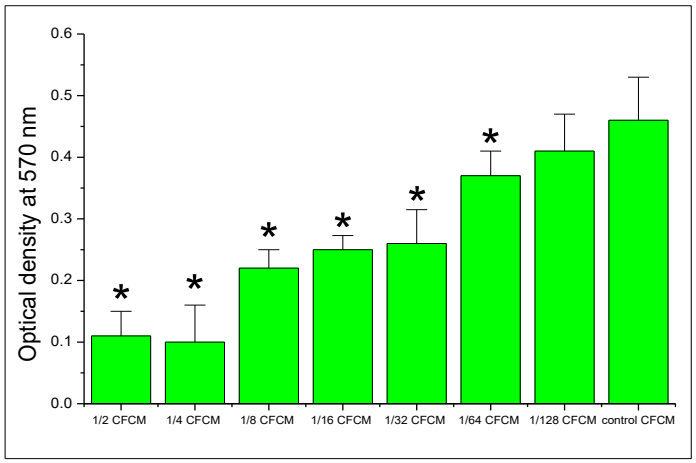

Fig. 1. Effect of different dilutions of Sterile Microbiota Growth Medium (SMGM) of ten *S. epidermidis* isolates that were isolated from the skin samples on the biofilm formation of *K. pneumoniae*. *: <0.05.

3.3. Effect of SMGM on *K. pneumoniae* growth

In the present study, the effect of $^{1}/_{16}$ SMGM from ten isolates of *S. epidermidis* on the growth curve of the planktonic form of *K. pneumoniae* was examined. As shown in Fig. 1, the presence of SMGM significantly inhibited ($p < 0.05$) bacterial growth compared to the control at several time points (4 h, 8 h, 12 h, 16 h, 24 h, and 48 h). While the control culture reached an optical density of approximately 0.7 at 24 h, the SMGM-treated culture showed a reduced growth rate, reaching only an optical density of 0.55. These results suggest that the antagonistic effect of metabolites secreted by *S. epidermidis* into the culture medium has inhibitory activity against *K. pneumoniae* in its planktonic form.

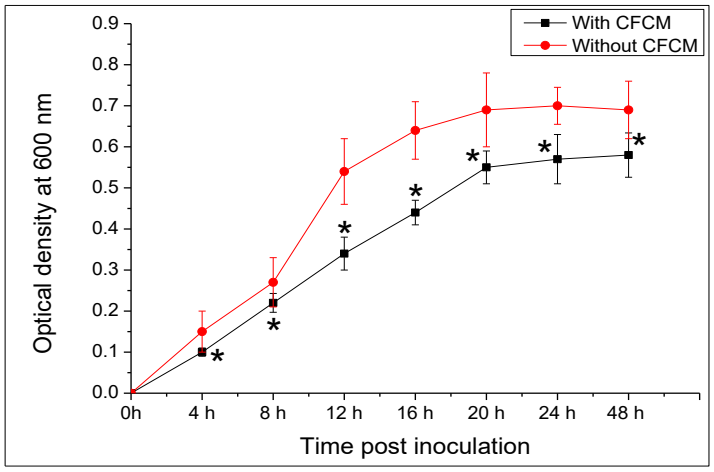

Fig. 2. The time course of inhibition of planktonic growth of *K.* pneumoniae by using Sterile Microbiota Growth Medium (SMGM) of *S. epidermidis*. *: P < 0.05.

4. DISCUSSION

Biofilm formation by pathogenic bacteria is a major virulence factor that contributes to antibiotic resistance and increases the burden on the healthcare system. That is raising treatment costs for bacterial infections [11]. The present study demonstrated that cell-free culture media (SMGM) derived from *S. epidermidis* isolates inhibit the growth and biofilm formation of *K. pneumoniae*. This provides evidence that metabolites secreted by bacteria, especially *S. epidermidis*, have a significant antagonistic effect on both the planktonic form and biofilm development of *K. pneumoniae*. The results also suggest that commensal staphylococcal species might influence the behavior of *K. pneumoniae*, offering new insights for antibacterial discovery.

The most important observation was the ability of SMGM in inhibiting biofilm formation of pathogenic bacteria (*K. pneumoniae*) at different dilutions. This effect was also seen at a high dilution ($^{1}/_{64}$), where a notable inhibition of biofilm formation occurred; however, at a $^{1}/_{128}$ dilution, the inhibitory effect was no longer significant. The results show that *S. epidermidis* secretes metabolites have antibacterial and antibiofilm effects and those effects remain active even at low concentrations. Biofilm inhibition is important because biofilm-associated *K. pneumoniae* infections are difficult to treat because the formation of biofilm is always associated with antibiotic resistance and overcomes the host immune response [12]. By disrupting biofilm formation, SMGM metabolites may prevent the development of chronic and persistent infections, reduce virulence, and enhance susceptibility to antibiotics.

The present study found that SMGM significantly reduced the planktonic growth of *K. pneumoniae*. At a $^{1}/_{16}$ dilution, growth was continuously inhibited at various intervals, unlike the untreated control during analysis. This supports the idea that *S. epidermidis* produces soluble antagonistic factors, which may include bacteriocins, antimicrobial peptides, or metabolic byproducts [13]. Previous research has indicated that coagulase-negative staphylococci like *S. epidermidis* can produce extracellular inhibitors that suppress Gram-positive organisms [6]. This study confirms these findings and suggests that these products may have therapeutic potential.

The ability of commensal organisms to curb pathogenic species through the secretion of metabolites has been reported in previous studies [6,14,15]. It was shown that commensal staphylococci control skin and mucosal microbiota composition by producing antimicrobial peptides that inhibit pathogenic bacteria colonization [6,14]. Here, our findings support the protective role of *S. epidermidis* in microbial communities. These ecological relationships can be used to develop probiotic or postbiotic treatments that take advantage of endogenous inhibitors against pathogens.

However, several limitations are evident in the current study. One of them is that the study was conducted *in vitro*, while the in vivo investigation may provide highly valuable results. The complexity of host environments, for example, immune responses, availability of nutrients, and interactions with microbes, can influence the functionality of pathogenic bacteria [16]. Another limitation of the current study is that we could not identify the exact compounds responsible for these inhibitory activities. Thus, future studies should focus on isolating, purifying, and characterizing such bioactive compounds. Providing the chemical structures of these materials that inhibit biofilm formation and bacterial growth is essential for determining their possible use as drugs in the future [17]. Moreover, using different isolates of S. epidermidis or other skin commensal bacteria isolates may provide more valuable findings.

5. Conclusion

Briefly, our new study demonstrates that SMGM derived from *S. epidermidis* significantly suppresses the growth of planktonic cells (all study dilutions) and biofilm formation (most dilutions except $^{1}/_{128}$) of *K. pneumoniae in vitro*. Our study highlights the potential of commensal staphylococci as a source of novel antimicrobial drugs and underscores the need for further investigation of microbial interactions in human microbiomes to identify alternative

measures for controlling pathogens resistant to a wide spectrum of antibiotics. Additional research examining the identification of bioactive metabolites and their efficacy in animal models can lay the foundation for developing new therapeutics for biofilm infections.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

This study was approved by the Ethics Committee of the University of Baghdad, Baghdad, Iraq (CSEF/1124/0113-1a; November 24, 2024). Since it was a retrospective analysis of routinely collected clinical data, individual patient consent was waived in accordance with national ethical guidelines.

CRediT authorship contribution statement

Barkat Ali Khan: Roles/Writing, Writing–review, Investigation; Project administration; Roles/Writing - original draft; and Writing-review & editing.

Muhammad Azhar Ud Din: Roles/Writing, Writing–review, Investigation.

Mohammed Talab Mohammed: Administration, Resources; Methodology, Supervision; Validation; Roles/Writing, Writing–review, Investigation; Project administration; Roles/Writing - original draft; and Writing-review & editing.

Habib Ullah: Conceptualization, Formal analysis, Writing – original draft.

Maimoona Batool: Formal analysis, Investigation, Writing-review & editing.

All authors have read and agreed to the published version.

Availability of data and materials

Data will be made available on request

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