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Research article

Bacteriocin Produced by *Lactobacillus acidophilus* Exhibits Antagonistic Activity Against Uropathogenic *Klebsiella pneumoniae*

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ABSTRACT

Antibiotic resistance in bacteria is a major problem facing the health system worldwide. The ability of bacteria to form biofilms enhances their resistance to antibiotics. Finding alternative antimicrobial agents is a crucial approach in the global fight against infectious diseases caused by multidrug-resistant bacteria. In this study, yogurt samples were cultured on Man–Rogosa–Sharpe agar (MRS) to isolate *Lactobacillus acidophilus*. Fifty urine samples were collected from patients with urinary tract infections (UTI). Eleven isolates of *Klebsiella pneumoniae* were obtained from these samples. The bacteriocin was extracted from *L. acidophilus* grown in MRS broth. Ammonium sulfate at 70% saturation was used for the partial purification of the bacteriocin. The gel diffusion method evaluated the antibacterial effect of both crude and partially purified bacteriocin on MDR-*K. pneumoniae*, which produced the highest level of biofilm. The ability of the 11 isolates of *K. pneumoniae* to produce biofilm was assessed using the microdilution method in a polystyrene microtiter plate. The results showed that out of eleven isolates, eight were MDR. All isolates produced biofilm at different levels. Both crude and partially purified bacteriocin (at dilutions $^1/_2$, $^1/_4$, $^1/_8$, $^1/_{16}$, and $^1/_{32}$) inhibited the growth of Kp9. The partially purified bacteriocin was more effective than the crude form in inhibiting Kp9 growth. Sub-MICs ($^1/_2$, $^1/_4$, $^1/_8$, and $^1/_{16}$ MICs) of both crude and partially purified bacteriocin inhibited biofilm formation of Kp9. The bacteriocin from *L. acidophilus* has the ability to inhibit MDR-*K. pneumoniae* and its capacity to prevent biofilm formation *in vitro*.

Keywords: Antibiofilm, Antagonism, Bacteriocin, *Klebsiella pneumoniae*, UTI.

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1. INTRODUCTION

Urinary tract infections (UTIs) are associated with a higher rate of morbidity worldwide, and this creates financial and technical pressure on the public health system, especially in developing countries [1,2]. *Klebsiella pneumoniae* is a Gram-negative opportunistic pathogen associated with a high rate of nosocomial UTIs, particularly in immunocompromised patients [3,4]. In recent years, the treatment of these pathogens has undergone significant changes for physicians because antimicrobial resistance (AMR), particularly the emergence of multidrug-resistant (MDR) isolates of *K. pneumoniae*, has increased dramatically. This pathogen has evolved multiple mechanisms to resist antibiotics, including the production of powerful enzymes such as extended-spectrum beta-lactamases (ESBLs) and carbapenemase [5]. The therapeutic options for resistant strains of *K. pneumoniae* are limited; therefore, it is urgent to discover and develop effective, non-traditional antimicrobial agents.

The new research projects focus on the use of probiotic isolates to produce safe and effective antimicrobial molecules. *Lactobacillus acidophilus* is an important member of the human microbiota, present in the gut, and is a widely recognized probiotic that exhibits

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strong antagonistic activity against various pathogens through multiple mechanisms [6]. These mechanisms include acidifying the environment through organic acid production (such as lactic acid) and producing antimicrobial peptides called bacteriocins [7]. Bacteriocins are natural products that are made by different microorganisms and known as novel therapeutics. They (bacteriocins) exhibit a potent, targeted mode of action by disrupting the membrane or cell wall components of pathogenic bacteria, making them less susceptible to multiple antibiotic resistance by reducing the effectiveness of resistant mechanisms employed by bacteria to resist the wide spectrum of antibiotics [8].

This study investigated the antagonistic activity of the bacteriocin produced by *L. acidophilus* against clinical isolates of *K. pneumoniae* from urine samples of patients with UTIs. The study hypothesizes that bacteriocin produced by *L. acidophilus* will exhibit antibiofilm and antibacterial activities against MDR *K. pneumoniae* isolates. This hypothesis is based on a review of the literature [9,10]. The objective of the current study is to examine the above hypothesis by evaluating the antibiofilm and antiseptic effects of the bacteriocin produced by *L. acidophilus* against MDR *K. pneumoniae* isolates that produced the highest level of biofilm in vitro. The findings of the current study will provide evidence for the potential of L. acidophilus-derived bacteriocin as a promising targeted biotherapeutic agent in the battle against antibiotic-resistant *K. pneumoniae* UTIs.

2. MATERIALS and METHODS

2.1. Bacterial Isolates

To isolate *K. pneumoniea*, 50 urine samples were collected from inpatients with UTIs. All patients signed the consent form before sample collection. Midstream urine samples were gathered from inpatients at various public hospitals in Baghdad, Iraq. The urine samples were cultured on MacConkey and nutrient agar. Biochemical tests were performed on mucoid, lactose-fermenting colonies. One milliliter of the yoghurt sample was spread onto De Man–Rogosa–Sharpe agar (MRS agar; pH 5.5; Hi-Media, India) and incubated at 37 ^{o}C in a CO_2 incubator for 24-48 h. Thereafter, smooth, convex, whitish-to-creamy colonies were collected. The Gram stain was performed to characterize bacterial cells. The biochemical tests were performed to assess the ability of bacterial isolates to ferment carbohydrates (glucose, lactose, sucrose, and mannitol) and to produce acid, thereby determining metabolic patterns characteristic of *Lactobacillus* species [10]. The purified isolates were stored for a short period by inoculating them on a slant of nutrient agar and kept at 4°C for 2 weeks. The isolates were stored long-term in nutrient broth containing 20% glycerol at -20 °C for 1 year [4, 9, 10].

2.2. Antibiotic Susceptibility

The Kirby–Bauer method was used to evaluate the susceptibility of *K. pneumoniae* to various antibiotics. Briefly, a standard inoculum of *K. pneumoniae* (10^8 CFU/ml) was spread onto Mueller-Hinton agar (MHA, Himedia) plates. Standard commercial antibiotic discs (six discs were put on each plate). The standard antibiotic discs imipenem (IMP 10 µg), tobramycin (TOB 10 µg), tetracycline (TE 10 µg), vancomycin (VA 30 µg), levofloxacin (LEV, 5 µg), Amikacin (AK 10 µg), gentamycin (GM, 10 µg), ceftriaxone (CRO 10 µg), cefepime (FEP 30 µg) were checked against all isolates of *K. pneumoniea*. The plate was then incubated for 18 h at 37°C. The diameters were compared with the measured diameters of the Clinical and Laboratory Standards Institute (CLSI) breakpoint charts [4].

2.3. Preparation of *L. acidophilus* Bacteriocin

Hundred milliliters of MRS broth were inoculated with 1 mL of a standard inoculum (10^8 CFU/mL) of *L. acidophilus*. The culture was incubated overnight at 37 ^{o}C in a CO_2 incubator. The bacterial cell-free supernatant was prepared by centrifuging the culture medium at 10000 x g for 30 min at 4°C. The supernatant was filtered through a 0.2-µm membrane filter (Millipore filter). Subsequently, the sterility of the supernatant was confirmed by culturing it on MRS agar. Thereafter, they were stored at -20 ^{o}C until use [10].

The bacteriocin in the supernatant of the growth medium was fractioned by ammonium sulphate precipitation (700 g/l) for overnight 4 °C. The precipitate was dissolved in 0.05 M sodium acetate buffer (pH 5.0) and dialyzed against the same buffer using a 1 kDa cutoff membrane at 4°C overnight. The biological activity was assayed against the sensitive isolated bacteria before and after purification. Protein concentration after each purification step was measured [10, 11].

2.4. Well-Diffusion Method

The well diffusion method was used to evaluate the antibacterial effect of crude and partially purified *L. acidophilus* bacteriocin against MDR *K. pneumoniae* isolates. Overnight cultures of MDR *K. pneumoniae* were washed three times and adjusted to 10^8 CFU/ml. Then, 100 µL was spread onto Mueller-Hinton agar (MHA, Himedia, India). Six wells of 6 mm diameter were punched in the MHA plates, and 50 µL of twofold dilutions of the crude and partially purified bacteriocin from *L. acidophilus* were added to each well. The plates were incubated for 24 hours at 37°C. The scale was used to measure the diameter of the inhibitory zones [10].

2.5. Biofilm Formation

The method of Al-Mutalib & Zgair (2023) was followed to evaluate biofilm formation in MDR *K. pneumoniae* isolates. In this method, the flat-bottom polystyrene microtiter plates we filled with 100 µl of sterile Tryptic Soy Broth (TSB, containing 0.2 g/L glucose, HiMedia). Overnight growth of MDR *K. pneumoniae* was washed three times with PBS (0.1, pH 7.2) and adjusted to 10^8 CFU/ml, which is equal to 0.1 at 600 nm (spectrophotometer, Bioevopeak, Jinan, China). Five microliters of *K. pneumoniae* was added to each well. After overnight incubation at 37 °C, the wells were washed three times with sterile distilled water. The wells were stained with Hucker crystal violet (0.4%) after drying. Hundred microliters of anhydrous ethanol were added. The optical density was measured at 590 nm using a microplate reader (BioTek 800 TS; Winooski, USA) [12].

2.6. Effect of Bacteriocin on Biofilm Formation

The anti-biofilm effect of different dilutions of crude and partially purified *L. acidophilus* bacteriocin on the biofilm formation of MDR *K. pneumoniae* isolates was evaluated. A similar method was used to assess biofilm formation, but instead of TSB, twice the dilutions of crude and partially purified *L. acidophilus* bacteriocin were prepared in TSB (Himedia) in the wells of a polystyrene microtiter plate (flat-bottom). The absorbance at 590 nm was measured using a microplate reader (BioTek 800 TS, Winooski, USA). The experiment was repeated three times [12].

2.7. Statistical analyses

In the current study, statistical analysis was performed using Origin v. 8.6 (OriginLab, Northampton, USA). The data were presented as means ± standard error (M ± SE).

3. RESULTS

3.1 Clinical Isolates and Antibiotic Susceptibility

Of 50 urine samples, only 11 *K. pneumoniae* isolates were identified. The Kirby–Bauer method was used to evaluate the susceptibility of *K. pneumoniae* to various antibiotics. The susceptibility of the isolates to different antibiotics were evaluated; imipenem (IMP 10 µg), tobramycin (TOB 10 µg), tetracycline (TE 10 µg), vancomycin (VA 30 µg), levofloxacin (LEV, 5 µg), Amikacin (AK 10 µg), gentamycin (GM, 10 µg), ceftriaxone (CRO 10 µg), cefepime. The results showed that eight isolates were MDR-*K. pneumoniae* (Kp1, Kp2, Kp5, Kp6, Kp7, Kp8, Kp9, and Kp10).

3.2 Biofilm

The biofilm was quantified by measuring the absorbance of the released crystal violet after the addition of absolute ethanol. The quantity of the biofilm corresponded to the absorbance of optical density at 590 nm. The results showed that isolates differed in their ability to form biofilm, with OD values ranging from 0.22 to 1.15. Kp9 showed the highest biofilm formation, indicating strong biofilm-forming ability. However, isolates such as Kp1, Kp4, and Kp11 showed weak biofilm formation. Most isolates demonstrated low standard deviations, suggesting high reproducibility of the assay. Notably, several MDR *K. pneumoniae*, including Kp2, Kp6, and Kp9, were moderate to strong biofilm producers. The results confirm a notable potential association between multidrug resistance and enhanced biofilm formation. Based on the results of the current study, isolate Kp9 was used in subsequent experiments and has shown resistance to a wide spectrum of antibiotics, producing the highest biofilm levels (a strong biofilm producer) (Fig. 1).

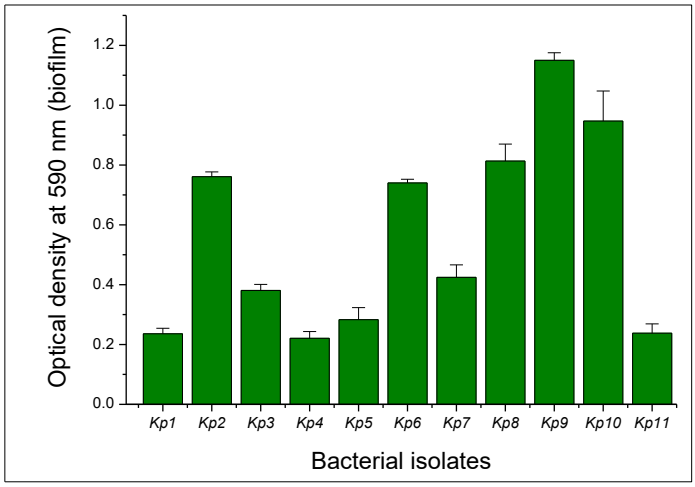

Fig. 1. Biofilm formation of 11 isolates of *K. pneumoniae*. The biofilm was quantified by measuring the absorbance of crystal violet released upon ethanol addition.

3.3 Bacteriocin antibacterial effects

To assess the effectiveness of bacteriocin in both preparations (crude and partially purified) on bacterial growth, the specific activity of bacteriocin was adjusted to an equal value after determining the particular activities of the crude and partially purified preparations, and protein levels were also assessed. The results showed that the antibacterial activity of the purified bacteriocin was higher than that of the crude bacteriocin (specific biological activity) at the ½, ¼, 1/8, 1/16, and 1/32 dilutions of both crude and partially purified bacteriocin. No antibacterial activity was seen in the high dilutions of crude and partially purified bacteriocin ($^1/_{64}$, $^1/_{128}$, and $^1/_{256}$ (Fig.2).

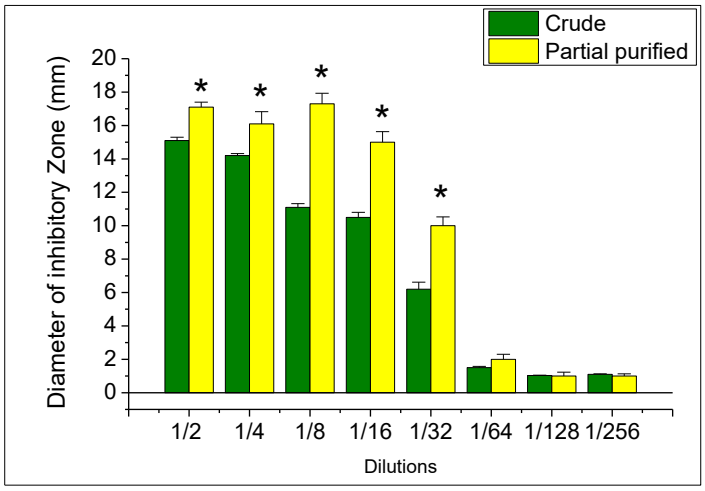

Fig. 2 Effect of different dilutions of crude and partially purified bacteriocin produced by *L. acidophilus*. Both have antibacterial effects against *K. pneumoniae* (Kp9) with different diameters. The partially purified preparation showed higher antibacterial activity than the crude bacteriocin (both had the same protein concentration). Asterisks indicate a significant difference from the diameter of the inhibitory zone produced by the effect of crude bacteriocin ($P<0.05$).

3.4 Antibiofilm effect of Bacteriocin

Fig. 3 shows the antibiofilm effect of sub-inhibitory concentrations (sub-MICs) of crude and partially purified bacteriocin on the biofilm formed by *K. pneumoniea* that produced the highest level of biofilm (Kp9). Both crude and partially purified bacitracin, prepared from *L. acidophilus*, caused a concentration-dependent reduction in biofilm formation. The partially purified bacteriocin shows higher inhibitory activity at higher concentrations. In the current study, one-way ANOVA followed by Tukey's post hoc test was applied; the statistical analysis showed significant differences relative to the control, Kp9 treated with TSB only ($P<0.05$). No significant difference between the effect of crude and partially purified bacteriocin on the ability of *K. pneumoniae* to form biofilm *($P>0.05$)*.

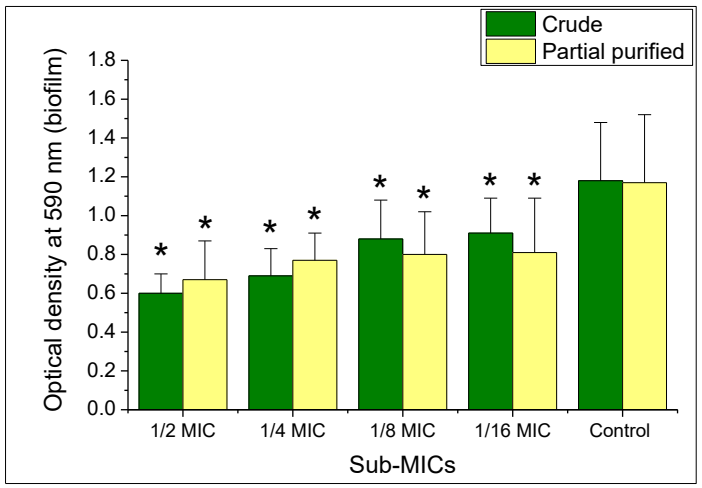

Fig. 3 Impact of different sub-inhibitory concentrations of crude and partially purified bacteriocin on biofilm formation of *K. pneumoniae* Kp9. The biofilm was quantified by measuring the absorbance of crystal violet staining at 590 nm. The control represents the biofilm formation of Kp9 treated with TSB only. Asterisks indicate a significant difference from the corresponding control.

4. DISCUSSION

The formation of biofilms leads to several clinical issues, including increased resistance of bacterial isolates to a wide spectrum of antibiotics [2, 12, 13]. The produced biofilm creates a physical barrier that reduces antibiotic permeability and the ability of antibiotics to enter bacterial cells [14]. The present study investigates the effect of a bacteriocin produced by *L. acidophilus* on biofilm formation by *K. pneumoniae* isolated from urine samples of patients with severe UTIs. The antibacterial impact of the different dilutions of the bacteriocin against the MDR *K. pneumoniae* that produced the highest level of biofilm on the polystyrene microtiter plate. The present study may provide a new concept for the potential use of bacteriocins as novel antimicrobial agents.

The current study showed that, among the 11 *K. pneumoniae* isolates from urine samples, 8 were MDR. This finding highlighted a very critical issue of the elevation of the MDR *K. pneumoniae* that is responsible for the UTIs, especially in Iraq. This occurs due to the widespread use of antibiotics, and most antibiotic treatments are not under the supervision of specialized physicians within hospitals [15]. The most important thing that these isolates were resistant to a broad panel of antibiotics, including imipenem, levofloxacin, and third-generation cephalosporins (ceftriaxone), highlights the critical need for alternative therapeutic strategies.

It was also observed that the isolated *K. pneumoniae* produced biofilm in a different range (0.22-1.15). The highest biofilm-forming bacteria also produced the highest biofilm mass, confirming the role of biofilm in conferring bacterial resistance to a wide spectrum of antibiotics. The Kp9 isolate that produced the highest biofilm level was resistant to the most antibiotics included in the current study; therefore, this isolate was used in subsequent studies in the current project. It is well known that biofilms are among the most important virulence factors in bacteria, which shield bacteria from antibiotics and the host immune system [16]. These types of bacteria may be responsible for chronic UTIs. That is why most *K. pneumoniae* isolates produced a high level of biofilm, owing to their resistance to antibiotics and their ability to overcome the host immune response [17].

The most notable finding is the antagonistic effect of *L. acidophilus* bacteriocin against the highly virulent Kp9 isolate. It was found that the *L. acidophilus* bacteriocin inhibits bacterial growth in a concentration-dependent manner, and that the partially purified form was more effective than crude protein at the same protein concentration. This indicates that the purified, effective material, attributed to a protein-based structure agent, was highly effective and can be further purified to yield an antimicrobial agent that can be used against highly resistant bacterial strains. The previous study supports our study, which reported that the metabolic extract of Lactobacillus has antibacterial and antibiofilm properties [9,10]. Other studies have also highlighted that the extracellular materials of different bacterial species exhibit antibacterial and antibiofilm activities [18]. The limitation of the current study is the small number of isolates (eleven) involved. Additionally, the study needs to examine the fully purified bacteriocin. An in vivo study may help improve the study's objectives.

The findings are limited by the small number of clinical isolates (N=11) and the partial purification of the bacteriocin, meaning the full *in vivo* efficacy and intrinsic activity require further comprehensive testing and structural elucidation.

This study opens the door to future research on the potential use of *L. acidophilus* metabolic extracts as a novel antibacterial agent. Moreover, these extracts can be used as a synergistic agent with other antibiotics to improve the activity of the antibiotic against pathogenic bacteria that are resistant to a wide spectrum of antibiotics. To improve the plan, the *in vivo* study is highly recommended.

5. CONCLUSION

The present study shows that a high percentage of *K. pneumoniae* isolates from UTI urine samples demonstrate multidrug resistance (MDR). Antibiotic-resistant isolates produce the highest levels of biofilm biomass. The metabolic cell extract of *L. acidophilus* has antibacterial and antibiofilm properties against *K. pneumoniae* isolates. The present study provides strong evidence for the potential use of this agent as an antibacterial and antibiofilm agent, based on several in vivo, cytotoxicity, and other clinical and biochemical experiments.

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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 (CSEC/1124/0098; November 8, 2024), since it was a retrospective analysis of routinely collected clinical data; individual patient consent was waived in accordance with national ethical guidelines and the Declaration of Helsinki.

CRediT authorship contribution statement

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

Zainab Hani Hatif: Roles/Writing, Writing–review, Investigation; Project administration; Roles/Writing - original draft; and 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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