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Inhibitory Effect of Bacteriocin on Antibiotic-Resistant Pseudomonas aeruginosa Isolated From patients in local hospitals

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Abstract

Pseudomonas aeruginosa is a Gram-negative opportunistic bacterium that is usually linked with wound and burn infections. The rising incidence of antibiotic resistant isolates has necessitated the search for new antimicrobial agents, including bacteriocins from lactic acid bacteria. The current investigation was aiming to investigate the antibacterial activity of concentrated partly purified bacteriocin from *Lactobacillus fermentum* against antibiotic resistant clinical isolates of *Pseudomonas aeruginosa*. A total of 200 clinical samples were obtained from patients with wound and burn infections from three hospitals in Iraq. Bacterial isolates were cultivated on nutrient agar , MacConkey agar and cetrimide agar and probable *P. aeruginosa* isolates were verified using VITEK 2 Compact system. Antibiotic susceptibility test was conducted using Kirby–Bauer disk diffusion technique on Mueller–Hinton agar as per CLSI standards. Bacteriocin from *L. fermentum* was concentrated and partly purified by ammonium sulfate precipitation, dialysis and DEAE-cellulose ion-exchange chromatography. Ten MDR *P. aeruginosa* isolates were chosen for screening the antibacterial activity of the active bacteriocin containing fraction utilizing agar well diffusion technique. Wells with a diameter of 8 mm were filled with 100 µL of bacteriocin fraction and the inhibition zones were measured in mm. Data were evaluated using one way ANOVA. *P. aeruginosa* was found in 83/200 (41.5%) clinical samples. The greatest rates of resistance were to Aztreonam 69 (83.1%) and Ceftazidime 53 (63.9%). The active bacteriocin fraction after DEAE cellulose chromatography has an activity of 320 AU/mL, protein concentration of 0.006 mg/mL, specific activity of 53,333 AU/mg protein, purification fold of 4.7 and yield of 81.6%. The bacteriocin showed varying levels of antibacterial activity against the chosen MDR isolates with inhibition zones ranging from 0.00±1.00 mm to 17.00±1.00 mm. The maximum susceptibility was against isolate P53 and no zone of inhibition was seen against isolates P52 and P71. One way ANOVA analysis showed substantial variation in bacteriocin activity among the tested isolates ($F = 121.08$, $p < 0.001$). The partly purified bacteriocin of *Lactobacillus fermentum* showed antibacterial activity against several of the MDR clinical isolates of *P. aeruginosa*. The results of the present study show that the purified bacteriocin fraction might be a suitable alternative or complementary antibacterial treatment for resistant *P. aeruginosa* isolates.

Keywords: Antibiotic resistance, burn infection, bacteriocin, MDR, wound infection, *Pseudomonas aeruginosa*

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Introduction

Pseudomonas aeruginosa, a Gram-negative, aerobic, rod-shaped bacterium is known for its comparatively large genome and high degree of genomic plasticity that together endow it with extraordinary ability to adapt to many ecological niches and resist multiple environmental- and physical-stressing conditions [1]. Clinically, *P. aeruginosa* being a pathogen of high medical importance that is strongly correlated with increased morbidity and mortality in patients with compromised immune defenses or pre-existing chronic diseases such as cystic fibrosis, malignancies, acquired immunodeficiency syndrome (AIDS), bronchiectasis, non-healing chronic wounds and recurrent urinary tract infections [2]. Antibiotic resistance is a major problem in treating and/or preventing illnesses caused by this bacterium. Multiple antibiotic resistance mechanisms, including efflux pump systems, have been demonstrated in *P. aeruginosa*, which can develop multidrug resistance [3]. In *P. aeruginosa*, the overexpression of efflux pumps has been suggested to be one of the main mechanisms responsible for multidrug resistance [4,5]. These pumps are a subclass of membrane-associated transport proteins that function in the energy-dependent efflux of diverse substrates from the bacterial cytoplasm to the extracellular environment [6]. Bacteriocins, in contrast, represent a separate class of ribosomally synthesized anti-microbial peptides made by bacteria that kill or inhibit both evolutionarily related and unrelated bacterial strains but do not harm the producing organism — via specific immunity proteins [7]. Gram-positive and Gram-negative bacterial are both capable of formation diverse range of bacteriocins, allowing the producing strains to inhibit growth of sensitive competitors. Such bactericidal activity is often viewed as an evolutionarily advantageous tactic that ultimately aids in niche dominance by decreasing competitive population levels and increasing their own access to locally available nutrients/spatial resources in the environment. One of the most striking differences between bacteriocins and traditional antibiotics relates to their biosynthetic origin; unlike most antibiotics that serve as secondary metabolites, bacteriocins are ribosomally synthesized products that are subject to protease degradation but largely non-toxic to the human host and wider ecosystem [8].

Materials and Methods

Study design and sample collection:

A total of 200 clinical specimens were collected from patients from burn and wound infections in three Iraqi hospitals such as Zaafaraniya General Hospital, Al-Kindi Teaching Hospital, and Ghazi Al-Hariri Hospital, during the period from 18 November 2025 to 3 March 2026. Samples were collected aseptically using sterile cotton swabs and transported to the laboratory as soon as possible for microbiological processing. The collected swabs were inoculated onto nutrient agar plates and incubated aerobically at 37°C for 24 h for primary bacterial isolation [9].

Identification of bacterial isolates

The clinical samples were cultivated on nutrient agar and MacConkey agar plates and incubated aerobically at 37°C for 24h. Potential colonies were chosen according to their cultural features, such as non-lactose-fermenting colonies on MacConkey agar and characteristic growth on cetrimide agar as a selective medium for *P. aeruginosa*. The bacterial isolates were identified and confirmed using the VITEK 2 Compact system according to the manufacturer's instructions. The selected isolate was identified as *Pseudomonas aeruginosa* with an identification probability of 93% [10].

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Antibiotic susceptibility test

The antibiotic susceptibility testing was done using the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar to assess the antibiotic resistance profile of the isolated *Pseudomonas aeruginosa*. Fresh bacterial culture was employed for preparing the bacterial suspension in sterile normal saline. The turbidity of the suspension was corrected visually to the 0.5 McFarland standard by comparison with a commercially manufactured McFarland turbidity standard under acceptable light condition. This process was done to ensure a consistent bacterial inoculum before inoculating the plates.The modified bacterial suspension was uniformly dispersed over the whole agar surface using sterile cotton swabs to inoculate the Mueller–Hinton agar plates. A few minutes after letting the agar surface to dry, antibiotic discs were aseptically put on the inoculated agar surface using sterile forceps. Plates were incubated aerobically at 37°C for 24 h. After incubation, the widths of the inhibition zones surrounding the antibiotic discs were measured in millimeters using a ruler and evaluated as sensitive, intermediate or resistant based on the CLSI recommendations (CLSI, 2025).

Table (1). List of the Antibiotic Discs Used in This Study According to (CLSI, 2025) guidelines

No.	Antibiotic	Abbreviation	Concentration (µg)
1	Amikacin	AK	30
2	Ceftazidime	CAZ	30
3	Ciprofloxacin	CIP	5
4	Aztreonam	ATM	30
5	Meropenem	MRP	10
6	Tetracycline	TE	30
7	Gentamicin	GEN	10
8	Trimethoprim / Sulfamethoxazle	COT	25
9	Piperacillin/ Tazobactam	PIT	100/10
10	Levofloxacin	LE	5
١١	Norfloxacin	NX	10

Preparation of Bacteriocin from *Lactobacillus fermentum*

In this investigation a concentrated partially purified preparation of bacteriocin from *Lactobacillus fermentum* was employed. *L. fermentum* was grown in de Man, Rogosa and Sharpe (MRS) broth and incubated at 37°C for 24 h in anaerobic conditions in an anaerobic jar. After incubation, the culture was centrifuged at 4000 rpm for 15 min at room temperature to remove bacterial cells and get the cell-free supernatant. The cell-free supernatant obtained was neutralized to pH 7.0 with sterile NaOH to remove the inhibitory action of organic acids.The supernatant was then neutralized and precipitated with protein by addition, with constant stirring until completely dissolved, of solid ammonium sulfate at 116 g/200 mL of supernatant (80% saturation). Proteins were precipitated by keeping the mixture at 4°C overnight. The precipitated protein was pelleted by centrifugation at 4000 rpm for 15 min and dissolved in sterile phosphate buffer.The redissolved protein fraction was put into dialysis tubing and dialyzed against phosphate buffer at 4°C for 24 h to eliminate excess ammonium sulfate. The dialysis buffer was exchanged throughout the dialysis phase to enhance the elimination of salts. After dialysis partial purification was done by DEAE-cellulose ion-exchange chromatography on a 30 cm column. The column was equilibrated and rinsed with phosphate buffer. The bound bacteriocin-containing proteins were eluted using 0.25 N NaCl prepared in 0.1 M Tris-HCl buffer. The pH of the Tris-HCl buffer was adjusted using 0.25 N NaOH or 0.25 N HCl when required. The eluted fractions

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were collected separately and were evaluated for antibacterial activity by agar well diffusion technique. Fractions with antibacterial activity were active bacteriocin containing fractions. The active fractions were kept at −20°C for one day before testing for antibacterial activity against several antibiotic resistant clinical isolates of *Pseudomonas aeruginosa*.The activity of bacteriocin was given in arbitrary unit/ml (AU/ml). The protein concentration was measured and represented in mg/ml. Specific activity was reported as a ratio of bacteriocin activity (AU/mL) to protein concentration (mg/mL) expressed as AU/mg protein. The overall activity was determined as the product of the bacteriocin activity (AU/mL) and the total volume of each component. Purification fold was calculated by dividing the specific activity of each purification step to that of the crude bacteriocin preparation and yield percentage was calculated by dividing the total activity of each purification step to that of the total activity of the crude preparation and multiplying by 100 [11].

Effect of Bacteriocin on *Pseudomonas aeruginosa*

The antibacterial activity of the concentrated partly purified bacteriocin from *Lactobacillus fermentum* was tested against selected antibiotic resistant clinical isolates of *Pseudomonas aeruginosa* by agar well diffusion technique . The isolates chosen were activated in Brain Heart Infusion broth and incubated at 37°C for 24 h. The bacterial suspension after incubation was compared visually with a commercially available McFarland turbidity standard under acceptable lighting circumstances to reach the 0.5 McFarland standard and to get a standardized bacterial inoculum [12]. Inoculation of prepared bacterial suspension on Mueller–Hinton agar plates using sterilized cotton brushes. Wells of 8 mm diameter were aseptically punched in the agar using a sterile cork borer. 100 µL of the concentrated partly purified bacteriocin fraction was poured into the wells. The plates were allowed to diffuse at room temperature and then incubated aerobically at 37°C for 24 h. The antibacterial activity was evaluated by measuring the inhibitory zone diameter surrounding the wells in millimeters [13].

Statistical Analysis

Data were analyzed using Microsoft excel and SPSS software, 16. Antibiotic susceptibility pattern was reported as frequency and proportion of resistant, intermediate and sensitive isolates. The antibacterial activity of the concentrated partly purified bacteriocin against the chosen antibiotic resistant clinical isolates of *Pseudomonas aeruginosa* was determined by measurement of inhibition zone diameters in millimeters. All bacteriocin activity assays were performed in independent triplicates, with results expressed as mean values accompanied by their corresponding standard deviations (± SD). Comparative analysis of inhibitory zone diameters across the tested isolates was carried out using one-way analysis of variance (ANOVA), whereby a p-value threshold of < 0.05 was adopted as the criterion for statistical significance.

Results and Discussion

The results showed that *Pseudomonas aeruginosa* was found in 83 isolates (٤١.0%) out of 200 clinical samples. Also, 48 (24.0%) of the isolates were identified as *Escherichia coli*, 34 (17.0%) were identified *as Klebsiella pneumoniae* and 10 (5.0%) were identified as *Staphylococcus aureus*. The other 25 isolates (١٣.0 %) were recognized as various bacterial species as illustrated in Fig. 1. The results showed *P. aeruginosa* was the most common bacterial isolate from the clinical samples examined (83 isolates from 200 samples). This finding highlights the important function of *P. aeruginosa* as an opportunistic bacterium associated with wound and burn infections especially in hospital environments [14].

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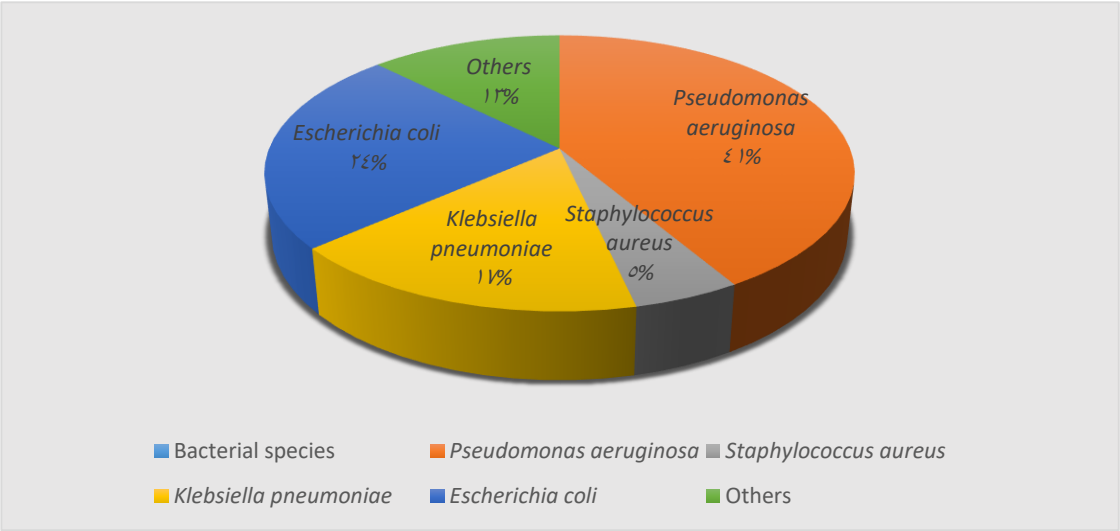

Figure (1). Distribution of bacterial isolates recovered from clinical samples.

The antibiotic susceptibility data indicate that the greatest resistance rate of *Pseudomonas aeruginosa* isolates was to aztreonam with 69 isolates (83.1%) followed by ceftazidime with 53 isolates (63.9%). Lower resistance rates were detected against levofloxacin and ciprofloxacin consisting of 3 isolates (3.6%) and 4 isolates (4.8%), respectively. On the contrary, the maximum sensitivity was shown against levofloxacin 78 (94.0%), meropenem 75 (90.4%), and norfloxacin 73 (88.0%).

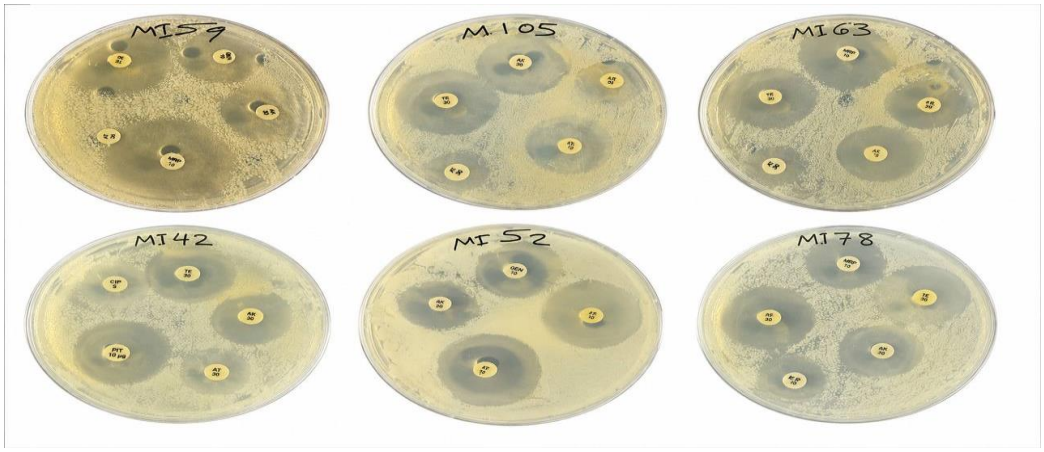

Figure (2). Antibiotic susceptibility test of *Pseudomonas aeruginosa* isolates on Mueller–Hinton agar using the Kirby–Bauer disk diffusion method after incubation at 37°C for 24 h.

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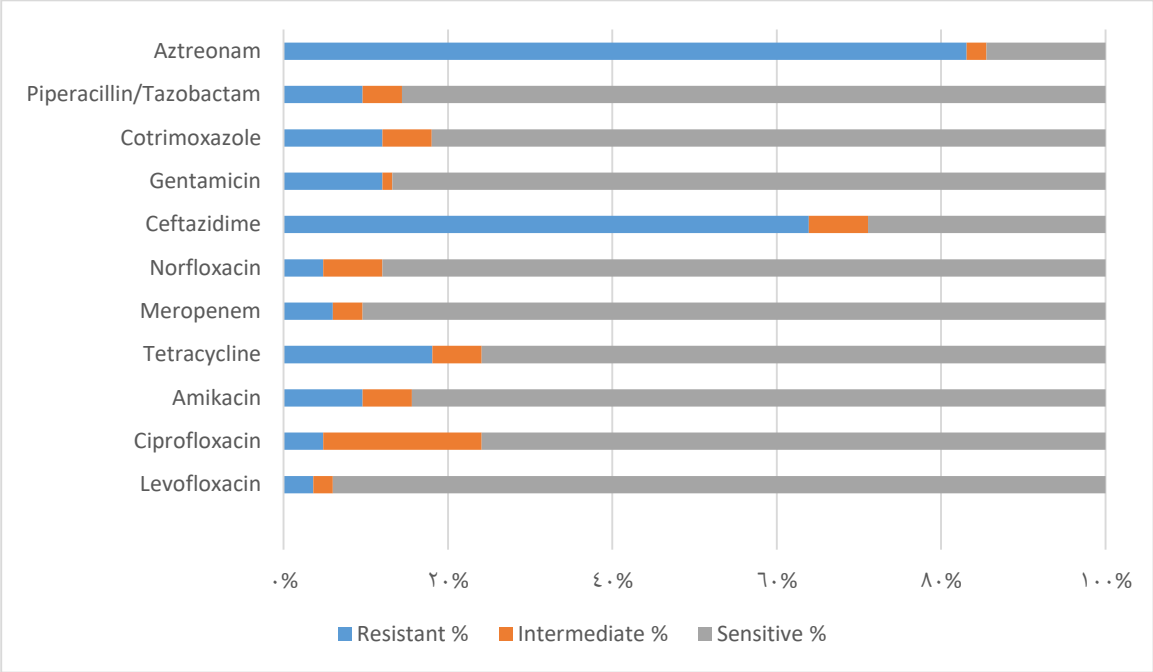

Figure (3). Antibiotic susceptibility pattern of *Pseudomonas aeruginosa* isolates against different antibiotics

Antibiotic susceptibility data of tested *P. aeruginosa* isolates indicated different resistance patterns. The maximum resistance was seen with aztreonam followed by ceftazidime. This observation may be attributable to the remarkable capacity of *P. aeruginosa* to acquire and deploy multiple resistance mechanisms, encompassing the enzymatic degradation of β-lactam antibiotics via β-lactamase production, reduction in outer membrane permeability, structural modification of antibiotic target sites, and the upregulation of efflux pump system [١٥,١٦]. Some antibiotics have high sensitivity rates, especially levofloxacin, meropenem, norfloxacin, gentamicin and piperacillin/tazobactam, however the existence of isolates resistant to various antibiotics is of clinical importance. Multidrug-resistant (MDR) isolates were defined as isolates showing resistance to at least one antimicrobial agent in three or more antimicrobial categories. Based on this definition, the most resistant *P. aeruginosa* isolates were selected for bacteriocin activity testing.Ten isolates of MDR were chosen on the basis of resistance profile to assess the antibacterial activity of bacteriocin [١٧]. The significance of this selection is that evaluating bacteriocin against highly resistant isolates is a stronger indicator of its potential value as an alternate or supporting antimicrobial agent [١٨, ١٩].

Table (2). The ten most antibiotic-resistant *Pseudomonas aeruginosa* isolates selected for bacteriocin activity testing.

Isolate No.	Resistant antibiotics	MDR
P79	5/11	
P52	4/11	
P68	4/11	
P34	3/11	
P٥١	3/11	MDR

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P64	3/11	
P71	3/11	
P53	3/11	
P27	3/11	
P46	3/11	

The purification efficiency of bacteriocin from *Lactobacillus fermentum* was examined by measuring bacteriocin activity, protein concentration, specific activity, total activity, purification fold and yield at each stage of the purification process . The crude production of bacteriocin had a volume of 250 mL, an activity of 80 AU/mL, a protein content of 0.007 mg/mL, a specific activity of 11,429 AU/mg protein, and a total activity of 20,000 AU. After ammonium sulfate precipitation, the volume was lowered to 18 mL, activity rose to 160 AU/mL, protein concentration was 0.020 mg/mL, specific activity was 8,000 AU/mg protein, total activity was 2,880 AU, purification fold was 0.7, and yield was 14.4%. The active fraction after DEAE-cellulose ion-exchange chromatography has a volume of 51 mL, 320 AU/mL activity, 0.006 mg/mL protein concentration and the greatest specific activity of 53,333 AU/mg protein. The total activity following DEAE-cellulose chromatography was 16320 AU with a purification fold of 4.7 and 81.6% yield. The bacteriocin preparation was concentrated around 4.9-fold by volume reduction from 250 mL crude supernatant to 51 mL of active purified fraction. The results show that DEAE-cellulose ion-exchange chromatography yielded a more efficient purification of the bacteriocin-containing fraction and boosted its specific activity The increase in specific activity following DEAE-cellulose ion-exchange chromatography demonstrates that this phase of purification was efficient to enhance the active bacteriocin-containing fraction and to reduce the non-active protein components. The increased activity of bacteriocin after purification could be caused by the elimination of interfering contaminants such as salts, residual medium components and unrelated proteins which may lower the apparent antibacterial activity in the crude formulation. Ammonium sulfate precipitation is a good method for concentrating proteinaceous antibacterial substances, but it may also precipitate other proteins which may account for the decreased purifying efficiency obtained after this step. In contrast, DEAE-cellulose chromatography shows greater separation of active fraction, giving higher specific activity and better purifying efficiency. These results suggest the need for a combination of precipitation, dialysis, and ion-exchange chromatography to create a more active and partly purified bacteriocin preparation suited for antibacterial testing against MDR *Pseudomonas aeruginosa* isolates [23] .

Table (3). Activity and Total Activity

Step	Volume (mL)	Activity (AU/mL)	Protein concentration (mg/mL)	Specific activity (AU/mg)	Total activity (AU)	Purification fold	Yield (%)
Crude	250	80	0.007	11,429	20,000	1	100
Precipitation by Ammonium sulfate	18	160	0.02	8,000	2,880	0.7	14.4
Purification by ion-exchange chromatography (DEAE-Cellulose)	51	320	0.006	53,333	16,320	4.7	81.6

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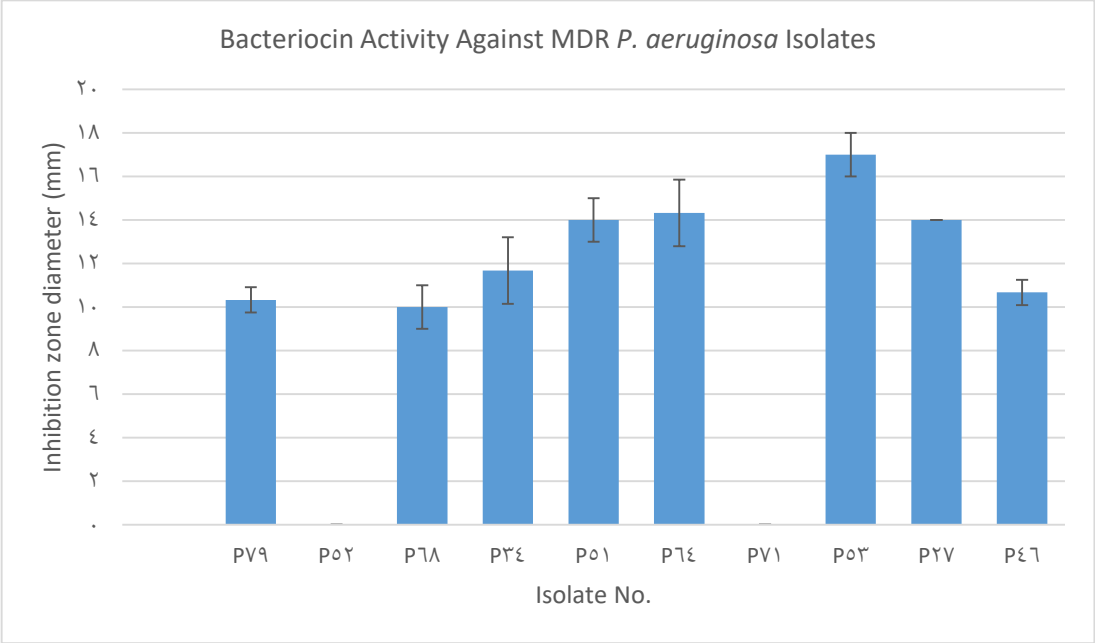

Figure (4). Antibacterial activity of bacteriocin against selected MDR *Pseudomonas aeruginosa* isolates. Values are means ± SD of three replicates.

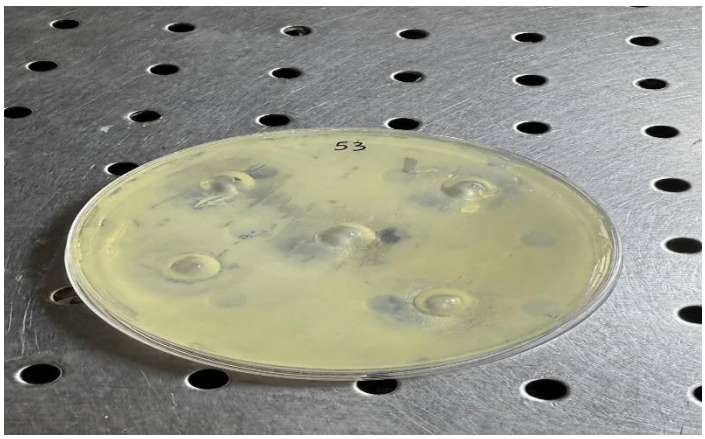

Figure (5). Antibacterial activity bacteriocin from *Lactobacillus fermentum* against MDR *Pseudomonas aeruginosa* isolate P53 characterized with agar well diffusion method.

The antibacterial activity of concentrated partly purified bacteriocin from *Lactobacillus fermentum* against ten chosen MDR isolates of *Pseudomonas aeruginosa* was determined using agar well diffusion technique. The diameter of the inhibitory zones was measured in mm and represented as mean ± SD of three experiments (Fig. 4). Inhibition zones varied from 0.00 ± 0.00 mm to 17.00 ± 1.00 mm. The maximum

antibacterial activity was obtained against isolate P53. However, no inhibition zone was seen against isolates P52 and P71. The bacteriocin exhibited varied antibacterial activity against the tested MDR *P. aeruginosa* isolates [20].The variability in sensitivity across isolates might be related to the changes in cell envelope shape, membrane permeability, resistance mechanisms or the capacity of certain isolates to tolerate antimicrobial peptides [21,22]. The antibacterial activity reported with majority of the tested MDR *Pseudomonas aeruginosa* isolates may be explained by many plausible processes relating to the proteinaceous structure of bacteriocin and its interaction with the bacterial cell membrane. *P. aeruginosa* is protected by an outer membrane as a Gram-negative bacteria. Still, the partly pure bacteriocin may suppress it by an interaction with surface receptors, outer membrane components, or membrane-associated proteins. This contact may disrupt the integrity of the bacterial envelope, enhance membrane permeability, and promote leakage of internal resources such as ions, nucleotides, and other important cellular components. Thus, ion equilibrium disturbance, loss of membrane potential and disruption of vital metabolic functions might occur, resulting in decreased bacterial proliferation and inhibitory zones surrounding the wells.The difference in inhibitory zone sizes between the studied MDR isolates may be explained by the outer membrane composition, lipopolysaccharide structure, membrane permeability, efflux pump activity and the existence of resistance-related mechanisms particular for each isolate. Strains having a less effective permeability barrier or greater sensitivity to antimicrobial peptides may enable a stronger contact of the bacteriocin with the bacterial surface, resulting in bigger inhibition zones. In contrast, isolates P52 and P71 did not produce any inhibitory zones, perhaps indicating a lower sensitivity to the bacteriocin preparation. This reduction in response might be the result of better outer membrane protection, lesser availability of appropriate surface binding sites, active efflux systems, or other adaptive resistance mechanisms that restrict bacteriocin access to its target site. Therefore, the inhibitory impact of bacteriocin was not uniform across the tested MDR isolates but was isolate-dependent. across the tested isolates, isolate P53 was the most susceptible to the concentrated partly purified bacteriocin [23,24]. Statistical analysis revealed substantial variation in inhibitory zone widths among the isolates tested. One-way ANOVA showed a significantly significant variance in bacteriocin activity among selected MDR *P. aeruginosa* isolates ($F = 121.08$, $p < 0.001$). This suggests that the reaction of the tested isolates to bacteriocin was not consistent and some isolates were more responsive than others. The present investigation concluded that the concentrated form of partly purified bacteriocin from *Lactobacillus fermentum* showed antibacterial activity against several MDR clinical isolates of *P. aeruginosa* [25].

Conclusion

The most isolated bacterial species from wound and burn samples in the present investigation was *Pseudomonas aeruginosa.* The isolates exhibited different patterns of antibiotic resistance with highest resistance to aztreonam and ceftazidime. Ten MDR *P. aeruginosa* isolates were chosen based on their resistance profile to investigate antibacterial activity of concentrated partly purified bacteriocin from *Lactobacillus fermentum*. The bacteriocin demonstrated different antibacterial activity against the chosen MDR isolates with inhibition zone from 0.00 ± ١.00 mm to 17.00 ± 1.00 mm. Isolate P53 exhibited maximum activity whereas isolates P52 and P71 showed no inhibition. Statistical analysis indicated substantial differences of tested isolates in bacteriocin activity. Our data indicate that the concentrated partly purified bacteriocin from *L. fermentum* might be a possible alternative or supporting antimicrobial agent against MDR *P. aeruginosa*. Further research is suggested to identify MIC values, to test various concentrations of bacteriocin, to include adequate control groups, and to study the mechanism of action. These data collectively underscore the significant potential of concentrated partially purified bacteriocin from *L. fermentum* as a novel therapeutic agent, presenting a promising avenue to combat the escalating challenge of multidrug-resistant *P. aeruginosa* infections.

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Author's Declaration

- We certify that all the Tables included in the document are original and developed by us.
-The research was authorized by the Medical Ethics Committee of Al-amal National Hospital for Cancer Treatment ethical review committee (No. REC.COB/2005/24 dated 20/5/2026). All members provided informed composed assent following checking on the review portrayal. [College of Biotechnology/Al-Nahrain University].

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