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Study of antibacterial and antibiofilm activity of Bacteriocin in patients with *Acinitobacter baumannii* chest infection

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Abstract

Acinetobacter baumannii is a Gram-negative opportunistic bacterium associated with nosocomial and community-acquired illnesses. This pathogen is rapidly expanding on a global scale and is linked to the development of resistance against numerous antibiotics. Therefore, this research aims to investigate the activities of bacteriocin as antibacterial and antibiofilm against *Acinetobacter baumannii*. Sputum specimens were collected from sixty patients with respiratory tract infections admitted to the respiratory care unit in three hospitals in Baghdad. *Acinetobacter baumannii* was isolated and identified using biochemical tests, and the diagnosis of the isolates was validated. An antibiotic susceptibility test was conducted using the VITEC 2 systems. Biofilm formation was detected using a microtiter plate assay. Bacteriocin extract from Lactobacillus was employed to assess antibiofilm activity via the microtiter plate method, while antibacterial activity was determined using the agar well diffusion method. The results showed that the prevalence of *Acinetobacter baumannii* was 30% among Iraqi samples. In this study, *Acinetobacter baumannii* showed higher overall resistance to most antibiotics (100%). In addition, the overall sensitivity for colistin antibiotic was (100%), accompanied with good inhibitory effect against *A. buamannii* growth that associated with a definitive effect on biofilm production by this bacteria. In conclusion, the study approved the validity of bacteriocin against *Acinetobacter baumannii*. The authors suggests the possibility of utilization of bacteriocine as effective adjuvants and bio control agents for treatment and eliminating multidrug bacteria using the novel technique.

Keywords:- *Acinetobacter baumnnii*, biofilm formation, antibiotic resistance, Bacteriocin.

Introduction

Acinetobacter baumannii is a gram-negative (G-ve), coccobacillus, strictly aerobic, catalase positive, negative oxidase and urease tests, nonmotile, and have ability to grow at both 37°C and 44°C, and non-lactose fermentative opportunistic pathogen (1). *A. baumannii* is regarded as a lethal pathogen responsible for both community-acquired and nosocomial infections. It is one of the six most significant multi-drug resistant bacteria (MDR) in hospitals globally. Consequently, high number of antibiotic resistant *A. baumannii* reduces effective therapies and raises mortality (2). *A. baumannii's* ability to form biofilm on abiotic and biotic

surfaces is the most essential characteristic that contributes to chronic and persistent, resistance to antimicrobial agents, and its ability to survive in the hospital setting, particularly in intensive care units (ICUs) (3). Strains of *A. baumannii* has the ability to produce the biofilm on the surfaces of endotracheal tube, which may present a significant concern to patients in need of mechanical ventilation. Consequently, this may result in elevated colonization levels in the lower respiratory tracts (4). Bacterial cells within biofilms are resistant to drugs, phagocytosis, and other elements of the innate and adaptive immune system, creating therapeutic challenges(5). Bacteriocins are antimicrobial peptides produced by bacteria by ribosome synthesis. They have multifunctional properties and are mostly used to inhibit the growth of comparable or closely related bacterial strains (6). Bacteriocins are regarded as the most significant category of antimicrobial peptides due to their efficacy and prospective applications in human health (7). The bacteriocins have been extensively reported for their capacity to effectively eliminate or inhibit pathogenic bacteria, including multidrug-resistant (MDR) strains, in laboratory environments (8). Bacteriocins can be secreted by both Gram-positive and Gram-negative bacteria. Specifically, bacteriocins generated from lactic acid bacteria, which belong to the group of Gram-positive bacteria, are highly intriguing (LAB)(9). Bacteriocins have demonstrated effectiveness over antibiotics. Antimicrobial peptides are regarded as offering superior protection without any adverse effects in comparison to antibiotics. Studies have found that bacteriocins can be used as supplements to antibiotics since they are highly stable, have minimal side effects, and have the potential to improve the effectiveness of antibiotics through a synergistic action (10). Bacteriocins exhibit a synergistic effect when used alongside antibiotics. They improve the effectiveness of antibiotics and prevent the development of antibiotic-resistant strains. Additionally, they mitigate the adverse effects of antibiotics by reducing the required dosage to eradicate bacteria. Bacteriocins can exert their effects through many mechanisms, such as blocking the synthesis of the cell wall, inhibiting cellular processes through DNase and RNase activities, and most frequently, creating gaps in the membrane of the target cell (11). Review of literature revealed scarce publications regarding bacteriocins and its application in Iraq, consequently this study intends to investigate the activities of bacteriocin as antibacterial and antibiofilm against *Acinetobacter baumannii*.

Materials and Methods

Ethical approval

This study has been approved by ethical research committee / college of medicine / Al-Iraqi university (6M.SA.T43 in 30 April 2024).

Isolation and identification of *Acinetobacter buamannii*

This study was conducted from October 2023 to March 2024. It included patients were admitted to the respiratory care unit at three hospitals in Baghdad: the Medical City/Martyr Gazi Al-Hariry hospital, Private Nursing Home hospital, and Al-Imamein Al-kadhimein medical City. Sputum samples were aerobically cultured on blood and MacConkey agar plates at 37°C for 24 hours, resulting in the growth of isolated bacteria on MacConkey agar, which manifested as purple colonies indicative of non-lactose fermenters. On blood agar media, the colonies manifest as translucent to opaque, non-hemolytic, and non-pigmented. The isolates of *A. baumannii* capable of growth at a temperature of 44°C. Following the manufacturer's guidelines, the bacterial isolates were identified using biochemical tests, confirming the diagnosis, and the antimicrobial susceptibility test was conducted using the VITEC 2 system.

The biochemical study of *Acinetobacter baumannii* indicated positive results for catalase and citrate production, while yielding negative results for haemolysis, indole, lactose, oxidase, and urease production **(**Figure.1).

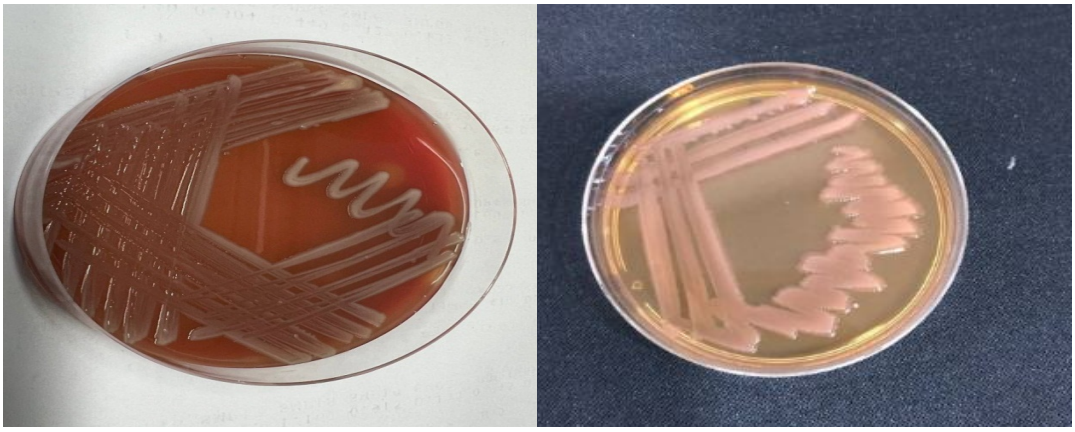


Figure.1: *Acinetobacter baumannii* growth on MacConkey agar and blood agar media

The detection of Biofilm Formation

The biofilm development experiments were conducted using quantitative microtiter plate methods, as described in the reference (12).
Each well of 96-well polystyrene microtiter plates was filled with 100 µl of *A. baumannii* broth exhibiting a turbidity of 0.5 McFarland, along with an equivalent volume of Muller Hinton Agar. The plates were subsequently incubated at 37°C for 24 hours. After three washing with phosphate buffered saline (PBS), all adhering biofilm was stained with 200 µL of 1% crystal violet for 30 minutes and thereafter allowed to dry at room temperature for 15 minutes. Once the dye had dried at room temperature, it was dissolved again using a mixture of 200 µL ethanol and acetone (in a ratio of 80:20, volume/volume). The concentration of the dye was then measured at a wavelength of 450 nanometres using an ELISA reader.

Bacteriocin

Bacteriocin was prepared and extracted from Lactic acid bacteria isolated from yogurt according to Salman, (2022) (13).

A-Identification of lactobacillus Bacteria

Lactobacillus bacteria were isolated from yoghurt samples collected from Canon yoghurt markets in Baghdad using De Man, Rogosa, and Sharpe (MRS) agar medium. A loopful of curd samples was inoculated onto a sterile MRS agar Petri dish with the streaking technique under aseptic circumstances, and incubated at 37°C for 48 hours and kept at 4°C (Menon *et al.,* 2013).Phenotypic characterization was performed based on morphological and biochemical characteristics. Microscopic examination was done using Gram stain. Biochemical tests included oxidase and catalase.

B-Preparation of Bacteriocin

In accordance with the manufacturer's guidelines, 200 ml of MRS medium was prepared in a flask and autoclaved at 121 °C and 15 psi for 15 minutes. Each flask was inoculated with 0.5 McFarland of Lactobacillus broth and incubated at 37 °C for 48 hours under anaerobic conditions. Subsequently, each 10 ml was transferred into a sterile test tube for cold centrifugation at 5000 rpm for 15 minutes. The precipitate was eliminated and the supernatant was taken. Then, the supernatant was filtered using a Millipore filter with a pore size of 0.22 nm. Following the filtration process, the loop containing the filtrate was streaked onto an MRS agar plate and incubated for 48 hours at 37℃ under anaerobic conditions to confirm the sterility of the filtrate (*Lactobacillus* extract). The *Lactobacillus* extract (bacteriocin) was preserved in sterile tubes and maintained at 4 °C until required.

Antibacterial activity of Bacteriocins against *A. baumannii*

The Kirby-Bauer disc diffusion method was used to determine the inhibitory effects of bacteriocins.

To prepare the inoculum from a pure culture, isolated colonies of the target organism were selected with a sterile wire loop and placed in a test tube with 3ml of sterile normal saline. The turbidity was adjusted to match 0.5 McFarland. The excess inoculum was eliminated by rotating the swab against the inner wall of the tube, followed by three applications across the medium, with the plate being rotated 60 degrees after each application. The swab was then moved along the agar periphery. Wells of 6 mm diameter were created on the plate using sterile corn borers and filled with 100 µl of bacteriocin. After incubating at 37 °C for 24 hours, the diameter of the inhibitory zone around each well was measured (Figure.2).

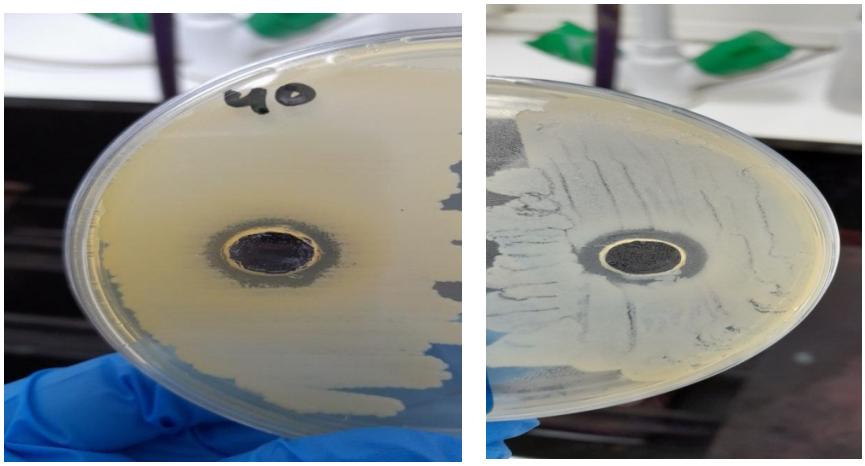


Figure-2: Antibacterial activity of bacteriocin on *Acinetobacter*

Quantitative assessment of antibiofilm activity via microtiter plate assay

Ten microliters of bacteriocin and one hundred microlitres of *A. baumannii* broth with a turbidity of 0.5 McFarland were added to each well of 96-well polystyrene microtiter plates. An equivalent volume of Muller-Hinton Agar was additionally included. The plates were subsequently incubated at 37°C for 24 hours. After three washes with phosphate buffered saline, any adhered biofilm was stained with 200 µL of 1% crystal violet for 30 minutes. The dyed biofilm was thereafter allowed to dry at room temperature for 15 minutes. Washed again with PBS. After the dye was dried at room temperature, it was dissolved again using 200 µL

of ethanol/acetone (80:20, v/v) and the optical density at 450 nm was measured using an ELISA reader to quantify the dye bound to the cells.

Statistical analysis

The SPSS/20.0 software was used to examine the meaning level, or p value, between the various elements included in this study, in addition to determination of percentage and chi-square values. The fisher test, with a 95% confidence interval, was employed to calculate the variation in drug resistance levels. The results were presented as the mean value ± standard deviation (SD). In the study of contingency tables, p values above 0.05 were considered statistically non-significant, whereas p values less than or equal to 0.05 were classified as significant.

Results

From October 2023 to March 2024, a total of 200 sputum samples were collected from patients suffered from RTI and admitted to the respiratory care unit in different hospital in Baghdad. The age of patients varied between 4 and 80 years of either sex. The results presented in this study were based on the analysis of 60 A. baumannii isolated from sputum samples (Figure. 3). The Vitek 2 System of A. baumannii antibiotic susceptibility test is displayed in Figure. (4). Significant resistance to most antibiotics was shown by Acinetobacter baumannii, including Ticarcillin (TIC), Ticarcillin-clavulanate (TIM), Piperacillin (PIP), Piperacillin-tazobactam (PTZ), Cefixime (CFX), Cefpodoxime (CFP), Cefotaxime (CTX), Ceftazidime (CEF), Ceftriaxone (CRO), Cefepime (CPM), Ciprofloxacin (CIP), Imipenem (IPM), Meropenem (MER), Gentamicin (GEN), Tobramycin (TOB), Tetracycline (TE), Amikacin (AK), and Levofloxacin (LEV) (each 100%). Conversely, it showed overall sensitivity for colistin(COL) antibiotic (100%), followed by minocycline(MIN) (83.3%), and trimethoprim- sulfamethoxazole(TMS) (23.3%) respectively.

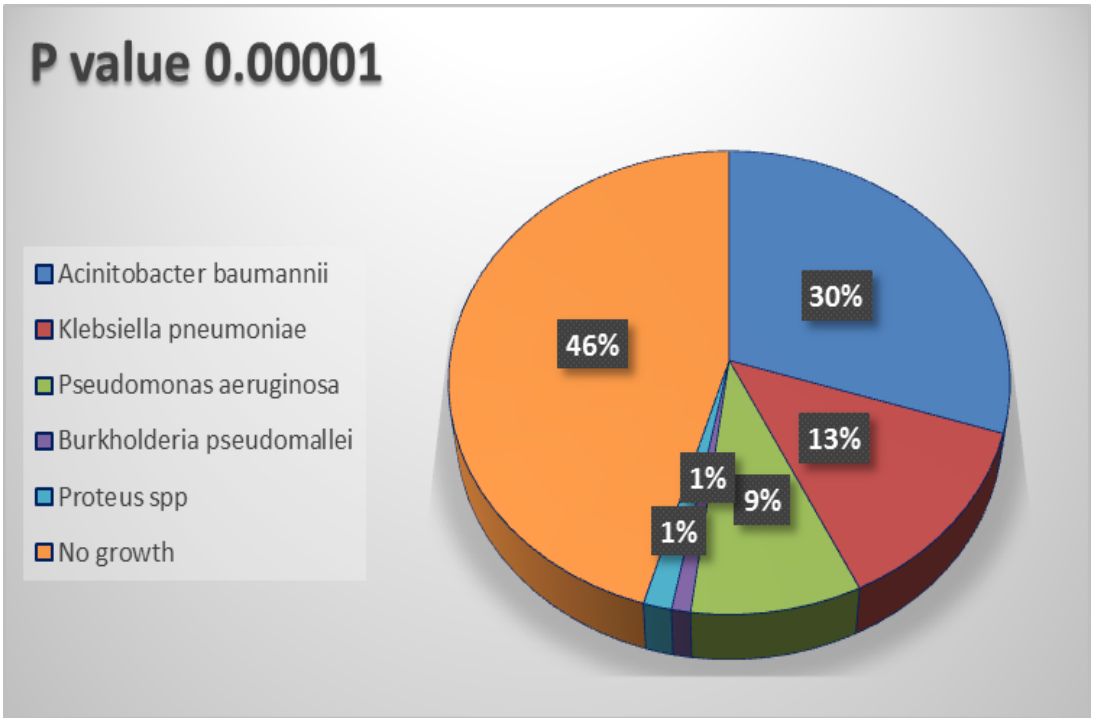
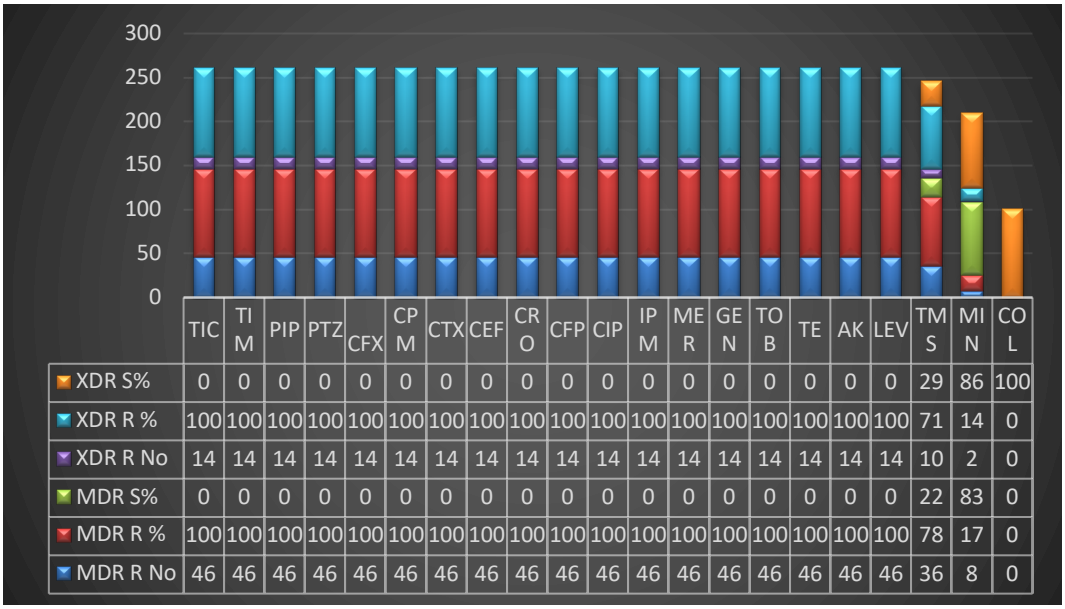


Figure. 3: Prevalence of bacteria among sputum specimens culture



	TIC	TIM	PIP	PTZ	CFX	CPM	CTX	CEF	CRO	CFP	CIP	IPM	MER	GEN	TOB	TE	AK	LEV	TMS	MIN	COL
XDR S%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	29	86	100
XDR R %	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	71	14	0
XDR R No	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14	10	2	0
MDR S%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22	83	0
MDR R %	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	78	17	0
MDR R No	46	46	46	46	46	46	46	46	46	46	46	46	46	46	46	46	46	46	36	8	0

Figure. 4: Antibiotic susceptibility by Vitek 2 System of *A. baumannii* isolates (n=60)

Additionally, according to classification of antibiotic resistance, *A. baumannii* shown a higher percentage of multidrug drug resistance (77%), followed by extensively resistance (23%), and no pan drug resistant bacteria, Figure (5).

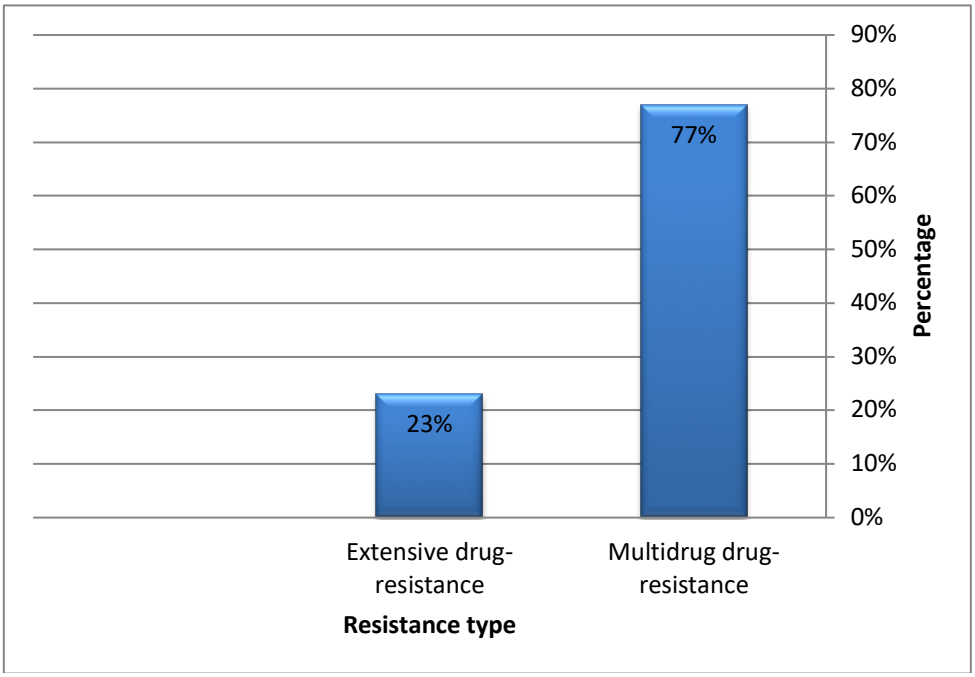


Figure. 5: Classification of isolated A. baumannii bacteria's susceptibility to antibiotics (n=60)

In the present study employed the microtiter plate method to assess biofilm formation in isolates of A. baumannii. The results indicated that all isolates exhibited a higher level of

biofilm production, with a mean value of 0.443 ± 0.146. Only two levels of biofilm development were found as strong and moderate (65% and 35%).

Table. 1: Biofilm formation profile for all tested isolates of *Acinetobacter baumnnii*

Isolation sources N(%)	**Non N(%)**	**Weak N(%)**	**Moderate N(%)**	**Strong N(%)**
Female	0	0	10(16.7)	21(35)
Male	0	0	11(18.3)	18(30)
Total	0	0	21(35%)	39(65%)
P value	The chi-square statistic is 0.212. The *p*-value is .645238. The result is *not* significant at $p < .05$.			

Additionally, the results indicate a correlation between biofilm production patterns and age, in accordance with bacterial resistance profiles (Table.2). The result reveals distinct patterns based on the level of drug resistance. Patients with MDR bacteria exhibited an average age of 40.94 ± 7.2 years in the moderate biofilm production group, compared to 48.46 ± 11.2 years in the strong biofilm production group, with a statistically significant difference (p = 0.04). This trend was even more pronounced in the XDR group, where those with moderate biofilm profiles had a significantly lower mean age 21.6 ± 6.9 years compared to those with strong biofilm profiles 41.9 ± 10.5 years, with a statistically highly significant different (p-value= 0.0001), also the result showed there is highly statistically significant difference (p = 0.0001 and 0.05) between strong and moderate biofilm formation groups in MDR and XDR bacteria isolates.

Table.2: Association between Biofilm formation profiles, age according to bacterial resistance profile

Association between Biofilm formation profiles, age according to bacterial resistance profile			
Biofilm formation profile	Moderate	Strong	P value
MDR (Mean ±SD)	40.94±7.2	48.46±11.2	0.04
XDR (Mean ±SD)	21.6±6.9	41.9±10.5	0.0001
P value	0.0001	0.05	

Regarding to Effect of bacteriocin against *A.buamannii* ,the result show that the diameter of inhibition zone formed by bacteriocin was ranged from 20 to 32 mm with mean of 27.3500 ± 2.82738 mm, so bacteriocin considered good inhibitory to *A. buamannii*.

Table (3):Antibacterial activity of bacteriocin

Inhibition zone diameter of Bacteriocine on MDR and XDR tested isc			
Bacteriocine (mm)	MDR	XDR	P value
Mean±SD	27.37±2.9	29.39±2.3	0.09
MIN	20	24	

MAX	32	30	
*Colistin (mm)	17.4±1.2	16.3±1.7	0.81
P value	0,0001	0,0001	
*Colistin resis.= ≤ 10 mm/*Colistin sens..= ≥ 11 mm			

The result in table. (4), revealed the effect of bacteriocin on biofilm as antibiofilm activity. The result showed that bacteria become (75%) weak biofilm production and (25%) moderate biofilm production after bacteriocin treatment (24 hrs.) so the result is high significant.

Table (4): Antibiofilm activity of bacteriocin

Biofilm formation profile after bacteriocin treatment (24 hr)				
Biofilm profile	Before		After	
	Moderate	Strong	Weak	moderate
	21 (35%)	39 (65%)	45 (75%)	15 (25%)
P value	The chi-square statistic is 14.1107. The *p*-value is 0.000172. The result is significant at $p < .05$.			

Discussion

Acinetobacter baumannii is a Gram negative bacteria that is commonly found in hospitals and offers significant danger to patients in intensive care units. It is linked to a higher rate of morbidity and more healthcare expenses (14). According to the present study, the prevalence of *Acinetobacter baumannii* in Iraqi samples was found to be 30%, which was higher compared to other microorganisms. These findings agree with a study conducted in Nepal by (15), where the prevalence was reported to be 20.65%.

The results of current study demonstrate that all *A. baumannii* isolates possess the capacity for biofilm formation (100%). Moreover, it was found that 21(35%) moderate and 39(65%) strong biofilm formation. The results agree with the many studies as (16) that 50 (100%) isolates were biofilm formation. A strong biofilm was identified in 40 (80.0%) of the examined isolates, whereas 10 (20.0%) of the isolates exhibited the capacity to generate moderate biofilms(16). A study conducted on 100 *Acinetobacter baumannii* isolates from immunocompromised hospitalised patients in the intensive care unit (ICU) revealed that all isolates possessed the ability to form biofilms, with 58% demonstrating a strong capacity for biofilm formation. In the absence of biofilm, there was no isolation because the biofilm formation is a characteristic of *A. baumannii* and plays an essential role in which bacteria can resist conditions in hospital environments. The ability of *A.baumannii* to produce biofilms plays a crucial role in the dangerous interactions between the host and the pathogen, as well as in infections associated with medical devices. Antibiotic resistance is a phenomenon that enables bacteria to resist the environmental conditions and promotes the development of biofilms. Conversely, biofilm significantly facilitates bacterial colonization in the context of diseases. It functions as a robust barrier that prevents dangerous compounds like antibiotics from diffusing and entering, hence offering substantial protection to *A. baumannii* strains. The strong survival ability of *A. baumannii* in challenging conditions and its high resistance to many antibiotics can be attributed to the production of biofilms, as demonstrated by numerous investigations. The current study investigated that there is relationship between antibiotic resistance and biofilm formation in the clinical isolates of *A. baumannii*, revealing a strong association between antibiotic resistance and biofilm forming capabilities. This results has

been reported in earlier studies as (17-20), In the current study, the predominant isolates of *A. baumannii* exhibited antibiotic resistance and demonstrated the capacity for biofilm formation. Especially, the strains obtained from ICUs had a more strong capacity to form biofilms, which is in line with the results of previous study (21), who found a significant association between biofilm formation capacity and antibiotic resistance.

The overuse of antibiotics has resulted in an increase of antibiotic resistance among pathogenic microbes. In this study, *Acinitobacter baumannii* revealed higher overall resistance to most antibiotics(100%), Furthermore, the overall sensitivity to the colistin antibiotic was 100%, followed by minocycline at 83.3%, and trimethoprim-sulfamethoxazole (23.3%). Numerous studies in this field have revealed that *A. baumannii* isolates demonstrate significant resistance to a broad spectrum of antibiotics. For instance, a study conducted in Iraq by (22) Increasing problem of multi drug-resistant pathogens and the quickly declining antibiotic resource may be one of the main concerns facing mortality in the 21st century. Researchers globally are looking for alternate approaches to challenge this problem. The elevated specific activity of the bacteriocin against clinical infections, particularly multidrug-resistant strains, suggestions a potential solution to this growing problem. Results of current study support the anti-biofilm activity of bacteriocin preducing Lactobacillus ssp, and suggest its use as suitable adjuvants and also biocontrol agents for treatment. Many researchers studied the antibiofilm effects of bacteriocin on pathogenic bacteria but few investigations reported their effects on *Acinetobacter buamannii* as (23) indicating that the inhibition of bacterial biofilm formation has been an attractive target for a therapeutic intervention. Studies on the antimicrobial activity of Bacteriocin against *A. baumannii* are currently limited (24, 25). In the present study, the results of present study show that bacteriocin considered good inhibitory to *A.buamannii*. These results in agreement with Iraqi study by (26), the results demonstrated that the isolates of *Lactobacillus* were tested using various techniques for their capacity to produce bacteriocins., the results by agar well diffusion assay showed strong antibacterial activity of Lactobacillus spp. against one isolate of *Acinetobacter baumannii* with an inhibition zone diameter (17.75 ± 0.49).Study by (27), which shows that the nosocomial strains used in the study, *K. pneumoniae* and *A. baumannii* have been shown to be sensitive targets of the bacteriocin isolated from *L. plantarum*. Two categories of bacteriocin antibacterial mechanisms can be identified: Bacteriocins function by creating pores in the cytoplasmic membrane, hence impairing membrane permeability and integrity. Bacteriocins, when functioning intracellularly, disturb normal cellular metabolism, resulting in the leaking of intracellular components such as potassium, ATP, and lactate dehydrogenase, which ultimately causes membrane depolarization and cell death (28).

Conclusion

Bacteriocin from lactic acid bacteria has a definitive effect on biofilm produced by *Acinetobacter baumnnii*. The results of current study showed the antibiofilm activity of bacteriocin producing *Lactobacillus ssp,* and suggest their use as suitable adjuvants as well as bio control agents for treatment.

DECLARATIONS

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The authors stated that this work received no funding.

Competing interests statement

The authors declare that they have no conflict of interest.

Ethics statement
The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have adhered to.

Author contributions
All authors contribute equally in this study.

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