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The Inhibitory Effect of Probiotics on the adeB Efflux Pump Gene Expression in Acinetobacter baumannii

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Abstract

When *Acinetobacter baumannii* infections become multidrug-resistant, there is an urgent need to restore the effectiveness of existing antibiotics work again. One promising approach is using efflux pump inhibitors. These inhibitors are compounds that restore bacterial sensitivity to antibiotics by blocking the efflux pumps. The discovery of probiotics as beneficial bacteria, has expanded worldwide as a preventative and therapeutic agent to eliminate potential pathogens, particularly where the need for new treatments is critical due to increasing antibiotic resistance**.** The study aims to evaluate the synergistic effect of probiotics on *adeB* efflux pump gene expression in *A. baumannii* as a treatment. This study was conducted in the Department of Biotechnology at Al-Nahrain University. Samples were collected from October 2024 to January 2025 from clinical settings, including patients of both sexes and all age groups. A total of 180 clinical samples were obtained. All collected samples were cultured on MacConkey and blood agar. Antibiotic susceptibility testing was performed using the Kirby-Bauer disc diffusion method against 12 antimicrobial agents representing 8 categories, and further confirmed using the AST card on the automated Vitek-2 compact system. Biofilm formation by *A. baumannii* isolates was quantified using the microtiter plate assay (96-well format). RNA was successfully extracted from two clinical isolates of *A. baumannii* using a commercial RNA extraction Kit. These two isolates were strong biofilm production and extremely high rates of multidrug resistance. Quantitative Real – Time PCR was performed before and after treating them with probiotics, antibiotics, and probiotic-antibiotic combinations, to estimate the gene expression of the Efflux pump gene (*adeB*) with a housekeeping gene *(rpoB*). A total of 50 *A. baumannii* isolates were identified. An extremely high rate of multidrug resistance was observed. The probiotic agent, used alone or in combination, consistently caused a downregulation (Fold Change < 1) of the *adeB* efflux pump gene in both *A. baumannii* isolates. This direct suppression of major resistance machinery is the molecular mechanism by which the probiotic re-sensitizes the bacteria, as it allows the sub-minimum inhibitory concentrations of the antibiotics to remain inside the cell.

Key word: Efflux pump, Biofilm, Multi-drug Resistance, Probiotics, *Acinetobacter baumannii.*

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Introduction

Acinetobacter baumannii is an aerobic, gram-negative coccobacillus bacterium that is a common cause of serious hospital-acquired infections (nosocomial infections), including pneumonia, infections of the bloodstream, meningitis, and urinary tract infections [1]. For patients in the intensive care units (ICU), particularly those requiring ventilator support, the danger of this infection is substantial, contributing to the high mortality rate seen in immunocompromised individuals in this setting [2]. *A. baumannii* infections are notoriously hard to treat because of the bacteria's high potential for epidemics, its ability to form a biofilm on abiotic surfaces and medical devices [3]. Clinical isolates of *A. baumannii* are widely reported to be resistant to many antimicrobial agents, including Aminopenicillins, Ureidopenicillins, broad-spectrum Cephalosporins, most Aminoglycosides, Fluoroquinolones, and chloramphenicol [4]. Multiple drug resistance in *A. baumannii* is caused by underlying molecular mechanisms, including changes in membrane permeability, alterations to antibiotic target proteins, the development of multidrug efflux pumps, and the production of beta-lactamase enzymes [5]. Bacterial efflux systems increase the minimum inhibitory concentration (MIC) of antibiotics by reducing the amount of antibiotic that can build up inside the bacterial cell [6]. The two families that make up the efflux system of *A. baumannii* are the resistance nodulation division (RND) superfamily and the multidrug and toxin extrusion (MATE) family [7]. When *A. baumannii* infections become multidrug-resistant, needed a way to make existing antibiotics work again. One promising approach is using efflux pump inhibitors. These inhibitors are compounds that restore bacterial sensitivity to antibiotics by blocking the efflux pumps. Efflux pumps are bacterial mechanisms that actively "pump out" antibiotics, making the treatment ineffective. The inhibitors stop this "outpour" activity, either by competing with the antibiotic for the pump's binding site or by non-competitive methods that still block the pump's function [8].

Although efflux pumps play a critical role in antibiotic resistance, the relationship between the expression levels of specific efflux pump genes and resistance antibiotic profiles varies geographically and among clinical isolates [9]. The discovery of probiotic lactobacilli, beneficial bacteria, has proliferated worldwide as a preventative and therapeutic agent to eliminate potential pathogens, where the need for new treatments is great for the treatment of infection that unaffected by antibiotics [10]. *Lactobacillus* which is generally recognized as safe, is an important member of the human intestinal microflora and has previously been reported as a potential candidate for competition with pathogenic bacteria and as a potential substitute for antibiotics [11]. Understanding these correlations is crucial for elucidating local resistance epidemiology, informing effective treatment strategies, and potentially identifying novel therapeutic targets to circumvent efflux pump-mediated resistance. The study aims to evaluate effect of probiotics on efflux pump gene expression in *A. baumannii*, a bacterium responsible for various infections.

Materials and Methods

Bacterial sampling:

A total of 180 clinical samples were collected from diverse clinical settings at the Baghdad Teaching Laboratories in Medical City, Baghdad/Iraq, for this study at the Biotechnology Department of Al-Nahrain University. Collection occurred from October 2024 to January 2025, and the samples, representing patients of all ages and both sexes, included sputum, wounds, blood, urine, and cerebrospinal fluid (CSF).

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Cultivation of suspected microbial samples:

Clinical samples were initially plated on MacConkey and Blood agar and incubated at 37°C for 24 hours. The resulting bacterial isolates were then characterized through morphological and microscopic examination, with final, accurate identification performed using the Vitek-2 system.

Detection of biofilm formation

Quantification of *A. baumannii* biofilm formation was achieved using the 96-well microtiter plate assay, which assessed the bacterial isolates' ability to produce biofilm, the method adapted according to [12].

Antibiotics susceptibility test:

The susceptibility of each pure isolate to the twelve antimicrobial agents listed in Table1 was determined using a dual-method approach. This included the conventional Kirby-Bauer disc diffusion method and the automated analysis provided by the Vitek-2 compact system using a specific AST card.

Table 1. Antibiotic discs used for the study.

No.	Name	Symbol	Cons.	Origin Company
1	Amikacin	AK	30 µg	Liofilchem / (Italy)
2	Cefotaxime	CTX	30 µg	Liofilchem / (Italy)
3	Ceftazidine	CAZ	30 µg	Liofilchem / (Italy)
4	Ciprofloxacin	CIP	5 µg	Liofilchem / (Italy)
5	Colistin	CL (CS)	10 µg	Liofilchem / (Italy)
6	Gentamicin	GM	10 µg	Liofilchem / (Italy)
7	Levofloxacin	LE	5 µg	Liofilchem / (Italy)
8	Meropenem	MRP	10 µg	Liofilchem / (Italy)
9	Piperacillin	PRL	100 µg	Liofilchem / (Italy)
10	Piperacillin-Tazobactam	TZP	10 µg	Liofilchem / (Italy)
11	Tetracycline	TE	30 µg	Liofilchem / (Italy)
12	Trimethoprime-Sulfamethazole	SXT	25 µg	Liofilchem / (Italy)

Activation of Probiotic:

The *Lactobacillus plantarum* was obtained from a commercial probiotic supplement, as indicated on the manufacturer's supplement facts label in order to study the effect of *L. plantarum* on the efflux pump gene expression in clinical *A. baumannii* isolates. The strain was cultured in de Man, Rogosa, and Sharpe (MRS) broth followed by incubation anaerobically in a candle jar at 37°C for 48 hours.

Preparation of Probiotic filtrates:

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The unconcentrated filtrate of the *Lactobacillus plantarum* isolate was obtained by inoculating (1ml) of the isolate culture into (9ml) of MRS broth and incubating the mixture at 37°C for 24 hours. Subsequently, (100ml) of MRS broth was inoculated with 2% of the grown culture and incubated in a candle jar at 37°C for 48 hours. The culture was then centrifuged at 4000 rpm for 15 minutes at 4°C, after which the supernatant was filtered using (0.22µm) Millipore filters. To prepare concentrated filtrates, (100ml) of the unconcentrated filtrate was evaporated in an oven at 40°C until the volume was reduced to (50ml), resulting in a one-fold concentrated filtrate. This process was repeated with the one-fold concentrated filtrate to obtain a two-fold concentrated filtrate (25ml) and further repeated to achieve a three-fold concentrated filtrate (12.5ml).

Determination of MIC of *Lactobacillus* supernatant:

The inhibitory activity of probiotic filtrate against the strains was determined by utilizing the well polystyrene plate method [13].

- Each of the *A. baumannii* strains were grown in MacConkey agar at 37 °C for 18-24 hours.
- After incubation, a single colony was transferred to a tube containing (5ml) of Mueller-Hinton broth and incubated overnight at 37 °C.
- the concentration of two isolates suspensions was equilibrated with 0.5 McFarland (1.5×10^8 CFU/ml) by using spectrophotometer.
- A portion of Mueller-Hinton broth (180µl) (enriched with 1% agarose) was put in each of the 96 wells, then (20µl) of bacterial suspension was added to it. The microtiter plate was incubated at 37°C for 18-24 hours.
- The supernatant was carefully removed, and each well was washed twice with 200µl of phosphate-buffered saline (PBS).
- The biofilms were treated with the probiotic (*L. plantarum*), *(*100µl) from the cured was added to the first well of the plate vertically, mixing and then (100µl) from was taken from it to the second well until reached the last well, (100µl) was discarded from the last well, to give the following final concentration (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78) µg/ml.
- Positive control was prepared by adding only broth to each isolate, and a negative control was prepared by adding only broth.
- After that, each well was stained with (20µl) of Resazurin. Resazurin changes from blue and non-fluorescent to pink and highly fluorescent when chemically reduced through aerobic respiration produced by cell multiplication, indicating cell viability. The plates were incubated at 37 °C for 18-24 hours.
- Stained biofilms were measured at 570nm using a microtiter reader.
- Each stain was examined triplicate.

Determination of MIC of Antibiotics

Each of the *A. baumannii* strains were grown in MacConkey agar at 37 °C for 18-24 hours. After incubation, a single colony was transferred to a tube containing (5ml) of Mueller-Hinton broth and incubated overnight at 37 °C and the concentration of two isolates suspensions was equilibrated with 0.5 McFarland (1.5×10^8 CFU/ml) by using spectrophotometer. A portion of Mueller-Hinton broth (180µl) (enriched with 1% agarose) was placed in each of the 96 wells, then (20µl) of bacterial suspension was added to well and then incubated at 37 °C for 18-24 hours. The supernatant was carefully removed, and each well was washed twice with (200µl) of phosphate-buffered saline (PBS). Stock solution was

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prepared by weighing (0.1g) Cefotaxime and Colistin powder then (10ml) distilled water was added to reach the concentration of (100000µg\ml). (100µl) from pure Muller Hinton broth was added to each well of polystyrene microtiter plate. Then treated with the antibiotics, (100 µl) from the working solution were added to the first well of the plate vertically, mixing, and then transferring (100 µl) from first well to the second well until reaching the last well, from which (100 µl) was discarded, the following final concentration of Colistin (128, 64, 32, 16, 8, 4, 2, 1) µg/ml and concentration of Cefotaxime (2048, 1024, 512, 256, 128, 64, 32, 16) µg/ml. Positive control was prepared by adding only broth to each isolate, and a negative control was prepared by adding only broth.

Determination of MIC of Probiotics- Antibiotics Combination:

A portion of Mueller-Hinton broth (180µl) was placed in each of the 96 wells, then (20µl) of bacterial suspension was added to it**,** then **(**100µl) from pure Muller Hinton broth was added to each well of polystyrene microtiter plate, then treated with the combinations of *L. plantarum* and antibiotics*,* (100µl) from the combination (50µl) from probiotic and (50µl) from working solution of the antibiotics was added to the first well of the plate vertically, mixing and then (100µl) from was taken from it to the second well until reached the last well mix and drop it. The plates were incubated at 37 °C for 18 hours. After that, each well was stained with (20µl) of Resazurin. Resazurin changes from blue and non-fluorescent to pink and highly fluorescent when chemically reduced through aerobic respiration produced by cell. multiplication, indicating cell viability. The plates were incubated at 37 °C for 4 hours. Stained biofilms were measured at 570 nm using a microtiter reader.

Molecular Study: RNA Extraction

RNA was successfully extracted from 2 clinical isolates of *A. baumannii* using a TRANS-GENE-BIOTECH/Cat.No:ER501 RNA extraction Kit procedure, and the samples were subsequently quantified and stored at -20 c.

Reverse Transcription for RNA samples

The procedure was used to convert RNA into first-strand cDNA using GoScript Reverse Transcription System kit according to the instructions of the manufacturer company.

Quantitative Real – Time (qRT) PCR

The isolates that were chosen before and after treating them with probiotics**,** antibiotics, and probiotic-antibiotic combinations, to estimate the gene expression of the Efflux pump gene (*adeB*) with a housekeeping gene (*rpoB*) (Table 2). The reaction mix was composed of the components and their quantities, as mentioned in (Table 3). The cycling parameters was set as in (Table 4).

Table (2): Primer Sequences of Efflux pump gene used in Quantitative PCR (qPCR).

Gene		Sequence of forward and reverse Primer (5´- 3´)	Size bp	GC%	Reference
adeB	F	ATTGTCGACGATGCCATTGTC	176 bp	48	NCBI
	R	ACAGAACCACTCGCAAATGC		50	

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rpoB	F	ACAAAGTTGCTCGTGGTGAC	85 bp	59	
	R	CACGCATGTTTTGACCAAGC		59	

Table (3): Components of quantitative real-time PCR used in gene expression experiment.

Component	Volume (µl)
cDNA Template	2
Forward Primer	0.4
Nuclease-Free Water	7.2
qPCR Master Mix, (SYBR)	10
Reverse Primer	0.4
Total volume	20

Table (4): qRT-PCR Cycling Program.

Steps	Temperature (°C)	Time (min)	Number of cycles
Initial denaturation	94	2	1
Denaturation	94	0.5	40
Annealing	60	0.5	40
Extension	72	0.5	40
Melting curve	65-95	0.5	1

Results and Discussion

Sampling and Isolation:

A total of (180) specimens had been collected across five distinct clinical sources (bloodstream, sputum, urine, wound and cerebrospinal fluid (CSF)) from clinical state which involving both sexes with different ages, from Baghdad Teaching laboratories in Medical City in Baghdad/ Iraq. Out of (180) specimens, collected a total of (120) (66.7%) were positive for culturing while (60) (33.3%) negatives for culture, the negative culture cases were attributed to the reasons that some patients were under antimicrobials therapy at the time of specimen collection. Critically, after final identification of isolates according to colonial morphology (Fig. 1), and confirmed by Vitek 2 compact device as a final

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identification of by using a GN (Gram-Negative) card. The distribution results among specific sources listed in (Table 5).

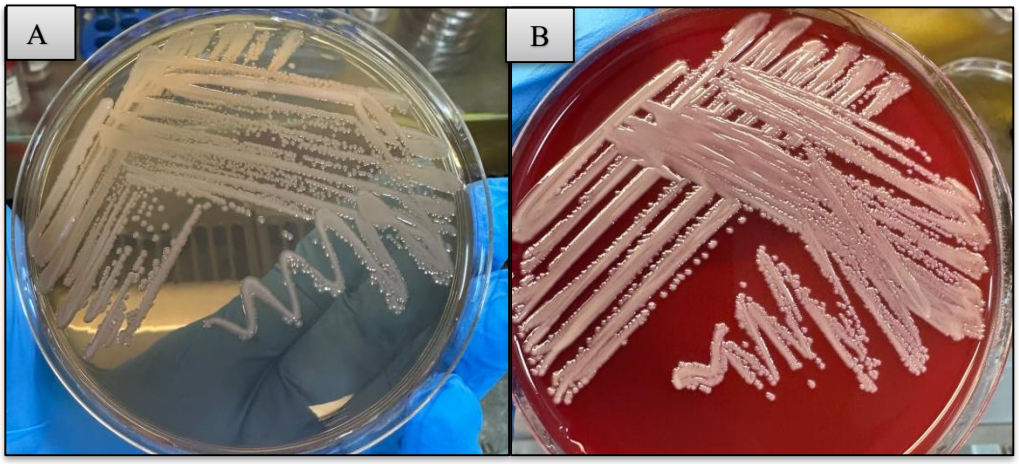

Figure 1: Colonial Morphology of isolates on: A- MacConkey agar, B- Blood agar.

Table (5): Distribution and Relative Prevalence of *Acinetobacter baumannii* Isolates Across Clinical Sample Sources.

Sample Source	Total Positive Cultures (N=120)	No. of *A. baumannii* Isolates (N=50)	Percentage
Blood	36	8	16%
CSF	9	2	4%
Sputum	40	20	40%
Urine	13	7	14%
Wound	22	13	26%
TOTAL	**120**	**50**	**100%**

A. baumannii accounted for the majority of these positive cultures, representing (50) out of (120) isolates, of all recovered bacteria, while other (70) indicate to other type of bacteria. The distribution among specific sources highlights its predilection for certain anatomical sites where *A. baumannii* was most frequently isolated from sputum (40%), a finding consistent with its role as a leading cause of hospital-acquired pneumonia. The organism also showed a significant presence in wound samples (26%) and bloodstream (16%), emphasizing its capacity to cause severe, invasive infections like sepsis and complicated soft-tissue infections. The presence in cerebrospinal fluid (CSF) (4%) also points to its ability to infect the central nervous system, albeit less frequently.

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Biofilm Formation:

After the final diagnosis of the isolates and obtaining (50) isolates of *A. baumannii*, these isolates were subjected to the biofilm formation assay using the microtiter plate method. The results as indicated in Table (6) showed that (9) isolates, representing (18.0%) of total were unable to form biofilms, and the result was negative when comparing the absorbance of these isolates with the absorbance of the control. Overall, (41) out of (50) isolates, representing (82.0%) were classified as biofilm producers (Weak, Moderate, or Strong). The largest proportion of isolates (48.0%) were classified as weak producers. While these strains form a less robust structure, their high prevalence suggests that biofilm production is a common, baseline characteristic among these clinical isolates.

Critically, (17) isolates 34.0% demonstrated a capacity for biofilm formation, (9) isolates that 18.0% moderate biofilm formation and (6) isolates that 16.0% strong biofilm formation. These strains represent the highest clinical risk, as robust biofilm production provides the bacteria with increased protection from host immune responses and antibiotic penetration, contributing significantly to chronic colonization and persistent infections. 48% of isolates demonstrated a weak biofilm formation. Only a small minority of isolates, 18.0% were classified as non-producers, further emphasizing that biofilm formation is a highly conserved virulence mechanism in this *A. baumannii* population.

This high frequency of biofilm-competent strains is highly relevant in the clinical context, as the extracellular matrix provides a substantial barrier against both host immune defenses and antimicrobial agents [14], thereby enhancing the recalcitrance of infections a threat amplified when combined with the extreme drug-resistant phenotypes observed in this study.

Biofilm formation is a major virulence factor in *A. baumannii* infections, particularly those associated with foreign materials like catheters, ventilators, and surgical implants. The presence of a biofilm makes bacteria encased in a biofilm matrix are notoriously difficult to eradicate where the matrix acts as a physical barrier, slowing the penetration of antibiotics [15]. Furthermore, metabolic changes within the biofilm (decreased growth rate) make the bacteria less susceptible to agents like β-lactams and aminoglycosides, which primarily target actively growing cells [16].

Table (6): Biofilm-forming ability of *Acinetobacter baumannii* by using micro-titer plate assay.

Biofilm Phenotype	Symbol	Number of Isolates (n=50)	Percentage
Strong Producer	S	8	16%
Moderate Producer	M	9	18%
Weak Producer	W	24	48%
Non-Producer	(Blank)	9	18%
Total Producers		50	100%

Antimicrobial Susceptibility Pattern

The Kirby-Bauer method, following Clinical and Laboratory Standards Institute (CLSI) recommendations, is a manual technique where antibiotic-containing disks diffuse into agar. The Vitek 2 Compact system is an automated method that uses specialized AST cards. The susceptibility pattern in

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current study adapted toward 8 categories of antimicrobial agents within 12 types of antibiotics and the results of the susceptibility data for these isolates highlights a critically high level of antimicrobial resistance (Table 7).

Table (7): Antibiotic Susceptibility Test of *A. baumannii* Isolates to Antibiotics.

Antibiotic (Class)	Antibiotic (12 Type)	NO. of Resistance Isolates	Percentage (%)
Aminoglycosides	Gentamicin	38	92.7%
	Amikacin	31	75.6%
Carbapenems	Meropenem	31	75.6%
Cephalosporins	Cefotaxime	40	97.6%
	Ceftazidine	36	87.8%
Fluoroquinolones	Ciprofloxacin	31	75.6%
	Levofloxacin	30	73.2%
Folate Inhibitors	Trimethoprime-Sulfamethazole	35	85.4%
Penicillins	Piperacillin	40	97.6%
	Piperacilin-Tazobactam	31	75.6%
Polymyxins	Colistin	36	87.8%
Tetracyclines	Tetracycline	24	58.5%

The non-susceptibility to β-lactam agents, which are the backbone of therapy for most Gram-negative bacteria, is near-universal. The rates of non-susceptibility to Piperacillin (97.6%) and Cefotaxime (97.6%). Notably, even with the β-lactamase inhibitor combination, Piperacillin-Tazobactam is ineffective against (75.6%) of the isolates, suggesting the presence of advanced resistance mechanisms. The high rate of non-susceptibility to Meropenem (75.6%) is the most critical feature defining this MDR population. Loss of carbapenems eliminates the primary empirical treatment option for severe Acinetobacter infections and immediately shifts treatment protocols toward more toxic or less reliable agents, reinforcing the severity of the MDR status.

Resistance in last-resort and alternative therapies where the MDR status is compounded by significant non-susceptibility in critical last-line classes, where the current results indicate to non-susceptibility to Colistin stands at (87.8%). As Colistin is often the single remaining effective agent against carbapenem-resistant strains, this concurrent resistance is a key factor driving many isolates into the PDR category. Its loss leaves clinicians with no available options in the tested classes. High non-susceptibility to Gentamicin (92.7%) and Amikacin (75.6%) removes another critical group of compounds often used in combination therapy to combat MDR pathogens. While the non-susceptibility to Tetracycline (58.5%) is the lowest among the tested classes, it still represents a substantial loss of activity.

The co-occurrence of high multidrug resistance (MDR) and robust biofilm formation in *A. baumannii* is a critical finding. The fact that the 41 isolates tested for antibiotic resistance were nominated as biofilm producers establishes a direct, causative link between this virulence factor and the observed severe MDR profile. The extremely high rate of Multidrug Resistance (MDR) observed in this study 92.9% of isolates classified as MDR can be directly linked to the isolates' capacity for biofilm formation.

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The finding that 82.0% (41) out of (50) of the overall isolates were identified as biofilm producers (Weak, Moderate, or Strong) provides the underlying mechanism for the high rates of antibiotic failure. Biofilms form a protective, matrix-encased community (composed of exopolysaccharides, proteins, and DNA). This matrix acts as a physical barrier that limits the diffusion and penetration of antibiotics [17], such as Gentamicin (92.7% resistant) and Colistin (87.8% resistant), preventing them from reaching the bacterial cells located embedded deep within the biofilm. Moreover, bacteria exist in a state of nutrient limitation and low oxygen, leading to a reduced metabolic rate. Antibiotics, particularly those that target active cell growth and replication such as Meropenem (75.6% resistant), become significantly less effective against these slow-growing or dormant cells (often referred to as persister cells). This phenotypic change alone can increase antibiotic tolerance by up to 1,000 times compared to planktonic (free-floating) cells [18]. The close proximity of bacterial cells within the biofilm matrix facilitates the horizontal gene transfer (HGT) of resistance genes, particularly those carried on plasmids or transposons [19], including those encoding carbapenem resistance. The biofilm environment serves as a "hotspot" for the rapid acquisition and dissemination of the resistance determinants responsible for the observed MDR phenotype [20].

Determination of Sub-MIC of Colistin and Cefotaxime in Combination with a Probiotic against *A. baumannii.*

The MIC values were initially determined to establish the appropriate sub-MIC concentrations. These sub-MIC concentrations were selected based on the MIC result to ensure bacterial viability, since MIC result in complete growth inhibition, which unsuitable for gene expression analysis. The results of Sub-MIC (Table 8, Figure2) for the antibiotic and probiotic agents alone and in combination against the two *A. baumannii* isolates no.15 and 50 were determined using the Polystyrene Microtiter Assay. For both isolates, the probiotic agent alone required a concentration of (12.5µg/ml) for the Colistin combination and (25µg/ml) for the Cefotaxime combination to inhibit growth. The Colistin concentration required to inhibit growth alone were (4µg/ml) of isolate-15 and (8µg/ml) of isolate-50. The combination treatment reduced the required Colistin dose to (2µg/ml) for both isolates. For Cefotaxime, both isolates were highly resistant, requiring a concentration of (1024µg/ml) when used alone. When combined with the probiotic, the inhibitory Cefotaxime concentration was drastically reduced to a sub-MIC concentration of (16µg/ml). This represents an impressive reduction in the antibiotic required to inhibit the highly resistant strains.

Table (8): Sub-MIC of Colistin and Cefotaxime in Combination with a Probiotic against MDR *A. baumannii*

No. of isolate (antibiotic)	MIC (µg/ml)	Sub-MIC (µg/ml)	Pro MIC (µg/ml)	Pro sub-MIC (µg/ml)	Combination MIC (µg/ml)	Combination Sub-MIC (µg/ml)
Isolate-15 Colistin	8	4	25	12.5	2/25	2/12.5
Isolate-50 Colistin	16	8	25	12.5	2/25	2/12.5

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Isolate-15 Cefotaxime	**2048**	**1024**	**50**	**25**	**16/50**	**16/25**
Isolate-50 Cefotaxime	**2048**	**1024**	**50**	**25**	**16/50**	**16/25**

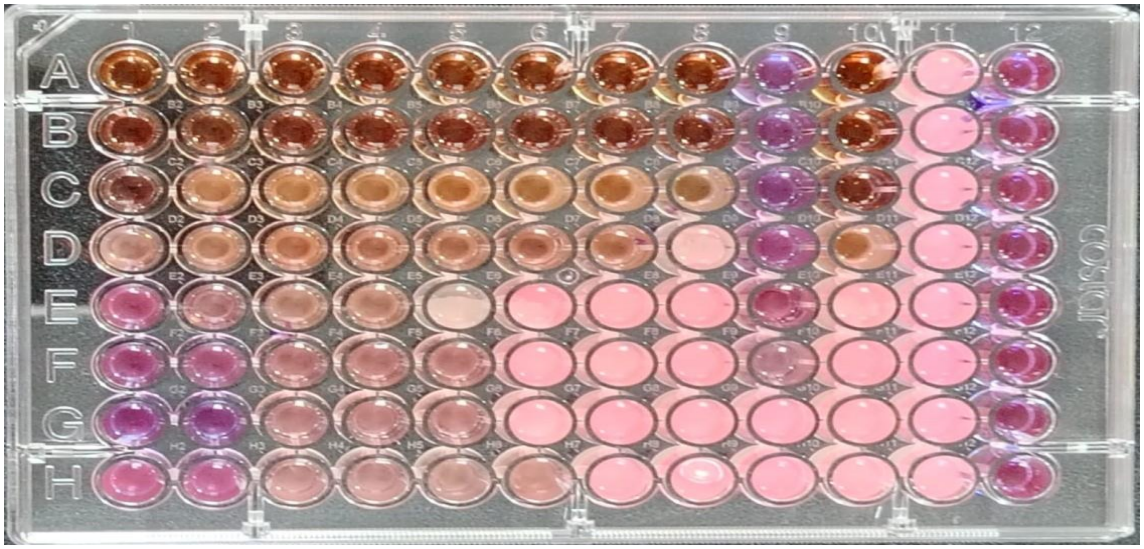

Figure 2: Determination of MIC of Probiotics, Colistin and their combination against isolate no.15

The observed reductions in the antibiotic concentrations required for bacterial inhibition demonstrate a significant potentiating effect of the probiotic agent on both tested antibiotics. For Colistin, a critical last-resort drug, the ability to achieve inhibition using a concentration of (2µg/ml) a dose below its standalone MIC is highly valuable. This outcome suggests that the combination therapy could permit a substantial reduction in the antibiotic dosage, which is a key strategy for mitigating the severe dose-dependent nephrotoxicity associated with Colistin [21]. Furthermore, the combination successfully restored the efficacy of Cefotaxime. Given that the single-agent MIC of (1024µg/ml) confirms high-level resistance, the ability to inhibit growth using (16µg/ml) concentration of Cefotaxime indicates that the probiotic is overcoming a major resistance mechanism. This strong re-sensitization suggests that probiotic compounds, such as organic acids or bacteriocins, may be acting as resistance modulators by interfering with crucial bacterial processes like beta-lactamase activity or efflux pump function. These findings strongly support the use of the probiotic combination as a promising strategy to treat multidrug-resistant *A. baumannii* infections by lowering the dosage of toxic drugs and reviving the clinical utility of previously ineffective antibiotics.

Gene Expression Analysis:

The investigation into the effect of the probiotic and antibiotics on efflux pump gene expression revealed clear opposing regulatory patterns across both *A. baumannii* isolates. Treatment with the MIC concentrations of antibiotics alone (Colistin and Cefotaxime) consistently caused a significant upregulation (increased expression) of the *adeB* efflux pump gene (Figure 3), indicating a strong bacterial

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defense response [22]. In stark contrast, treatment with the probiotic agent alone consistently resulted in the downregulation (decreased expression) of gene. Most importantly, the combination of a sub-MIC antibiotic dose with the probiotic maintained this powerful downregulation of *adeB* across all tested conditions. This suppression of efflux pump activity is the likely molecular basis for the observed potentiated antimicrobial effect and the successful re-sensitization of the highly resistant strains.

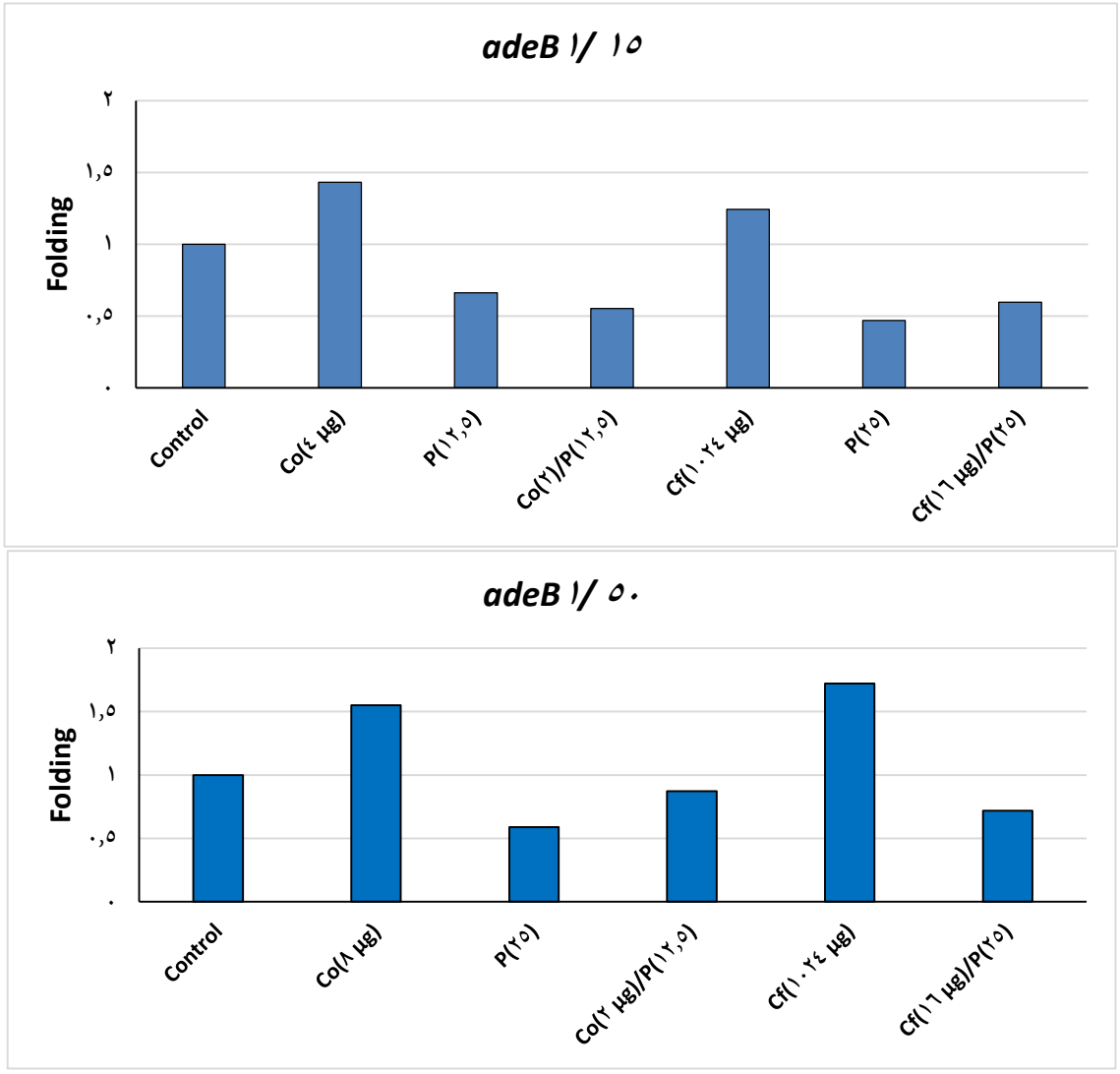

Figure 3: Gene expression of *adeB1* gene in both isolates (15 and50). Where CO: Colistin, P: Probiotic, CO/P (combined of antibiotic with probiotic), CF: cefotaxime.

The gene expression results provide a robust mechanistic explanation for the previously observed potentiating effect of the probiotic agent on the antibiotics, particularly the dramatic re-sensitization to Cefotaxime. Efflux pumps, such as those encoded by the *AdeABC* systems (*adeB*), are major mechanisms of Multidrug Resistance (MDR) in *A. baumannii*, working by actively pumping antibiotics out of the bacterial cell. The finding that Colistin and Cefotaxime alone trigger the upregulation of *adeB* confirms that the bacteria activate this primary defense mechanism in response to antibiotic stress. This

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upregulation is a key contributor to the high MICs seen for the antibiotics, especially the resistance to Cefotaxime (Sub-MIC1024µg/ml).

The probiotic agent consistently and powerfully downregulated efflux pump gene, whether used alone or in combination. This suggests the probiotic, or its active compounds, are acting as efflux pump inhibitors (EPIs). By suppressing the expression of *adeB*, the probiotic effectively disables a major line of defense for the bacteria. When the antibiotics are used in combination with the probiotic, the downregulation of efflux pumps persists where the *adeB* gene in strain15 that treated with (Cf/P combination) the gene expression fold was downregulation to (0.596 µg/ml) while the gene expression of same gene was (1.24 µg/ml) in same isolate. This genetic suppression allows the sub-MIC concentration of the antibiotic (16 µg/ml for Cefotaxime) to remain inside the bacterial cell long enough to be lethal, even though the same concentration would be immediately expelled if the efflux pumps were running at full capacity (as they are when the antibiotic is used alone). Therefore, the probiotic's ability to modulate the expression of the *adeB* efflux pump gene is the likely primary mechanism underlying the clinical outcome of antibiotic re-sensitization and dose reduction. Treatment with high concentrations of Colistin and Cefotaxime alone triggered a significant upregulation (Fold Change > 1) of the efflux pump gene, confirming the bacteria actively defend against the drugs. The combination therapy successfully overrode this bacterial defense response, maintaining the gene in a downregulated state. This demonstrates that the probiotic's modulating effect is dominant over the stress-inducing effect of the antibiotics, validating the use of the combination to achieve efficacy where antibiotics alone fail.

Conclusion

The study concludes a high rate of multidrug resistance representing 92.9% of isolates classified as MDR. The finding that 82.0% (41 out of 50) of the overall isolates are biofilm producers which provides the underlying mechanism for the high antibiotic failure rates. The probiotic agent, used alone or in combination, consistently caused a downregulation (Fold Change < 1) of the *adeB* efflux pump gene in both *A. baumannii* isolates. This direct suppression of major resistance machinery is the molecular mechanism by which the probiotic re-sensitizes the bacteria, as it allows the sub-MIC concentrations of the antibiotics to remain inside the cell.

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Author's Declaration

- We hereby confirm that all the Tables in the manuscript are original and have been created by us.

- This study was approved by the Ethics Committee of the College of Biotechnology, Al-Nahrain University.

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