Synergistic Effects of Probiotics and Ciprofloxacin on Gene Expression in Klebsiella Pneumoniae Isolated from Urine and Sputum Specimens

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KEYWORDS

Klebsiella pneumoniae, Gene expression, MDR, Probiotic, q-Rt PCR.

ABSTRACT

Background: Antibiotic resistance is associated with efflux pumps and resistance genes, among the significant threats to health systems worldwide. The efflux pumps and resistance genes that distinguish many antibiotics are called Multidrug Resistance (MDR), and probiotics are essential treatments for some bacterial inflammation caused by *K. pneumonia*. Our research, which takes a unique approach by investigating the prevalence of *Klebsiella pneumoniae* bacteria that cause different clinical infections, has the potential to impact the field of antibiotic resistance studies significantly. By identifying the (*Acr*A and *gyr*A) genes using the polymerase chain reaction and exploring the synergistic effect of probiotics and Ciprofloxacin antibiotics on some virulence genes in *K. pneumoniae*, we aim to contribute to the understanding of antibiotic resistance mechanisms and potential new treatment strategies. **Our methods** were meticulously designed and executed, underscoring the reliability of our results. From December 2022 to June 2023, we collected 199 samples from patients with urinary tract infections (UTIs) and sputum at Baquba Teaching Hospital and Al-Batoul Maternity Hospital in Diyala Governorate, Iraq. The nucleic acid DNA of the bacterial isolates under study was carefully extracted, and then the polymerase chain reaction (PCR) was performed using specialized gene primers (*Acr*A and *gyr*A). The RNA of the bacterial isolates was extracted, and then quantitative real-time (q-Rt PCR) was performed to detect gene expression by the *Acr*A and *gyr*A genes. **Results:** The results of antibiotic sensitivity showed that all isolates were 100% resistant to Ampicillin, 87.71% Cefoxitin, 64.91% Trimethoprim-sulfamethoxazole, 50.87% Amikacin, 35.08% Levofloxacin, 26.31% Imipenem, and 22.80% Ciprofloxacin. The study results showed that all bacterial isolates (22) obtained the genes (*Acr*A and *gyr*A) in percentage 100%. The current study showed an inhibition in the gene expression of the *Acr*A and *gyr*A genes; there was an inhibition in gene expression for the two isolates (U4, S2) after treatment with probiotics and a synergistic of probiotics and ciprofloxacin and compared to Control. **Conclusion**: Most *Klebsiella pneumoniae* isolates under study were isolated from patients with (UTIs) and sputum with multiple antibiotic resistance (MDR). All bacterial isolates possessed the genes (*Acr*A and *gyr*A) in a percentage of (100%), and there was inhibition in the gene expression of the *Acr*A and *gyr*A genes after treatment with probiotics and a synergistic of probiotics and ciprofloxacin.

1. Introduction

Klebsiella pneumoniae is a gram-negative bacterium that belongs to the Enterobacteriaceae family and is described as a non-motile encapsulated bacterium found in different environmental niches and has been associated with patient populations with pneumonia, diabetes mellitus, or alcohol use disorder. Mucosal surfaces of the gastrointestinal (GI) tract and the oropharynx of humans are typically colonized by the bacterium. It can display high virulence and antibiotic resistance when the bacterium enters the body ((1. Klebsiella pneumoniae is an opportunistic, nosocomial pathogen bacterium; it often infects immunocompromised individuals in hospitals and is characterized by a selective agar medium; it grows readily without motility and can ferment lactose (2). Klebsiella species are Gram-negative, facultatively anaerobic, encapsulated, non-spore-forming, Non-flagella, catalase positive, and oxidase negative. Though they may develop in various temperatures, the ideal temperature is 37°C, and the IMViC test (Indol, Methyl red, Voges-Proskauer, Citrate) and urease tests vary across the species. (3) Klebsiella pneumoniae exists as a saprophyte in the intestinal tract and nasopharynx of the human body (4). The typical location of contamination is the urinary tract. Klebsiella is responsible for 6 to 17% of all nosocomial urinary tract infections (UTI). Definite sets of victims, e.g., individuals having diabetes mellitus or neuropathic bladders, show an even greater risk of incidence (5). K. pneumoniae infections have significantly increased morbidity and death in people with chronic illnesses. The ability of K. pneumoniae to cause illness depends on various virulence factors; Thus, the coexistence and expression of virulence factors and genetic drivers of antibiotic resistance complicate treatment results. Thus, the evolution of pathogenic multi-drug-resistant (MDR) K. pneumoniae poses a significant danger to the healthcare system (6). In recent years, the United States reported that K. pneumoniae is the most common cause of hospital-acquired pneumonia, representing 3% to 8% of all nosocomial

bacterial infections. This activity reviews the treatment and evaluation of K. pneumonia patients and the inter-professional team's role in managing this condition of patients (7).

Gene expression refers to how information is conveyed kindly from others, which is later translated into protein production. One of the axioms of mRNA affects DNA. Blessed life taught all types of expression to be functionally effective; the first step of that is complementary RNA) the Expression of a gene being transcribed into a complementary RNA strand, which are (rRNA) and ribosomal RNA (tRNA) and some encode for transfer RNA Clones are essential. Still, they are not translated into protein products, while clones for other uses are translated into Protein molecules.

2. Methodology

Specimens Collection

After the specialist doctor examined the patients, 199 samples were taken from patients with UTIs and sputum, both sexes from 1 to 75 years of age, and from Al-Batoul Teaching Hospital, Baquba General Hospital, and educational laboratories between December 2022 and June 2023. During this period, they included the patients who were reviewed and hospitalized. To isolate the bacteria and determine their original identity, the samples were inoculated on various media and then incubated for 24 hours at 37 °C.

Bacterial isolation

The samples were cultured on MacConkey Agar, methylene Eosin Blue Medium, and HiCrome Agar and incubated for a full day at 37 °C. *Klebsiella pneumoniae* was identified based on the morphological features of the culture media, such as size, shape, color, texture, and edges. In addition, manual biochemical tests used Gram staining, Catalase, Oxidase, and the Urease Enzyme Production Test[(8)]-for final confirmation biochemical tests embedded in the VITEK2 compact system.

Antibiotics susceptibility test

The susceptibility of isolates to antimicrobial agents was tested using the disks method of Mueller-Hinton agar based on CLSI [(9)]. The antibiotics in this study are Ampicillin (10 µg), Cefoxitin (30 µg), Imipenem (10 µg), Amikacin (30 µg), Levofloxacin (5 µg), Ciprofloxacin (5 µg), and Timethoprime-sulfamethouxazole (1.25/23.75 µg).

Molecular identification by *16SRNA* gene

Klebsiella pneumoniae was diagnosed using microscopic identification, cultural identification [(10)], and biochemical identification [(11)]. To confirm the diagnosis at the species level of *K. pneumoniae* at the molecular level by using a pair of DNA primers to amplify a specific *16SRNA* gene by polymerase chain reaction (PCR), the nucleic acid (DNA) was extracted using the Nanodrop device, where the concentration of the nucleic acid ranged between (122-187) ng/µ1, while its purity was from (1,8-2,1) As the gene (*16SRNA*) was amplified to the size of (95) base pair.

Extraction of total DNA and PCR amplification

DNA was extracted from bacterial isolates of *Klebsiella pneumoniae* bacteria under study using Gene aid Genomic DNA Purification, which were previously grown on nutrient broth medium and incubated for 24 hours at 37 °C. The gene *Acr*A (495bp) was amplified using the specific primer [(12)] F (ATGAACAAAAACAGAGG) R (TTTCAACGGCAGTTTTCG). The gene *gyr*A (441 bp) was amplified using the specific primer [(13)] F (CGCGTACTATACGCCATGAACGTA) and R-(ACCGTTGATCACTTCGGTCAGG) and the PCR Master Mix Components (Geneaid Accupower®Profi Taq PCR Premix) that Containing every other Component which necessary for Polymerase Chain Reaction (PCR) reaction, Taq DNA Polymerase, $MgCl_2$, Tris- HCl, PH_9, KCl as land sliding in Table (1)

Table 1: Components for the polymerase chain reaction (PCR).

Components	**The volume of the reaction mixture for one tube (µL)**
Master Mix PCR solution	10
Forward primer	1
Reverse primer	1
Template DNA	2
Deionized Nuclease Free Water ~~14~~	6
Total volume	20

PCR tubes were prepared under the manufacturer's instructions. Then, it was transferred to the centrifuge at 1300 revolutions per minute (rpm) for 3 minutes and placed in the PCR Thermocycler. PCR condition for ***AcrA*** gene included. The denaturation at 95°C for 5 minutes to *Acr*A followed by 30 cycles, Annealing at 52°C for 30 seconds, Extension at 72°C for 1 minute, and final extension cycle for 7 minutes at 72°C. For the ***gyr*A** gene, the reaction conditions are included: denaturation at *95*°C for 5min for ***gyr*A** followed by 30 cycles, Annealing at 55°C for 30 sec, Extension at 72°C for 1 min, and Final Extension cycle for 7 min at 72°C. The PCR product was transferred onto an agarose gel with a concentration of 1.5% and then photographed by UV. A digital camera captured the image and documentation.

RNA purification and extraction

RNA was extracted from 2 isolates: (U4) isolated from Urine and (S2) isolated from Sputum, according to the detector instructions T. Rizol™. The RNA extract concentration was determined by measuring absorbance using the Quants fluorometer.

Detection of gene expression by quantitative real-time PCR of *Acr*A and *gyr*A gene

To confirm and select the highest and lowest resistance isolates through gene expression, which is determined and based on comparing the values of mean CT of *Acr*A and *gyr*A genes and confirmed normalized by Folding = $2^{-\Delta\Delta CT}$, where the ΔCT is the difference in CT threshold cycle between the target and reference gene obtained from quantitative real-time -PCR for *Acr*A and gyrA (target gene) and 16SRNA (reference gene). The standard protocol was applied on extracted RNA with TRIzol™ GoTaq®(Syber green) Master Mix (1-Step RT-qPCR) System used according to manufacturer's instructions and applied to a single reaction-where only template. RT-PCR performed using q PCR Master mix 5µl, 0.25µl of each RT mix and $MgCl_2$, 0.5µl of each forward and reverse primer, 2.5µl of nuclease-free water and 1µl of RNA. *16S* rRNA (reference gene) [14] F: 5′ GACGATCCCTAGCTGGTCTG-3′and R: 5′ GTGCAATATTCCCCACTGCT-3′. The RT-PCR was performed using the following conditions: RTenzyme activation at 37 for 15 min, one cycle. Initial denaturation (95 °C, 10 min), the 40 cycle of denaturation (95 °C, 20s). The annealing temperature is for the 20s, and the extensional temperature is 72oC for the 20s. Calculate gene expression using the equation.

Folding = $2^{-\Delta\Delta CT}$

$$\Delta\Delta CT = \Delta CT_{Treated\ with\ probiotic} - \Delta CT_{Un\ treated\ with\ probiotic}$$

$$\Delta CT = CT_{Targetgene} - CT_{referencegene}$$

3. Result and Discussion

Isolation and Identification of Klebsiella pneumoniae

Our study samples were collected from urine and sputum at Baquba Teaching Hospital, Al-Batoul.

The results of the bacterial culture on the MacConkey medium were based on the source of the sample. In the urine, out of a total of (121) samples showed (86) were positive for culture at a rate of (71%), and in the sputum, out of a total of (78) samples, showed (40) at a rate of (51.3%) samples were positive for culture. The number of Klebsiella pneumoniae bacteria isolated from urine samples was 35 (28.92%), and the number of K. pneumoniae bacteria isolated from sputum samples was 15 (19.23%).

Antibiotic susceptibility tests for bacterial isolates of Klebsiella pneumoniae

All isolates of Klebsiella pneumoniae under study were tested for antibiotic susceptibility patterns and tested seven antibiotics: Ampicillin, Cefoxitin, Imipenem, Amikacin, Levofloxacin, Ciprofloxacin, Trimethoprim-sulfamethoxazole, which is most commonly used to treat K. pneumoniae infections. The bacterial isolates were resistant to Ampicillin (100%), Cefoxitin (87.71%), Imipenem (26.31%), Amikacin (50.87%), Levofloxacin (35.08%), Ciprofloxacin (22.80%), Trimethoprim-sulfamethoxazole (64.91%).

Molecular diagnosis of Klebsiella pneumoniae using the 16S rRNA gene

The results of the current study showed that 22 (100%) bacterial isolates carrying the (16S rRNA) gene, which depends on the primer used for the gene (16S rRNA), as the gene has the size of (95bp) compared with the DNA ladder that has bundles of known molecular size.

Molecular diagnosis of Klebsiella pneumoniae using the acrA gene The results of the current study showed that 22 (100%) bacterial isolates carrying the (acrA) gene, which depends on the primer used for the gene (acrA), as the gene has the size of (495bp) compared with the DNA ladder that has bundles of known molecular size. As in Figure (1).

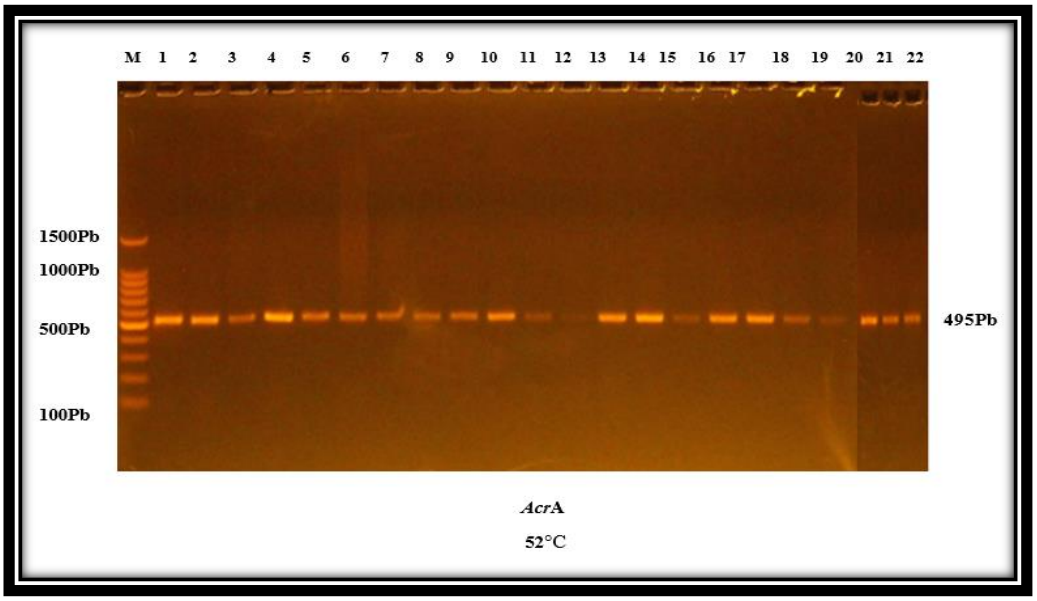

Fig 1: Electrophoresis of the *acr*A gene in *K. pneumoniae* bacteria using an agarose gel concentration of 1.5% containing 1µ of Ethidium bromide dye and by using a DNA ladder from (100-2000) base pairs, as the path is shown in the two figures The presence of the *acr*A gene in all samples.

Molecular detection of the DNA polymerase chain reaction product of the *gyr*A gene

The results of the molecular detection of the *gyr*A gene, which has a size of (441) base pairs, using the PCR technique and the Thermocycler showed that 22 (100%) bacterial isolates possess the *gyr*A gene, as shown in Figure (2).

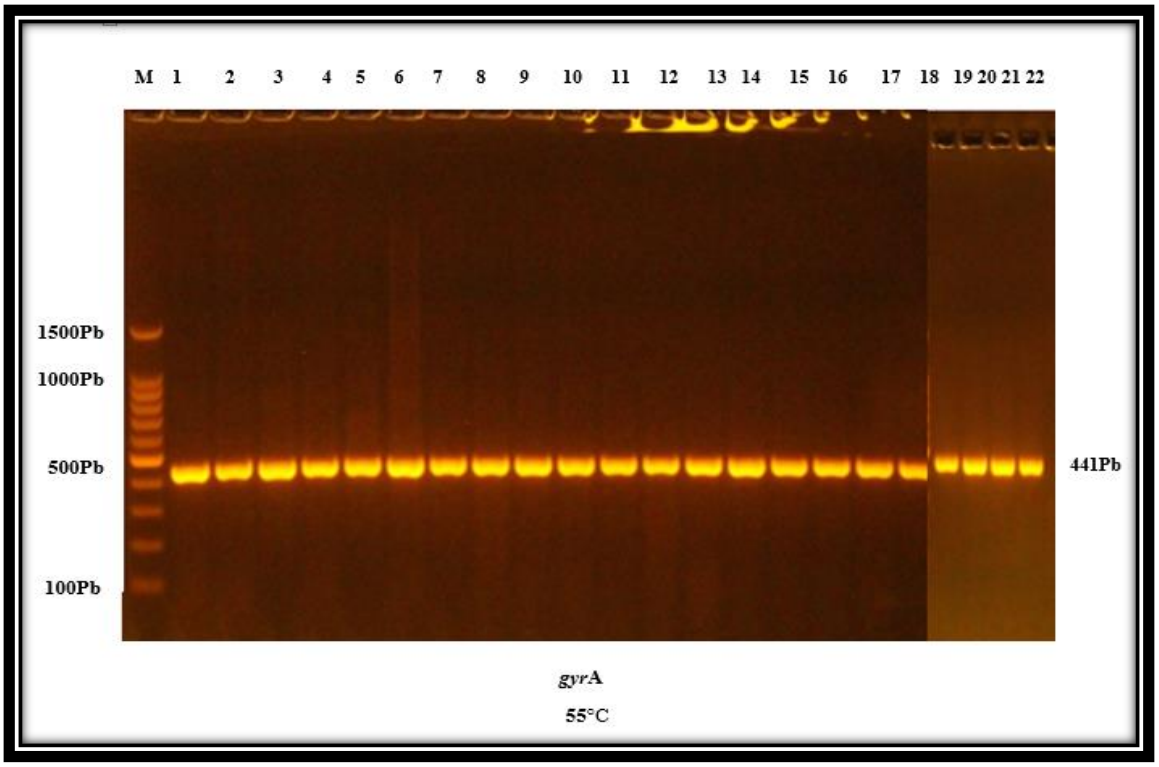

Fig 2: Electrophoresis of amplification products of the *gyr*A gene in 1.5% agarose gel and a voltage of 100 volts for an hour using a DNA Ladder (100bp-2000bp) as it appears in the M track and start from 100 base pairs, the bands represent the gene packages of *K. pneumoniae* bacteria with a size of 441 bp.

Real-time PCR Quantification of *Acr*A and *gyr*A Genes Expression

The experiment of the quantitative RT-PCR reaction

was completed by selecting (2) of the (22) *K. pneumoniae* isolates. The source of these isolates was urine and sputum. The gene gave a high degree of expression in these two isolates. This variation refers to the source of the isolates. The cycle of threshold (Ct) value of the *Acr*A and *gyr*A genes in the present study is shown in Tables (1a, b and 2a, b).

Table (1a): Ct values and fold of gene expression of *Acr*A gene of *K. pneumoniae* treated Probiotics

SOURS	Klpn-16s	*Acr*A	ΔCT	ΔΔCt	Folding Change2^-ΔCt
S2	6.95	16.41	9.46	0.00	1.00
S2P	7.98	23.16	15.18	5.72	0.02
U4	7.08	20.95	13.88	0.00	1.00
U4P	8.18	25.75	17.58	3.70	0.08
P-value	**P<0.05***				

Table (1b): Ct values and fold of gene expression of *Acr*A gene of *K. pneumoniae* treated with Synergistic Ciprofloxacin antibiotic and Probiotics.

SOURS	Klpn-16s	*Acr*A	ΔCT	ΔΔCt	Folding Change2^-ΔCt

U4	10.55	21.58	11.02	0.00	1.00
UC+P1	10.06	23.18	13.11	2.09	0.23
S2	9.02	23.33	14.32	0.00	1.00
SC+P1	8.18	23.36	15.18	0.86	0.55
P-value	**P<0.05***				

Table (2a): Ct values and fold of gene expression of *gyr*A genes of *K. pneumoniae* treated with Probiotics.

SOURS	**Klpn-16s**	***gyr*A**	**ΔCT**	**ΔΔCt**	**Folding Change2^-ΔCt**
S2	6.95	18.62	11.67	0.00	1.00
S2P	7.98	19.99	12.01	0.34	0.79
U4	7.08	18.08	11.00	0.00	1.00
U4P	8.18	21.74	13.57	2.57	0.17
P-value	**P<0.05***				

Table (2b): Ct values and fold of gene expression of *gyr*A gene of *K. pneumoniae* treated with Synergistic Ciprofloxacin antibiotic and Probiotics

SOURS	**Klpn-16s**	***gyr*A**	**ΔCT**	**ΔΔCt**	**Folding Change2^-ΔCt**
U4	10.55	20.21	9.65	0.00	1.00
UC+P1	10.06	21.77	11.70	2.05	0.24
S2	9.02	23.46	14.45	0.00	1.00
SC+P1	8.18	23.65	15.47	1.02	0.49
P-value	**P<0.05***				

DISCUSSION

The results of the current study were higher than the percentage obtained by the researcher [15]. The rate of *Klebsiella pneumoniae* bacteria isolated from urine samples was (10%).

The results of the current study were less than the percentage obtained by the researcher [16]**.** The percentage of *K. pneumoniae* bacteria in urinary tract infections (UTIs) was (46%), which is close to the results of the current study. The reason for the differences in the results of the percentage distribution of *K. pneumoniae* isolates is due to a variety of variables, including differences in the environment from which the isolate was collected, the health conditions of the patients, the type of samples, the source of the isolate, the time of sampling, the geographical region, and other causal variables. For the differences between readings of this type.

The results of the current study regarding the antibiotic ampicillin were consistent with the findings of the researcher [17] in Diyala Governorate, where her study included 50 isolates of *K. pneumoniae* bacteria to verify

their ability to resist antibiotics, and the results showed that the rate of resistance to the ampicillin is 100%. The results of the current study also agreed with the results of the researcher [18], as the percentage of resistance to ampicillin was (100%). The antibiotic ampicillin targets the manufacture of the cell wall. At the same time, the results of the current study were inconsistent with the findings of the researcher [19] in Indonesia, where the rate of ampicillin resistance was (75%). The resistance of this bacteria to antibiotics converges, consistent with the study's findings [20]. The percentage of resistant *K. pneumoniae* bacteria to Cefoxitin and Imipenem antibiotics was (83.3 % and 33.3%), respectively.

The results of the study regarding Amikacin were inconsistent with the findings of the researcher [21,22]. as the rate of resistance to Amikacin was (100%) and (10%) respectively. It was in agreement with the results reached by the researcher [23]. where the percentage of resistance to the antibiotic Amikacin was (47.82%).

The results of the current study for Levofloxacin and Ciprofloxacin antagonists are consistent with the results reached by the researcher [24]. where it was found that *Klebsiella pneumoniae* was resistant to Levofloxacin and Ciprofloxacin antagonists, with a resistance rate of (56%) and (32%); however, it did not agree with the results reached by the researcher [25], as the percentage of bacterial resistance to Levofloxacin was (62.7%).

The results of the current study about the Trimethoprim- Sulfamethox antibiotic, the percentage of *Klebsiella pneumoniae* resistance to this antibiotic was (64%), as the results of this study were not consistent with the results reached by the researcher [26]. as the resistance rate to the Trimethoprim- Sulfamethox antibiotic in *Klebsiella pneumoniae* was (38.2%). The results of the current study were consistent with those reached by the researcher [27], as the percentage of resistance to this antibiotic was (60%).

The current study's results could have been more consistent with the researcher's [13] results, as the *gyr*A gene was present in *Klebsiella pneumoniae* (100%). However, the study's results disagreed with many other researchers, such as the findings of the researcher [28], who discovered that the presence of the *gyr*A was (80%). The results of the molecular detection of the *acr*A gene show that all isolates possess this gene. This result agrees with a researcher [29] Since the ratio of its isolates was 100%. However, this study's findings disagreed with the researcher's (30) opinion, with the percentage of this gene being 50%.

According to the quantitative real-time polymerase chain reaction (qRT-PCR) results of the genes (*gyr*A and *Acr*A), it is noticed there was a change in the Ct of the gene after the treatment, and this led to a decrease in the expression in these genes. Gene expression using this technique for selected multi-resistant *K. pneumoniae* isolates from different sources. These isolates were (U4) Isolated from Urine and (S2) isolated from sputum, and after calculating the value of Folding = $2^{-\Delta\Delta CT}$, the results showed that all of these genes showed gene expression, as the average value of Folding was (1). To determine the effect of probiotics on the gene expression of the gene *gyr*A for the two isolates, and after treating them with a series of dilutions of the probiotic, the minimum sub-inhibitory concentration (subMIC) value was calculated. The current study showed an inhibition in the gene expression of the *Acr*A gene; there was an inhibition in gene expression for the two isolates (U4, S2) after treatment with probiotics and compared to Control. Gene expression results for the identical two isolates after treatment with probiotics and a synergistic of probiotics and ciprofloxacin were compared to the two isolates before treatment, as there was an inhibition in gene expression for the isolate (U4, S2). After treating them with probiotics and a synergistic of probiotics and ciprofloxacin, the average value was Folding= 2^ (- ΔΔCT) for the two isolates before treatment (1) After treating the two isolates (U4, S2) with probiotics 0.02 and 0.08, after treating the identical two isolates with a synergistic of probiotics and ciprofloxacin 0.55 and 0.23.

As for the *gyr*A gene for the two isolates (U4, S2) after treatment with probiotics and compared to Control, gene expression results for the identical two isolates after being treated with probiotics and a synergistic of probiotics and ciprofloxacin were compared to the two isolates before treatment, as there was inhibition in gene expression for the isolate (U4, S2), after treating them with probiotics and a synergistic of probiotics and ciprofloxacin. The average value of Folding = 2^ (- ΔΔCT) for the two isolates before treatment (1) and after treating the two isolates (U4, S2) with probiotics was 0.79 and 0.17, and after treating them with a synergistic of probiotics and ciprofloxacin 0.49 and 0.24.

To our knowledge, this is the first study to report the detection of gene expression for the (*gyr*A and *Acr*A) gene in Iraq after being treated with probiotics and a synergistic combination of probiotics and ciprofloxacin, so there are no available results for comparison. On the other hand, the current study's results were partially consistent with [31], as it found that *Escherichia coli* bacteria were inhibited after treatment with the probiotic.

4. Conclusion and future scope

most isolates of Klebsiella pneumoniae bacteria under study were isolated from patients with urinary tract infections (UTIs) with multiple antibiotic resistance (MDR). All bacterial isolates possessed the genes (gyrA and AcrA) in a percentage of (100%), and the two isolates (U4, S2) were inhibited after treatment with the probiotic and a synergistic of probiotics and ciprofloxacin.

Recommendations

additional molecular research will be performed to identify changes in the local K. pneumoniae patterns, and the gene expression of capsular and beta-lactam genes will be studied in the local isolates of K. pneumoniae in clinical and environment in Iraq. Moreover, we are Studying phylogenetic analysis (tree of genomics) of K. pneumoniae isolated from different environmental cases for detected mutation.

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