ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 18, Issue 11, 2025

Online - 2455-3891
Print - 0974-2441

Research Article

SYNERGISTIC ANTIBACTERIAL EFFECTS OF CIPROFLOXACIN, CEFTAZIDIME, AND BACTERIOCIN COMBINATIONS AGAINST MULTIDRUG-RESISTANT *ESCHERICHIA COLI*

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Received: 16 August 2025, Revised and Accepted: 27 September 2025

ABSTRACT

Objectives: Antibiotic-resistant bacteria are an irritating health issue that has killed lots of people in recent years. The developing resistance of bacteria to new antibiotics, including misuse by patients, is a major issue for medication industries. Researchers are discovering alternatives to developing new antibiotics, such as generating combinations of antibiotics or biological mixtures to inhibit bacterial growth. The study aimed to determine the effect of the combination of antibiotics (ciprofloxacin [CIP] with ceftazidime [CAZ]) and each antibiotic CAZ/CIP with bacteriocin on the *Escherichia coli.*

Methods: A total of 30 samples were collected from patients with urinary tract infections and burn injuries for this study. After determining all isolate species via the Vitek2 system, the minimum inhibitory concentration values were determined for CIP/CAZ and CIP/bacteriocin, CAZ/bacteriocin combination on 10 Multidrug-resistant (MDR) *E. coli*. Moreover, the expression level of *Gyrase A* and *Gyrase B* genes from 10 selected isolates of *E. coli* was measured using quantitative real-time polymerase chain reaction.

Results: The antibacterial activity showed a highly significant difference in isolates treated with CIP/CAZ and CIP/bacteriocin, CAZ/bacteriocin combination compared to those treated with each antibiotic alone ($p \leq 0.05$). The gene expression for *Gyrase A* and *Gyrase B* revealed a significant reduction when using CIP/CAZ and bacteriocin with each antibiotic compared to control isolates ($p \leq 0.05$).

Conclusion: The combination of CIP/CAZ and bacteriocin with CIP and CAZ exposed a significant effect against MDR isolates; initial new approaches in combating MDR *E. coli*, particularly when combined with highly effective biological agents such as peptides or adjuvant.

Keywords: Multidrug-resistant *Escherichia coli*, Ciprofloxacin, Ceftazidime, Bacteriocin, Combination therapy, Gyrase A and Gyrase B expression, Antimicrobial resistance, Synergistic antibacterial activity.

 DOI: http://dx.doi.org/10.22159/ajpcr.2025v18i11.56546. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Multidrug-resistant (MDR) bacteria are usually recognized as one of the most significant public health problems of our day. Increasing rates of antibiotic resistance affect all aspects of modern medicine and risk the results of surgical, transplant, and cancer treatment [1,2]. Bacteria are classified as MDR when resistant to at least one agent from three or more antimicrobial classes [3]. *Escherichia coli* is the most prevalent type of MDR bacteria. *E. coli* is also the most common pathogen related with urinary tract infections (UTIs) and burn injuries [4].

Extensive research has shown that the most promising approaches to creating multidrug formulations to combat antimicrobial resistance are dual drug delivery strategies [5]. The most effective way to restore the efficacy of existing antibiotics while reducing the required drug concentrations is to combine the multidrug formulation approach with topical drug delivery [6]. Ciprofloxacin (CIP) is an antimicrobial agent belonging to the fluoroquinolone class. It is an antibiotic with bactericidal properties, indicating that it eliminates bacteria rather than merely inhibiting their growth. CIP inhibits enzymes that play a crucial role in the central dogma [7]. Ceftazidime (CAZ), a third-generation cephalosporin antibiotic, interacts with penicillin-binding proteins (PBPs) – particularly PBP3 – leading to disruption of the cell wall structure [8].

MDR had developed different mechanisms action one of these is gained another trait from another bacteria for example (resistance genes were acquired by horizontal gene transfer) [9], change in active site in protein that make cell wall which prevent antibiotic activity for example (dihydrofolate reductase site modification produce trimethoprim resistant bacteria) [10], another type in bacteria is inactivation of some particular genes which may be a major target for the antibiotics for example alterations in DNA gyrase and topoisomerase IV [11]. The combination of two or more antibiotics with adjuvants or with other molecules that have no antibiotic activity has an extensive effect against MDR bacteria [12]. Combining antibiotics with adjuvants is a prominent approach to prevent the challenges posed by MDR [13]. The combination of CIP and CAZ, when utilized at subminimal inhibitory concentrations (sub-MICs), effectively delayed the emergence of resistance in comparison to the use of either drug independently[14].

In this research, we carried out extensive investigations using an MDR *E. coli* strain. Through *in vitro* testing for antimicrobial susceptibility, we assessed the MIC values for the following combinations: Antibiotic–antibiotic and antibiotic with biological compounds with antibiotic activity. To assess the effects of the specified combinations, we isolated the mRNA, assessed its purity and concentration, and then converted it into cDNA. Gene expression was measured using real-time quantitative polymerase chain reaction (RT-qPCR).

METHODS

Sampling

Patients with symptoms of UTIs and wound discharge provided 30 samples for collection. All the isolates were transferred under cooling

conditions by transport media for bacterial transfer and diagnosis. All isolates were identified by selective mediums such as eosin methylene blue and MacConkey agar, and then the isolates were cultured on nutrient broth to be identified using the Vitek2 Compact System. After bacteria were identified, an antibiotic sensitivity test was performed on all identified samples to determine the numbers and types of bacteria resistant to antibiotics [15].

Production and purification of bacteriocine

The process of selecting the isolate capable of producing bacteriocin, as well as the detailed process of its production, extraction, purification, and measuring its effectiveness against pathogenic bacteria, was carried out and published in the previous research paper [5].

Determination of MICs for each combination

Following the antimicrobial susceptibility test for six types of antibiotics against 30 *E. coli* samples, 10 samples were identified as MDR. Notably, the resistance of bacterial isolates to CIP and CAZ was 100% across the six types of antibiotics. First, these two antibiotics were mixed at varying concentrations (20, 30, 40, 50, 60, 70, 80, and 90 µg/mL) to obtain MICs. The test was performed on microtiter plates. The plates were kept in an incubator overnight at 37°C. After incubation, at 260 nm, the results were read on the ELISA reader. Second, each one of the antibiotics was tested in concentrations (30, 40, 50, and 60 µg/mL) combined with bacteriocin (125 µg/mL), this concentration of bacteriocin considered the MICs according to our previous research [5]. The test was also performed on microtiter plates. The plates were also kept in the incubator overnight at 37°C. After incubation, the results were read on the ELISA reader at 260 nm.

Determination of fractional inhibitory concentration (FIC) for each antibiotic combination

The synergistic effect between CIP and CAZ was evaluated using the checkerboard microdilution method. The MIC of each antibiotic was determined individually using broth microdilution in 96-well plates. Serial dilutions of both antibiotics were combined in a checkerboard layout. Each well contained a unique concentration pair and was inoculated with a standardized bacterial suspension. Plates were incubated at 37°C for 18–24 h.

FIC calculation

- $FIC_{CIP}=MIC_{CIP}$ in combination/MIC_{CIP} alone
- $FIC_{CAZ}=MIC_{CAZ}$ in combination/MIC_{CAZ} alone.

Interpretation

A ΣFIC ≤ 0.5 indicates synergism, confirming enhanced activity when CIP and CAZ are used together.

***E. coli* gene expression study under each combination**

The expressions of the (*GyrA* and *GyrB*) genes were examined under the influence of each combination treatment. The analysis of the molecular expression was done using RT-qPCR. In summary, the RNeasy Mini Kit (Qiagen) was used to extract the total RNA from each isolate in compliance with the significantly altered manufacturer's operating instructions. Fermentas, USA's DNase was used on the extracted RNA to remove genomic DNA. RNA purity was evaluated using formaldehyde-denaturing 1.2% (w/v) agarose gel electrophoresis.

Nucleic acid concentrations and absorbance ratios at A260/A280 and A260/A230 were measured using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). Follow the manufacturer's instructions to convert 0.5 µg of total RNA into single-stranded cDNA using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and random hexamer oligonucleotides (Fermentas). From the produced cDNA, the *GyrA* and *GyrB* genes were amplified. The primer sequences are indicated in Table 1. A Bio-Rad MiniOpticonTM device was utilized to perform real-time PCR using the Fermentas SYBR Green qPCR Master Mix. The General thermocycler program and reaction conditions used in this study are initial

Table 1: The study used primers, along with their oligonucleotide sequences and amplicon sizes

Gene names	Sequences (5'-3')	Product size (bp)
GyrA	F: GCCATGAACGTACTAGGC R: GGATATACACCTTGCCGC	180 bp
GyrB	F: AGAAATTATCGTCACCATTCACGC R: GTACACCGTGTTCGTAGATCT	278 bp
GAPDH Housekeeping gene	F: ACTTACGAGCAGATCAAAGC R: AGTTTCACGAAGTTGTCGTT	170 bp

denaturation at 95°C for 3 min, denaturation at 95°C for 10 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min for each primer. The comparative Ct approach ($2^{-\Delta Ct}$ formula) was used to evaluate the expression levels of the *GyrA* and *GyrB* genes after normalization with the *GADPH* gene.

Statistical analysis

Statistical analysis and visual representation were done using SPSS 27 and GraphPad Prism 10, and a t-test was used to find significance between the two groups. In comparison, analysis of variance is used to find significance among three or more groups, and the p-value is considered significant if $p<0.05$.

RESULTS

Isolated bacteria

The laboratory culture results indicated that all the samples were *E. coli*, whereas the others were not. For more accuracy, after culturing the bacterial isolates, they were diagnosed by the Vitek2 compact system. The results obtained from this system agreed with those obtained from culture identification in our study, which indicated that they were *E. coli* at a probability of 99%.

The synergistic effect between CIP and CAZ

The MIC values of CIP and CAZ were determined individually and in combination using the checkerboard assay. The MIC of CIP alone was 200 µg/mL, whereas CAZ alone showed an MIC of 260 µg/mL. When used in combination, both antibiotics exhibited reduced MIC values of 50 µg/mL each.

The calculated FIC values were 0.25 for CIP and 0.1923 for CAZ, resulting in a total FIC index of 0.4423. According to standard interpretive criteria, this value indicates a synergistic interaction between the two antibiotics against the tested bacterial strain.

Antibacterial activity determination for each combination

Ten isolates that showed MDR against antibiotics were tested for the combination of CIP and CAZ at various concentrations against bacteria. The MICs of the CIP and CAZ combination were determined to be 50 µg/mL (Table 2). This concentration was chosen after testing in three replicates on the same sample. Notably, bacteriocin was already used at 125 µg/mL. The MIC values for the CIP/CAZ combination showed a clear dose-dependent reduction in bacterial growth. Starting from 0.193±0.05 at 20 µg/mL, the MIC progressively decreased to 0.099±0.04 at 90 µg/mL. The control group exhibited a significantly higher MIC (0.492±0.90), and the overall p-value (<0.001) confirms the statistical significance of the observed differences. These results demonstrate that the combination therapy is markedly more effective than monotherapy, suggesting a synergistic interaction between the two antibiotics.

In Fig. 1, the MIC analysis followed by *post hoc* testing revealed statistically significant differences between treatment groups, with a $p<0.001$. The use of alphabetical lettering to denote statistical similarity or difference underscores the precision of the comparisons: Groups sharing the same letter are statistically indistinguishable,

whereas those with different letters exhibit meaningful variation in antimicrobial efficacy. This suggests that CIP and CAZ differ in their inhibitory power, possibly due to distinct mechanisms of action or resistance profiles in the tested *E. coli* strain.

As well as in Fig. 2, which explores the impact of combining bacteriocin (125 µg/mL) with either CAZ or CIP at varying concentrations (30, 40, 50, and 60 µg/mL). The results demonstrate a clear dose-dependent inhibition of bacterial growth, with statistical significance marked by *** (p<0.05) and **** (p<0.001). These findings strongly support the hypothesis that bacteriocins enhance the antibacterial activity of traditional antibiotics, particularly at higher concentrations. The observed synergy may stem from complementary modes of action – bacteriocins disrupting membrane integrity while antibiotics target intracellular processes – leading to amplified bacterial suppression.

Fold of *Gyrase A* and *Gyrase B* gene expression of *E. coli* under the effect of each combination

The fold of gene expression was calculated for all bacterial isolates using ($2^{-\Delta Ct}$ formula).

Expression levels of *GyrA* were assessed across various treatment groups. The untreated control (1A) and single antibiotic treatments (1B and 1C) showed relatively high expression (ΔΔCt values of 1.05–1.33), indicating minimal suppression. In contrast, combination therapies – CIP/CAZ (0.25±0.12), CIP/bacteriocin (0.07±0.04), and CAZ/bacteriocin (0.16±0.13) – resulted in significant downregulation of *GyrA*, with p-values consistently below 0.05. These findings suggest that the combined treatments interfere with DNA replication by targeting the *GyrA* subunit, contributing to bacterial inhibition, as shown in Table 3.

Similar trends were observed for *GyrB* expression. The control and single treatments showed elevated levels (1.24–1.4), whereas combination therapies significantly reduced expression: 0.22±0.1 for CIP/CAZ, 0.11±0.1 for CIP/bacteriocin, and 0.12±0.09 for CAZ/bacteriocin. All comparisons yielded highly significant p-values (<0.001), indicating strong transcriptional suppression. Given *GyrB*'s role in ATP-dependent DNA supercoiling, its inhibition further compromises bacterial viability and supports the efficacy of the treatment combinations as shown in Table 4.

Figs. 3 and 4 delve deeper into the molecular consequences of these treatments by examining the relative expression of *Gyrase A* and *Gyrase B* genes using the 2-(ΔΔCt) method. Control groups included untreated bacteria and bacteria treated with either CIP or CAZ alone, whereas treatment groups combined these antibiotics with bacteriocins. The data reveal significant downregulation of both *Gyrase A* and *Gyrase B* in the combination therapies, again marked by *** and **** to denote statistical strength. These enzymes are essential for DNA replication and supercoiling, and their suppression indicates that the treatments not only inhibit growth but also interfere with vital genetic functions. The molecular evidence aligns with the phenotypic observations, reinforcing the therapeutic promise of these combinations. The decrease in gene expression indicates that the combinations used had a strong and effective role in reducing the growth or multiplication of *E. coli* compared to the gene expression of untreated isolates.

Table 2: Results of ciprofloxacin (CIP)/ceftazidime (CAZ) minimal inhibitory concentration (MIC) determination

Concentration (µg/ml)	MIC (Mean±SD)
	CIP/CAZ
20.00	0.193±0.193
30.00	0.169±0.169
40.00	0.147±0.147
50.00	0.105±0.105
60.00	0.103±0.103
70.00	0.101±0.101
80.00	0.100±0.100
90.00	0.09900.099
Control	0.492±0.90
p-value	<0.001

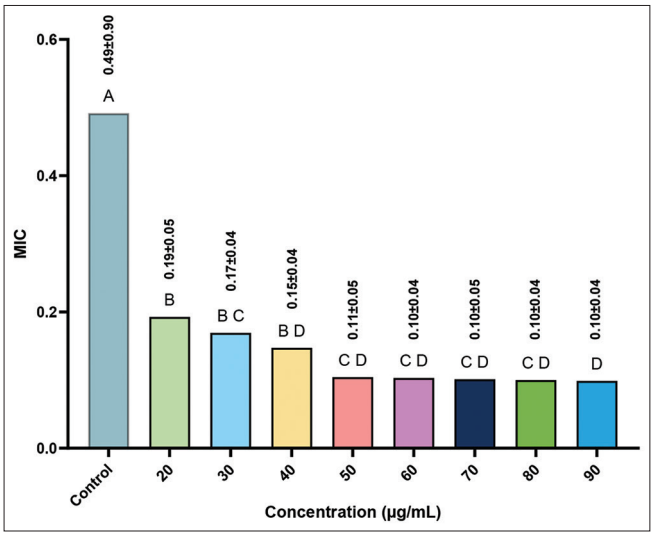

Fig. 1: Minimal inhibitory concentration means a *post hoc* test for ciprofloxacin/ceftazidime. p<0.001; Similar letters indicate no significant difference, whereas different letters indicate a significant difference

DISCUSSION

Antibacterial activity

The antibacterial activity results for the combination of CIP and CAZ showed a noticeable increase in bacterial inhibition against *E. coli* [15]. The MIC for the CIP/CAZ combination was determined to be 50 µg/mL. This concentration was selected after testing the combination in three replicates and performing statistical analysis. The combination therapy demonstrated a clear dose-dependent reduction in bacterial growth, with the MIC progressively decreasing from 0.193±0.05 at 20 µg/mL to 0.099±0.04 at 90 µg/mL. In comparison, the control group showed significantly higher MIC values (0.492±0.90), with a highly significant p-value (<0.001), confirming the statistical importance of the observed differences. These results indicate that the combination therapy is more

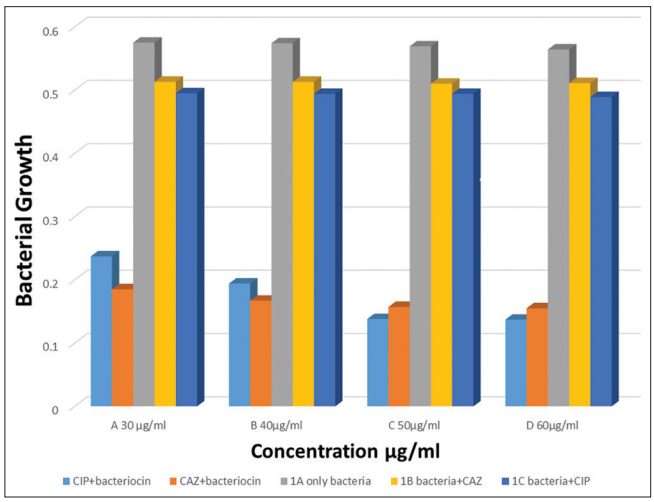

Fig. 2: Results of the combination between bacteriocin at concentration 125 µg/mL+ceftazidime and bacteriocin+ciprofloxacin at concentration (a) 30; (b) 40; (c) 50; and (d) 60 on the MDR *E. coli* growth

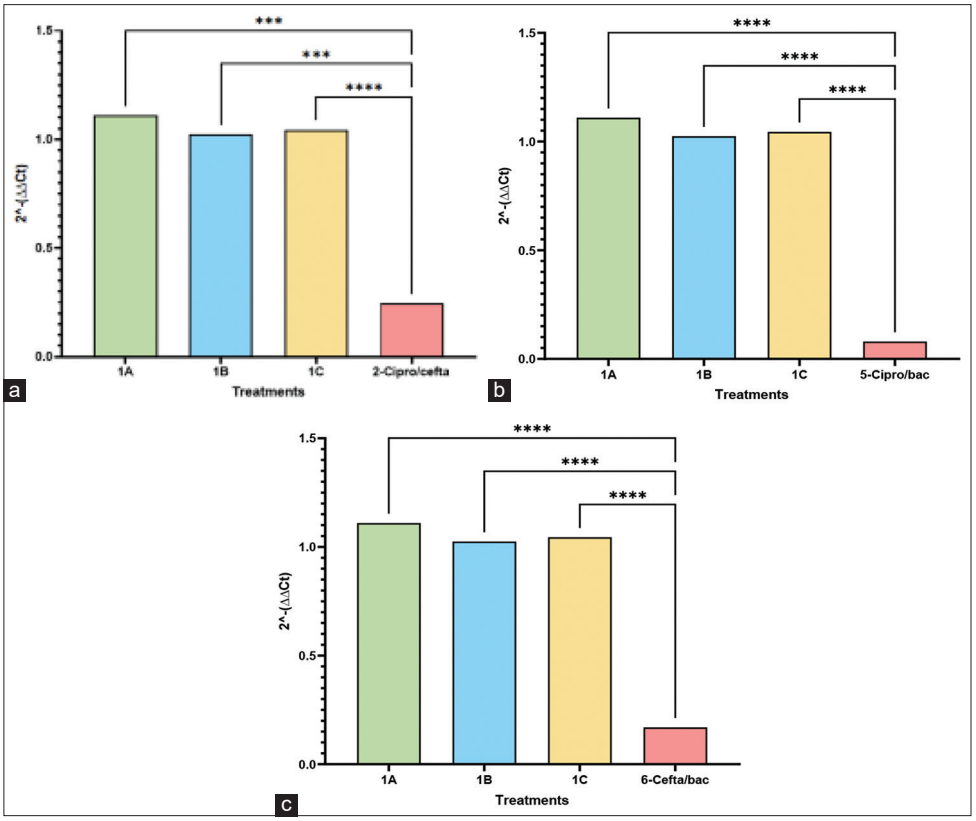

Fig. 3: Multiple comparison of mean $2^{-(\Delta\Delta Ct)}$ for *Gyrase A* between control groups, a: Only bacteria; b: bacteria+ceftazidime (CAZ); c: bacteria+ ciprofloxacin (CIP) with Treatments. Multiple comparisons between CIP/CAZ (a) CIP/bacteriocin, (b) CAZ/bacteriocin, (c). Note: The value no.1 represents the value of the variable itself and does not give meaningful values

Table 3: Comparison of treatments with controls for *Gyrase* A gene

Treatment	$2^{-(\Delta\Delta Ct)}$ Mean±SD	p-value*	p-value**	p-value***
1A	1.05±0.47	1.0	0.868	0.222
1B	1.07±0.51	0.869	1.0	0.280
1C	1.33±0.85	0.222	0.280	1.0
2-CIP/CAZ	0.25±0.12	0.025	0.027	0.013
3-CIP/bacteriocin	0.07±0.04	0.006	0.008	0.006
4-CAZ/bacteriocin	0.16±0.13	0.028	0.030	0.013

*Comparison of each treatment with 1A, **Comparison of each treatment with 1B, ***Comparison of each treatment with 1C, Note: the value no. 1 represents the value of the variable itself and doesn't give meaningful values

Table 4: Comparison of treatments with controls for *Gyrase* B gene

Treatment	$2^{-(\Delta\Delta Ct)}$ Mean±SD	p-value*	p-value**	p-value***
1A	1.24±0.66	1.0	0.872	0.205
1B	1.28±0.58	0.872	1.0	0.120
1C	1.4±0.9	0.205	0.120	1.0
2-CIP/CAZ	0.22±0.1	0.004	<0.001	<0.001
3-CIP/bacteriocin	0.11±0.1	0.004	<0.001	<0.001
4-CAZ/bacteriocin	0.12±0.09	0.004	<0.001	<0.001

*Comparison of each treatment with 1A, **Comparison of each treatment with 1B, ***Comparison of each treatment with 1C, ***Indicate *P*<0.05, ****Indicate P<0.001

effective than monotherapy, supporting the hypothesis of synergistic interaction between CIP and CAZ [16].

Synergistic interaction between antibiotics

The calculated FIC value for the CIP/CAZ combination is 0.4423, which is below 0.5, confirming a synergistic effect between the two antibiotics in combating *E. coli*. This means that the interaction between CIP and CAZ is more effective than using each antibiotic individually [17]. The synergy is likely due to the complementary mechanisms of action – CIP inhibits DNA replication, whereas CAZ targets bacterial cell wall synthesis [18]. Together, these antibiotics provide enhanced antibacterial activity against resistant strains of *E. coli* [19].

Effect of bacteriocin on antibiotic activity

The addition of bacteriocin (125 µg/mL) to either CIP or CAZ at varying concentrations (30, 40, 50, and 60 µg/mL) significantly enhanced antibacterial activity [20]. The dose-dependent inhibition observed with bacteriocin is noteworthy, showing that bacteriocin has a complementary effect when combined with antibiotics. The statistical significance ($p<0.05$ and $p<0.001$) indicates that bacteriocins enhance the antibacterial effects of traditional antibiotics. This synergy likely results from bacteriocins disrupting bacterial membrane integrity while antibiotics target intracellular processes, thus leading to stronger bacterial suppression [21].

Gene expression analysis

The gene expression results show that combination treatments significantly reduced the expression of GyrA and GyrB, which are essential for DNA replication and supercoiling in *E. coli*. For GyrA, the untreated control and single antibiotic treatments showed relatively high expression (ΔΔCt values of 1.05–1.33), indicating minimal suppression [22]. In contrast, combination therapies (CIP/CAZ, CIP/bacteriocin, and CAZ/bacteriocin) resulted in significant

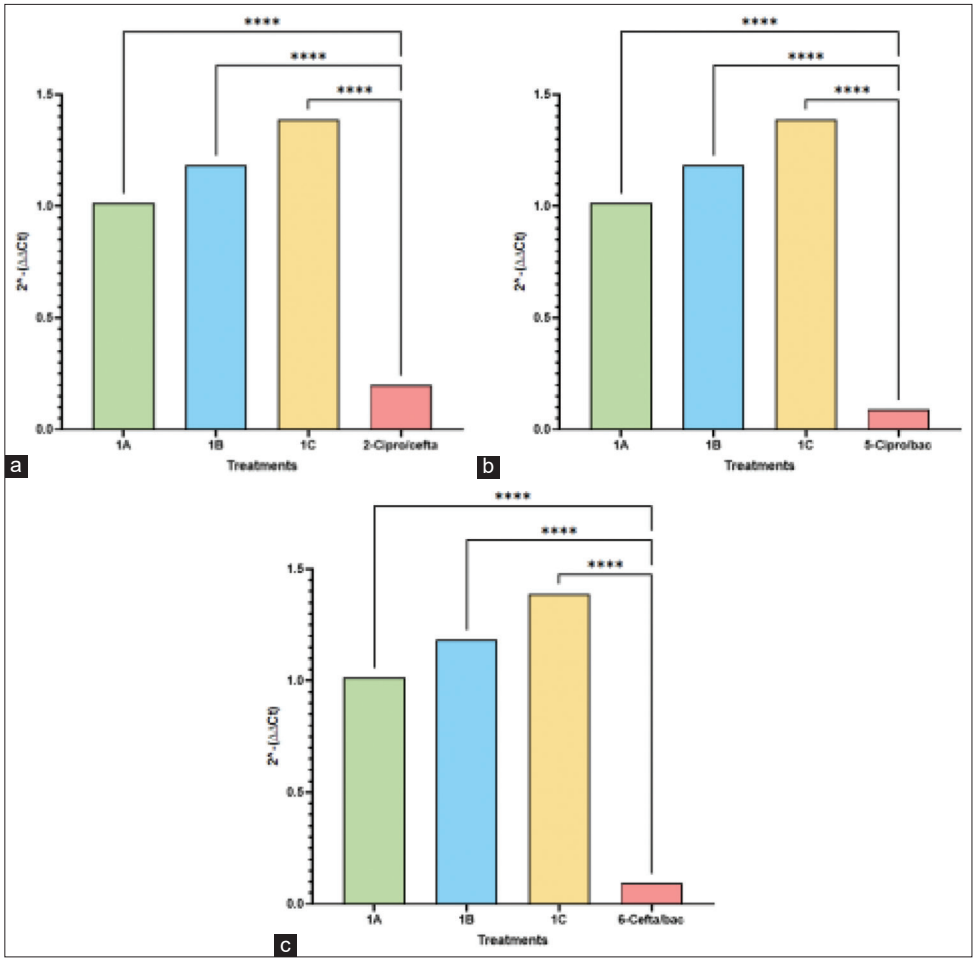

Fig. 4: Multiple comparison of mean $2^{-(\Delta\Delta Ct)}$ for *Gyrase B* between control groups (a: only bacteria; b: bacteria+ceftazidime [CAZ]; c: bacteria+ciprofloxacin [CIP]) with Treatments CIP/CAZ (a) CIP/bacteriocin, (b) CAZ/bacteriocin, and (c). ***indicate p<0.05; ****indicate p<0.001

downregulation of GyrA, with p-values consistently below 0.05. This suggests that the combined treatments interfere with DNA replication by targeting the GyrA subunit, leading to bacterial inhibition.

A similar pattern was observed for GyrB expression. The control and single treatments showed elevated levels (1.24–1.4), whereas the combination therapies (CIP/CAZ: 0.22±0.1, CIP/bacteriocin: 0.11±0.1, CAZ/bacteriocin: 0.12±0.09) significantly reduced expression, with highly significant p-values (<0.001). These results indicate that the combination treatments suppress the ATP-dependent DNA supercoiling, further compromising bacterial viability [23].

Molecular impact of combination treatments

Figs. 3 and 4 provide further evidence of the molecular consequences of these treatments by analyzing the relative expression of Gyrase A and Gyrase B using the 2-(ΔΔCt) method. Both control groups (untreated and single treatments) showed elevated expression levels, whereas combination therapies resulted in substantial downregulation of these key genes. The statistical significance (denoted by *** and ****) emphasizes the effectiveness of these treatments in reducing gene expression related to DNA replication [24]. The suppression of GyrA and GyrB further indicates that the treatments not only inhibit bacterial growth but also interfere with critical genetic functions essential for bacterial survival [25].

CONCLUSIONS AND CLINICAL IMPLICATIONS

The results from this study suggest that the CIP/CAZ combination is more effective in treating *E. coli* infections, especially those that exhibit MDR. The synergistic effect observed between CIP and CAZ supports the potential use of combination therapy to overcome antibiotic resistance. Furthermore, the enhancement of antibiotic activity by bacteriocins provides a promising therapeutic strategy that combines traditional antibiotics with biological agents for more effective treatment.

The downregulation of *GyrA* and *GyrB* in the combination treatments aligns with phenotypic observations, reinforcing the molecular mechanism by which these combinations inhibit *E. coli* growth. Gene expression analysis confirms the molecular consequences of these treatments and provides insight into their action at the genetic level.

ACKNOWLEDGMENTS

For their partial assistance, the authors would like to acknowledge their affiliations.

AUTHOR CONTRIBUTION

MAHA D. designed the study. MAHA D. and RIYAM H. carried out the laboratory work. RIYAM and NADHIM analyzed the data. RIYAM, MAHA, and NADHIM wrote the manuscript. All authors read and approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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