RESEARCH ARTICLE

Targeting Antimicrobial Resistance in *Salmonella* Enteritidis: Synergistic Action of N-acetyl cysteine with Florfenicol and CRISPR-Cas System Involvement

Mehmood Ahmad *[1], Muhammad Ovais Omer *[1], Zahid Iqbal [2], Adeel Sattar[1] and Muhammad Abu Bakr Shabbir [3]

[1] Department of Pharmacology and Toxicology, Faculty of Bio-Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan;[2] Department of Pharmacology and Toxicology, Faculty of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan; [3] Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.
*Corresponding author: drovaisomer@uvas.edu.pk; mehmood.ahmad@iub.edu.pk

A B S T R A C T

Salmonella enterica subsp. *enterica* serovar Enteritidis (SE) is one of the significant public health threats, mainly due to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains. Along with several known factors, such as irrational antibiotic use, horizontal gene transfer, and the possible involvement of the CRISPR-Cas system, these have necessitated the exploration of alternative treatment strategies. The present study aimed to identify the antibiogram of SE isolates from poultry and evaluate the synergistic effects of florfenicol (FF) and N-acetylcysteine (NAC) against MDR SE as well as explore the interplay of the CRISPR-Cas system in the emergence of AMR. Two hundred and seventy-one samples were collected. The samples included both cloacal and fecal samples from poultry birds in the Bahawalpur and Lahore Regions of Punjab, Pakistan. Identification and isolation were performed through conventional bacteriological methods followed by PCR. Phenotypic assays, including minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), mutant prevention concentration (MPC), and fractional inhibitory concentration index (FICI), were performed. Genotypic confirmation was performed by identifying AMR genes. Out of 271 samples, thirty (n=30) were confirmed positive for SE with IE gene-based PCR. Results revealed that the isolates were resistant against most of the antibiotics including, (86.67%), AMP (96.67%), AMX (96.67%), CTX (83.33%), GEN (83.33%), CH (60%), CLI (90%), TET (96.67%), SMZ/TMP (66.67%), VAN (56.67%), ENR (63.33%), CIP (53.33%), and CO (96.67%). Conversely, FF was effective against 56.67% of isolates. All of the resistant isolates (n = 30) exhibited the presence of antimicrobial resistance genes, including mphA, aac(3)-IIa, tetA, Sul1, gyrA, floR, qnrA, and mcr-1. The co-administration of NAC with FF demonstrated a significant synergistic effect against XDR SE isolate and the MIC of FF was notably reduced when combined with NAC (FICI; 0.2 to 0.5). Additionally, a significant (P<0.05) downregulation of CRISPR-Cas 3 genes was also recorded through qRT-PCR in both resistant and ATCC13076 strains after treatment with co-administration of FF and NAC compared with isolates treated with FF alone. It was concluded that the combination of NAC enhances the antimicrobial activity of FF, reduces biofilm formation, and potentially mitigates the antimicrobial resistance associated with the CRISPR-Cas system.

INTRODUCTION

Salmonella is a rod-shaped, gram-negative bacteria that belongs to the Enterobacteriaceae family. This bacterium is one of the major foodborne pathogens affecting commercial poultry and causing sporadic outbreaks on poultry farms worldwide (Yu *et al.*, 2021; Galán-Relaño *et al.*, 2023). The poultry sector plays a

crucial role in poverty alleviation and food security in underdeveloped countries through revenue generation and meat and egg production (Ahmed *et al.*, 2021; Cartoni Mancinelli *et al.*, 2022). In Pakistan, the poultry sector contributes 1.5 percent to the country's Gross Domestic Product (GDP), and the country ranks as the world's 11th largest chicken producer with an annual production of 48.83 million layers, 1.02 billion broilers, and 11.8 million breeding stocks (Magsi *et al.*, 2021; Khan *et al.*, 2022). As the human population rises, consumer demand for economical and hygienic meat has also increased exponentially worldwide (Haque *et al.*, 2020; Magsi *et al.*, 2021). However, this sector is regularly threatened by zoonotic bacterial infections leading to substantial economic losses and public health concerns. Among these infections, Salmonellosis is one of the prevalent bacterial infections reported in poultry birds with public health and economic concern globally (Hossain *et al.*, 2021; Galán-Relaño *et al.*, 2023). It is the most common cause of gastroenteritis in humans, accounting for over 90.3 million cases each year with 155,000 fatalities (Gong *et al.*, 2022; Kabantiyok *et al.*, 2023). Among the *Salmonella*, *Salmonella Enterica* Subsp. *Enterica* Serovar Enteritidis (SE) is the leading cause of avian salmonellosis with significant morbidity and mortality in poultry (Wang *et al.*, 2020; Siddiky *et al.*, 2022).

Since antibiotics are recommended to treat avian salmonellosis, multidrug resistance in *Salmonella* has developed, posing a significant public health threat. The rising antimicrobial resistance (AMR) is a prime concern that needs to be addressed from a One Health perspective, recognizing the interactions between causative agents, animals, humans, and their environment (Eriksen *et al.*, 2021). The irrational use of antibiotics in poultry birds enhances the emergence of multidrug resistant (MDR) and extended drug-resistant (XDR) SE. The MDR and XDR *Salmonella* is a potential threat to humans as it is a communicable pathogen and is transmitted by consuming contaminated chicken (Elshebrawy *et al.*, 2022). MDR SE raises one of the most serious public health concerns globally by displaying resistance to the last resort antibiotics and limiting the treatment options. As a consequence, the World Health Organization (WHO) regarded the MDR SE bacterium as a primary health concern (Monte *et al.*, 2019; Aung *et al.*, 2022). Antimicrobial stewardship and the development of alternative agents to existing antibiotics should be employed to overcome the global issue of antimicrobial resistance (Mann *et al.*, 2021; Murugaiyan *et al.*, 2022).

Besides reported factors of antibiotic resistance, including misuse, irrational use, and horizontal gene transfer; researchers also believe that the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas system might have been involved in the development of antibiotic resistance in bacteria (Tao *et al.*, 2022; Tao *et al.*, 2023). This system is an adaptive immune system of bacteria, protecting against foreign invaders. A typical CRISPR-Cas locus comprises three main components: CRISPR arrays, a cluster of cas genes, and an AT-rich leader sequence. The direct repeat sequences within the CRISPR arrays are 21 to 48 bp in size while the spacer sequences vary between 26 and 72 bp. These spacers are sequence motifs that evolved from the previous

interactions with mobile genetic elements (MGEs). The system is categorized into three primary types: I, II, and III, and the target bacteria of the current study possess the type-I CRISPR-Cas system. Noteworthy, this system apart from defending the bacteria against invaders, also increases the virulence or pathogenicity of bacteria. Additionally, in the light of several studies, it is also believed that the CRISPR-Cas system might play a role in the development of antibiotic resistance (Tao *et al.*, 2022; Suvvari *et al.*, 2023).

Antimicrobial resistance was observed against most antimicrobial groups, including beta-lactam antibiotics, aminoglycosides, glycopeptides, macrolides, chloramphenicol, tetracyclines, lincosamides, and quinolones. Relatively high resistance among pathogens against these antimicrobials in poultry production could produce public health concerns to consumers as potential vehicles of foodborne infection (Mthembu *et al.*, 2019; F. Rabello *et al.*, 2020; Yu *et al.*, 2021). Therefore, there is a dire need to find a solution of efficient treatment with minimizing the antibiotic resistance problem.

The use of N-acetyl cysteine (NAC) is an alternative approach to reduce biofilm-associated bacterial infections that are resistant to conventional antibiotics. Different studies reported its synergistic effect when co-administered with other antibiotics *in vitro* (Aiyer *et al.*, 2021; Sahasrabudhe *et al.*, 2023). It has been observed that NAC has a remarkable antibiofilm activity against different bacterial colonies when co-administered with different antibiotics (Aiyer *et al.*, 2021). NAC reported to potentiate the efficacy of different anti-tubercular drugs along with FQs *in vitro* against the different strains of *Mycobacterium tuberculosis* (Vilchèze and Jacobs Jr, 2021; Shee *et al.*, 2022). Therefore, NAC can be used alone or in conjunction with other antibiotics to effectively treat bacterial infections and restrict the emergence of resistance.

Therefore, the current study was designed to not only optimize the dose and develop the effective dose regimen against MDR SE but also to find out the drug interactions between the FF and NAC. To achieve this, we performed phenotypic (MIC, MBC, MPC, FICI) and genotypic experiments (PCR). Additionally, potential role of CRISPR-Cas system in antimicrobial resistance was also determined through qRT-PCR.

MATERIALS AND METHODS

Sample size calculation: The sample size was calculated as 271 using the formula previously described by Thrusfield (2007).

$$Sample\ size\ (n) = \frac{Z^2 pq}{d^2}$$

Where $Z^2 = 1.645^2$ at a 90% confidence level, p is the expected prevalence (50%), q= 1-p (50%), d is the desired absolute precision (5%) (Ahmad *et al.*, 2025).

Sample collection: A total of samples (n=271) from poultry birds were randomly collected in this study. Of these, 135 poultry samples [(cloacal swabs (45), droppings (45), meat (45)] were collected randomly from a live bird market located in the Lahore region. Likewise, 135 poultry samples [(cloacal swabs (45), droppings (45), meat (45)]

were also collected from the Bahawalpur region. All the samples were collected aseptically in sterile plastic bags and transported in a chilled insulated container (Khan *et al.*, 2019; Shivaning Karabasanavar *et al.*, 2020), to the University Diagnostic Laboratory (UDL), University of Veterinary and Animal Sciences (UVAS), Lahore for further processing.

Isolation and biochemical identification of SE: The samples were pre-enriched in buffered peptone water (BPW) and incubated at 37 °C overnight. A volume of 0.1ml from BPW was inoculated into 10ml of Selenite broth, incubated at 37 °C, and into Rappaport-Vassiliadis (RVS) broth at 37 °C for 24 hours. Samples were sub-cultured on Xylose Lysine Deoxycholate (XLD) culture media plate and Brilliant Green agar plates (BGA) through streaking. The plates were incubated for 24 hours at 37 °C and suspected *Salmonella* colonies were identified using conventional bacteriological methods described (Mthembu *et al.*, 2019; Elshebrawy *et al.*, 2022).

Genotypic confirmation of SE: Polymerase Chain Reaction (PCR) was employed to confirm the genomics of SE. For this purpose, bacterial DNA was extracted using a commercially available GeneJET DNA purification kit (Catalogue No. KO721, Thermo Scientific, Waltham, MA, USA).

The quality of the DNA was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed on seventy biochemically confirmed *Salmonella* isolates using genus-specific primers (*invA* gene) and species-specific primers (*IE* gene) as described previously (Rodríguez-Hernández *et al.*, 2021; Ansari-Lari *et al.*, 2022; Ahmad *et al.*, 2023).

Phenotypic confirmation of MDR SE: From seventy samples, thirty isolates were confirmed as SE by PCR and subjected to antibiogram testing. Antimicrobial resistance was determined by Minimum inhibitory concentration (MIC). Based on clinical relevance and standard veterinary and poultry farming practices, sixteen antibiotics were selected from seven different groups. Micro broth dilution was performed to determine the MIC. Various antibiotics, including penicillin (PEN), amoxicillin (AMX), ampicillin (AMP), cefotaxime (CTX) streptomycin (ST), tylosin (TY), nalidixic acid (NA), ciprofloxacin (CIP), tetracycline (TET), erythromycin (ER), clindamycin (CLI), gentamicin (GEN), sulphamethaxazole/trimethoprim (SMZ/TMP), enrofloxacin (ENR), azithromycin (AZT), florfenicol (FF), chloramphenicol (CH) and colistin (CO), were assessed through the broth microdilution technique. *Salmonella* Enteritidis (ATCC 13076) served as a quality control. The isolates were declared sensitive (S) or resistant (R) by comparing the resulting MIC values with the cut-off values as per the guidelines of CLSI (CLSI, 2016). The isolates demonstrating resistance to more than three antibiotics from at least three different classes were classified as MDR *Salmonella*.

The multiple antibiotic resistance (MAR) index was also calculated according to the formula described by (Elshebrawy *et al.*, 2022).

MAR index = n/N.

n indicates "number of resistance antibiotics," whereas N is the total number of antibiotics tested. All the *in vitro* experiments (MIC, MBC, FICI) were carried out in triplicate.

Mutant prevention concentration (MPC): The Mutant Prevention Concentration (MPC) is defined as the lowest antibiotic concentration that prevents the growth of the least susceptible first-step resistant mutant subpopulation after 120 hours of incubation (μg/ml). It serves as an upper boundary of the mutant selection window and is used to evaluate the potential of an antibiotic to suppress the development of resistance (Drlica and Zhao, 2007).

Drug interactions: The checkerboard microdilution method was used to investigate the drug interactions between Florfenicol (FF) and n-acetylcysteine (NAC). The fractional inhibitory concentration index (FICI) indicates the nature of interaction between two antimicrobial agents. It is interpreted as follows: FICI≤0.5 indicates synergism; FICI>0.5 to≤1.0 indicates an additive (or partial synergistic) effect; FICI>1.0 to≤4.0 indicates indifference (no interaction); and FICI>4.0 indicates antagonism (Fatsis-Kavalopoulos *et al.*, 2024).

Genotypic confirmation of antimicrobial-resistant genes: Phenotypically, antimicrobial-resistant SE isolates were analyzed for antibiotic-resistance genes using PCR. The antibiotic groups selected to recognize the resistance genes (mphA, aac(3)-IIa, tetA, Sul1, gyrA, floR, qnrA, mcr-1) included beta-lactams, macrolides, aminoglycosides, tetracyclines, sulfonamides, amphenicols, fluoroquinolone, and polymyxins (colistin), respectively. Amplified PCR products were separated by gel electrophoresis and then analyzed under a gel documentation system (Omega Fluor [plus] Aplegen Inc., California, USA).

Identification of CRISPR-cas system: Specific primers for cas1, cas2 and cas3 were designed online through primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Subsequently, PCR products were amplified and analyzed by using Omega Fluor[TM] Plus Gel Documentation System (Aplegen Inc., California, USA).

Detection of CRISPR-cas3 Gene Expression qRT-PCR: A single MDR SE isolate (Sample ID; MAL 91, MAR Index; 0.93) with its quality control strain (ATCC13076) was treated with FF alone and its combination with NAC at the concentration of 1/2 MPC. RNA was extracted from each sample using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), and cDNA was synthesized with the RevertAid First-Strand cDNA Kit (Thermo Scientific, USA). PCR amplification was performed on the CFX96 real-time PCR thermocycler (Bio-Rad, Singapore), with an initial preincubation at 95°C for 3 minutes, followed by 45 cycles of 10 seconds at 95°C annealing 60°C and 40 seconds at 52°C. Specific primers F-5'-CTCTCCCGTTAGCGGTGTTT-3' and R-5'-TCTGGTTCAATGCCTGCGAT-3' with an amplicon size of 123bp were used. The housekeeping gene 16S rRNA was used for normalization and the experiment was repeated three times to calculate the mean fold change.

Statistical analysis: The qualitative results regarding categorical variables were analyzed using the descriptive statistics in Minitab® 21.4.2 64-bit (Minitab, LLC.). Normality assumptions were tested using Shapiro-Wilk test prior to applying parametric tests. Two-way ANOVA was used to compared the relative expression of Cas3 gene in treated groups for both ATCC 13076 and clinical isolates. Chi-square test with Montecarlo simulation at 5000 bootstrapping and p-value adjustment with Bonferroni method in SPSS version 27 was used to compare the proportions of resistant and susceptible isolates based on phenotypic testing. Graphs were generated using GraphPad Prism version 9.5.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was considered at p-value less than 0.05.

RESULTS

Isolation and biochemical identification: Results of the conventional biochemical tests showed 48 samples (17.78%; 95% CI; 13.68 - 22.78) of the total samples were found positive for *Salmonella* spp. Further molecular testing revealed that 41 samples were positive for *invA* gene. However, out of these 41 samples, thirty (*n*=30; 73.17%, 95% CI; 84.30–58.07) were confirmed positive for SE with *IE* gene. Of these thirty SE confirmed isolates, 18 were from the Lahore region (including cloacal swabs (6), fecal samples (10), and poultry meat samples (2)). Whereas, 12 isolates were from the Bahawalpur region (including cloacal swabs (5), fecal samples (5), and poultry meat samples (2).

Phenotypic confirmation of MDR SE: Significant (P<0.05) proportion of SE isolates revealed phenotypic resistance against commonly used antibiotics including, NA (86.87%, 95% CI: 92.16–78.82), AMP (96.97%, 95% CI: 99.17–91.47), AMX (96.97%, 95% CI: 99.17–91.47), CTX (83.84%, 95% CI: 89.80–75.35), GEN (83.84%, 95% CI: 89.80–75.35), CH (60.00%, 95% CI: 69.06–50.20), CLI (90.00%, 95% CI: 94.48–82.56), TET (96.97%, 95% CI: 99.17–91.47), SMZ/TMP (66.67%, 95% CI: 75.18–56.91), VAN (56.57%, 95% CI: 65.90–46.74), ENR (63.64%, 95% CI: 72.44–53.82), CIP (53.54%, 95% CI: 63.04–43.76), and CO (96.97%, 95% CI: 99.17–91.47). Conversely, FF was found to be effective against 56.57% of isolates (95% CI: 65.90–46.74) (Fig. 1).
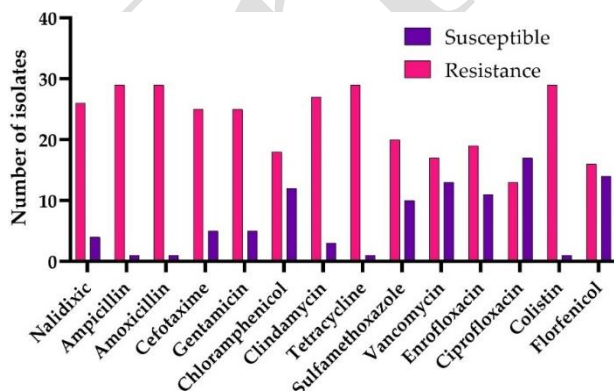

Fig. 1: Antibiotic susceptibility profile of *SE* isolates (*n*=271). Fig. shows the number of isolates exhibiting susceptibility and resistance to various antibiotics including NA, AMP, AMX, CTX, GEN, CH, CLI, TET, SMZ, VAN, ENR, CIP, COL, and FF. Significant (P<0.05) difference was observed among the proportions of susceptible and resistant isolates against the tested antibiotics, as tested by Chi-squared test.

Against cloacal sample isolates from Lahore, the median MIC values recorded for Nalidixic acid, Ampicillin, Amoxicillin and Gentamicin were 256µg/ml (IQR; 50-512), 80µg/ml (IQR; 25-512) 192µg/ml (IQR; 98-448), 48µg/ml (IQR; 12-256), respectively. In comparison, isolates from the Bahawalpur region showed comparatively higher resistance against Amoxicillin (MIC; 512µg/ml, IQR; 260-1024) and Ampicillin (MIC; 128µg/ml IQR; 48-768). Additionally, the fecal samples isolate from the Lahore region showed a higher resistance pattern against Ampicillin (MIC; 384µg/ml, IQR; 112-640) and Amoxicillin (MIC; 96µg/ml, IQR; 26-512). In comparison, those from the Bahawalpur region exhibited elevated median MIC values for Ampicillin (512µg/ml) and Sulfamethoxazole (256µg/ml). Moreover, higher median MIC values of Amoxicillin (512µg/ml), Ampicillin (320µg/ml), and Nalidixic acid (260µg/ml) were recorded against SE isolates of meat samples from the Lahore region. In comparison, significant (P<0.05) resistance was noted against Clindamycin (768µg/ml) and Sulfamethoxazole (320µg/ml) in Bahawalpur isolates. Detailed results of sample-wise MIC values are presented in Table 1.

Table 1: Median (IQR) antibiotic susceptibility profile and multiple antibiotic resistance (MAR) index of *SE* isolates from cloacal swabs, fecal samples, and meat samples against a panel of 16 antibiotics, including florfenicol (FF) and N-acetylcysteine (NAC).

	Cloacal Swab Samples	Fecal Samples	Meat Samples
Nalidixic acid	256 (64 - 512)	256 (64 - 256)	160 (22 - 448)
Ampicillin	128 (32 - 512)	512 (128 - 512)	256 (160 - 448)
Amoxicillin	256 (128 - 1024)	64 (32 - 512)	96 (19 - 800)
Cefotaxime	16 (4 - 32)	8 (4 - 16)	20 (5 - 56)
Gentamicin	64 (16 - 256)	16 (16 - 64)	96 (17.5 - 128)
Chloramphenicol	32 (4 - 64)	32 (4 - 64)	4 (2.5 - 25)
Clindamycin	64 (2 - 64)	64 (64 - 256)	288 (64 - 896)
Tetracycline	64 (32 - 128)	64 (32 - 128)	48 (20 - 112)
Sulfamethoxazole	128 (32 - 256)	128 (32 - 256)	192 (44 - 448)
Vancomycin	2 (2 - 128)	32 (2 - 128)	2 (2 - 24.5)
Enrofloxacin	2 (0.125 - 8)	4 (0.125 - 8)	5 (0.5938 - 8)
Ciprofloxacin	0.125 (0.125 - 16)	0.25 (0.125 - 8)	4 (1.094 - 4)
Colistin	4 (1 - 8)	4 (1 - 8)	2.5 (0.625 - 13)
Florfenicol	2 (2 - 16)	8 (2 - 16)	2 (0.875 - 6.5)
NAC	8 (4 - 16)	8 (4 - 16)	4 (2.5 - 7)
MAR Index	0.79 (0.71 - 0.86)	0.79 (0.71 - 0.86)	0.715 (0.64 - 0.79)

Nalidixic acid exhibited MBC from 8 to 1024µg/ml (median; 256µg/ml). Ampicillin and amoxicillin showed similar ranges from 8 to 2048µg/ml (median; 512 and 256µg/ml, respectively). MBC of Cefotaxime ranged from 0.5 to 128µg/ml (median; 12µg/ml). MBC of Colistin ranged from 1 to 32µg/ml (median; 6µg/ml), while that of florfenicol was recorded as 0.125 to 256µg/ml (median; 4µg/ml) (Fig. 2a). Moreover, the MPC of colistin ranged from 2 to 64µg/ml (median; 16µg/ml), while that of florfenicol was recorded as 0.25 to 256µg/ml (median; 8µg/ml). NAC showed MPCs ranging from 4 to 64mg/ml (median; 32mg/ml) (Fig. 2b).

Drug interactions: The results of the FICI of combined Florfenicol and NAC against SE isolates exhibited synergistic effects with FICI values ranging from 0.188 to 0.375. The synergistic effect of the drug combination was recorded against twenty-seven isolates (90%). In contrast,

additive effects were observed against 6.67% of the isolates, including MAL43 and MAB170 (FICI values of 0.75 and 0.625, respectively). Furthermore, an indifferent interaction was observed against MAL98 (FICI, 1.016), indicating no significant enhancement in activity (Table 2).

Genotypic confirmation of antimicrobial resistance genes: All the SE isolates phenotypically resistant to the antibiotics were confirmed genotypically for the presence of AMR genes. All of the resistant isolates showed the presence of antimicrobial resistance genes including *mphA, aac(3)-IIa, tetA, Sul1, gyrA, floR, qnrA, mcr-1* against beta-lactams, macrolides, aminoglycosides, tetracyclines, sulfonamides, amphenicols, fluoroquinolone, and polymyxins (colistin), respectively (Fig. 3).
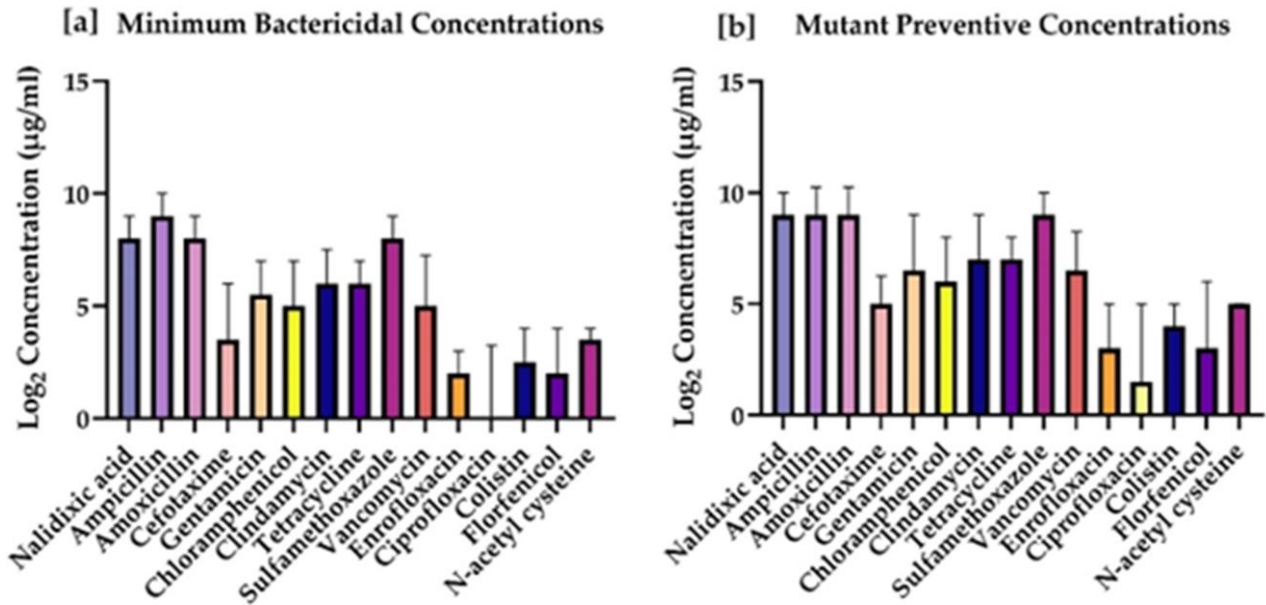

Fig. 2: Median (IQR) log$_2$ transformed antibiotic concentrations against *SE* isolates showing (a) minimum bactericidal concentrations (b) Mutant preventive concentrations including Nalidixic acid, Ampicillin, Amoxicillin, Cefotaxime, Gentamicin, Chloramphenicol, Clindamycin, Tetracycline, Sulfamethoxazole, Vancomycin, Enrofloxacin, Ciprofloxacin, Colistin, Florfenicol, and N-acetylcysteine. The error bars represent standard error of mean.
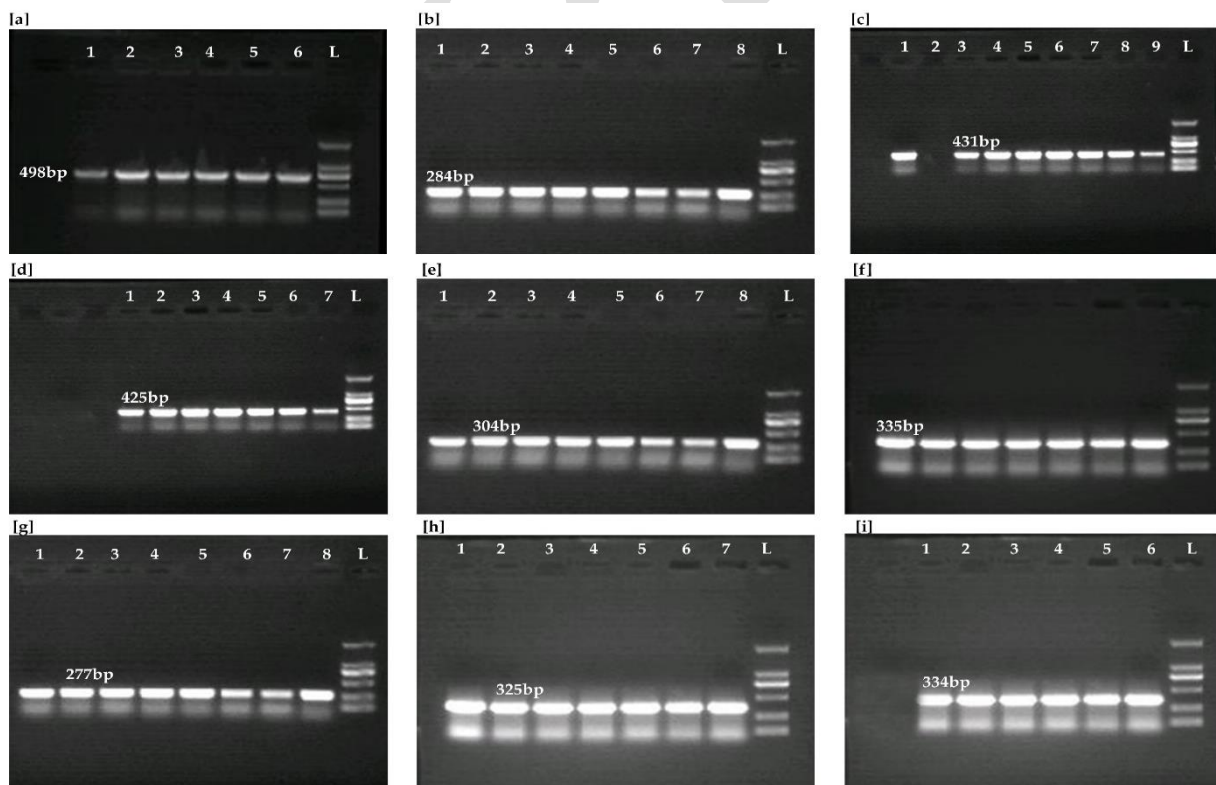

Fig. 3: PCR amplification of antibiotic resistance genes in *SE* isolates. (a) *gyrA* gene (498bp; lanes 1–6), (b) *sull* gene (284bp; lanes 1–8), (c) *tetA* gene (431bp; lanes 1–9), (d) *aac(3)-IIa* gene (425bp; lanes 1–7), (e) *floR* gene (304bp; lanes 1–5), (f) *mphA* gene (335bp; lanes 1–6), (g) *qnrA1* gene (277bp; lanes 1–8), (h) *mcr-1* gene (325bp; lanes 1–7), and (i) *ampC* gene (334bp; lanes 1–6). L: molecular weight ladder.

Table 2: Minimum inhibitory concentrations (MICs) of florfenicol and N-acetylcysteine (NAC) and fractional inhibitory concentration index (FICI) values for *SE* isolates. The FICI values indicate synergistic (S, FICI≤0.5), additive (A, 0.5<FICI≤1), and indifference (I, FICI > 1) interactions between florfenicol and NAC. Where, A; Additive, I; Indifferent, and S; Synergistic.

Sample ID	Combined MIC of florfenicol	Combined MIC of NAC	FICI	Sample ID	Combined MIC of florfenicol	Combined MIC of NAC	FICI
MAL3	0.5	0.5	0.375(S)	MAL88	0.5	0.5	0.375(S)
MAL29	0.25	2	0.375(S)	MAL89	4	2	0.375(S)
MAL38	0.25	1	0.375(S)	MAL91	8	4	0.375(S)
MAL43	8	4	0.75(A)	MAL98	2	0.125	1.016(I)
MAL52	0.125	0.5	0.375(S)	MAL109	0.03125	0.125	0.313(S)
MAL59	8	2	0.375(S)	MAL112	4	4	0.375(S)
MAB138	0.125	1	0.188(S)	MAB179	0.03125	0.125	0.313(S)
MAB150	4	2	0.375(S)	MAB185	0.5	0.125	0.281(S)
MAB158	2	1	0.375(S)	MAB188	0.5	0.5	0.313(S)
MAB170	1	0.5	0.625(A)	MAB197	4	1	0.313(S)
MAB174	0.5	2	0.375(S)	MAB215	0.03125	0.125	0.313(S)
MAL61	4	2	0.375(S)	MAL125	0.5	0.125	0.281(S)
MAL64	32	2	0.375(S)	MAL133	2	0.125	0.266(S)
MAL69	4	1	0.313(S)	MAB244	0.125	0.0625	0.281(S)
MAL84	2	0.5	0.313(S)	MAB257	0.5	0.125	0.281(S)

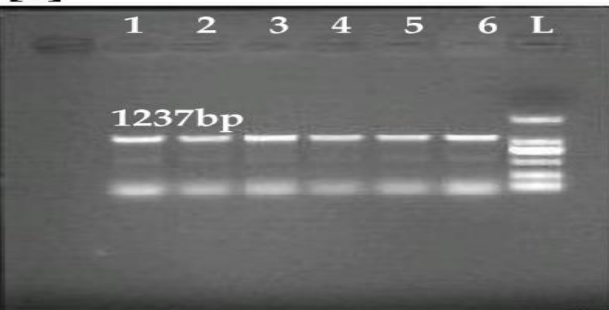**[a]**

362bp

[b]

241bp

[c]
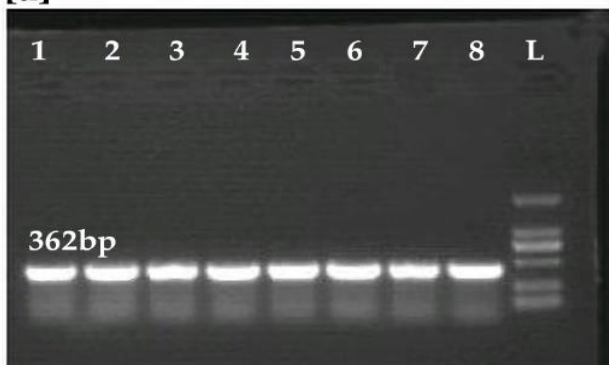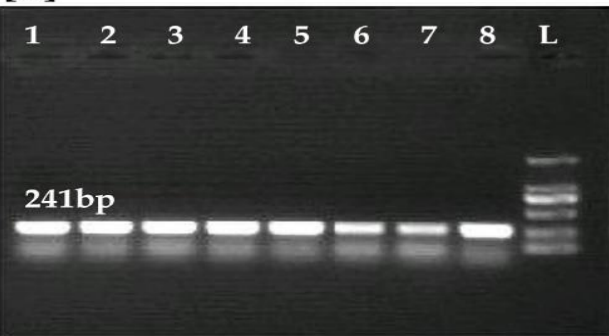
1237bp

Fig. 4: PCR products gel electrophoresis showing the presence of (a) *cas1*; 362bp, (b) *cas2*; 241bp, and (c) *cas3*; 1237bp genes in all *SE* isolates. Lane L represents the molecular weight marker.

Identification of CRISPR-cas system: To explore the probable genetic basis of antimicrobial resistance (AMR), we investigated the presence of CRISPR-Cas system components, specifically the cas1, cas2, and cas3 genes, in all SE isolates. All of the SE isolates confirmed the presence of cas 3, cas2, and cas1 genes and revealed the PCR products of 1237, 241, and 362bp, respectively (Fig. 4). As the CRISPR-Cas system was found to potentially influence bacterial defense mechanisms and may play a regulatory role in resistance development.

Time killing curves: For the static time-kill curve, the FF and its combination with NAC were evaluated against SE throughout 24 hours. The control group had a high $Log_{10}CFU/mL$ count by maintaining stable bacterial growth. Florfenicol alone at varying concentrations (1/4MIC, 1/2MIC, and 1MIC) showed moderate to significant reductions in bacterial load. Noteworthy, at 1MIC the bacterial count decreased sharply until 12 hours but regrowth occurred afterwards. However, when FF was combined with NAC in different ratios, the synergistic effect was observed. The most efficient bacterial control was observed for combinations at 8µg/ml + 4mg/ml and 16µg/ml + 8mg/ml, resulting in complete eradication at 18 hours, as shown in Fig. 5.
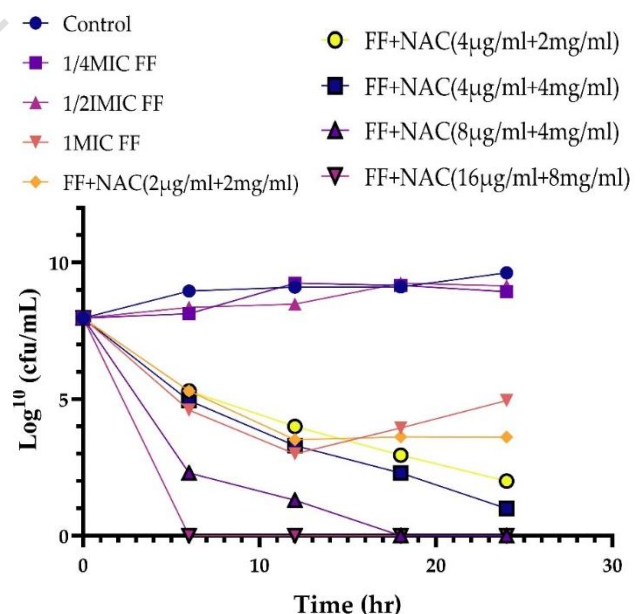

Fig. 5: Time-kill curves showing the antibacterial effect of Florfenicol (FF) alone and in combination with N-acetylcysteine (NAC) against XDR *SE isolate.* FF- NAC combination demonstrated enhanced bacterial eradication compared to FF alone (calculated in triplicates (*n*=3) at each concentration).

Detection of CRISPR-cas3 gene expression qRT-PCR:
The results of relative expression of the cas3 gene in ATCC 13076 and SE isolates regarding the Florfenicol treatment alone, ATCC 13076 had a mean expression of 1.38±0.03 compared with 2.09±0.07 in the SE isolates (P<0. 0001). Moreover, the mean fold change of cas3 expression in ATCC 13076 under the NAC treatment was 1.22±0.02, while in SE isolates was 1.58±0.03. Additionally, the combination of Florfenicol and NAC revealed that the SE isolates had a significant (P<0.0001) higher mean expression (2.18±0.05) compared with the ATCC 13076 strain (1.62±0.04) (Fig. 6) as compared using Two-Way ANOVA and Tukey's post hoc test.
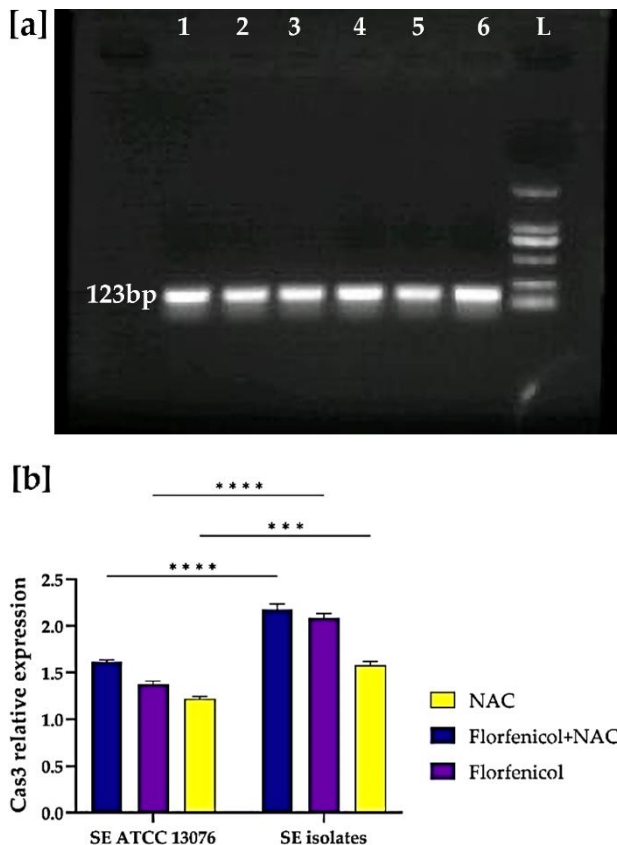
DISCUSSION

The findings of the current study revealed significant insights about the prevalence, molecular identification, and antimicrobial resistance patterns of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (SE) isolated from Lahore and Bahawalpur regions in Pakistan. In our research, the overall prevalence of *Salmonella* was 17.78% in poultry samples from various regions of Pakistan. The SE occurrence in this study was higher compared to a previous report from Pakistan, which showed a rate of 12% (Siddique *et al.*, 2021). In contrast to our results, another study conducted in Lahore, Pakistan, reported a high prevalence (45.83%) of *Salmonella* in poultry samples. Among the predominant *Salmonella* serovars, SE was 18.1% (Fatima *et al.*, 2023). This highlights the need for continuous and strict monitoring of *Salmonella* at both poultry farms and retail markets in Pakistan.

Reducing the possibility of the emergence of resistant pathogens requires vigilant antibiotic stewardship and the

urgent identification of optimal dosage regimens to target multidrug-resistant (MDR) strains of bacteria. The biofilm formation by *Salmonella* species plays a crucial role in the resistance to antimicrobial therapies (Dai *et al.*, 2021; Aleksandrowicz *et al.*, 2023). Consequently, this study explores the *in vitro* potential of FF against resistant strains of *SE* and its combination with NAC. NAC has the potential to alter the antibacterial efficacy of various antibiotics leading to synergistic, neutral, or additive effects.

Our findings on MDR *Salmonella* align with global trends (Rodríguez-Hernández *et al.*, 2021; Ansari-Lari *et al.*, 2022). About twenty-three antibiotics from ten different groups including quinolones, penicillin, and cephalosporins were found resistant to *Salmonella* which is obtained from poultry isolates (Castro-Vargas *et al.*, 2020; Lenchenko *et al.*, 2020). We have observed that both, Lahore and Bahawalpur origin samples were positive for SE. The region-specific differences in antibiotic usage practices highlight the importance of region-specific policies to combat antimicrobial resistance effectively (Khan *et al.*, 2022), our study also unraveled this approach, however, further studies are needed to validate this.

Our results further showed that significant proportions of the *Salmonella* isolates had resistance against antibiotics including amoxicillin, ampicillin, tetracyclines, colistin, clindamycin, nalidixic acid, cefotaxime, gentamicin, sulfamethoxazole, chloramphenicol, vancomycin, and ciprofloxacin. The molecular detection of MDR *Salmonella* species isolated from broiler farms in Bangladesh revealed high resistance to commonly used antibiotics as well (Mridha *et al.*, 2020; Hossain *et al.*, 2021). Various studies conducted in South Africa reported similar findings, showing high resistance among MDR *Salmonella* isolates from livestock production systems (Mthembu *et al.*, 2019; Mthembu *et al.*, 2021; Dlamini *et al.*, 2024). These findings align with our study, highlighting the need for better antibiotic stewardship and alternative treatment options in poultry production. Therefore, an alternate treatment approach is the need of the hour to tackle the global issue caused by MDR *Salmonella*.

The genomic characterization provides valuable insights manifesting that SE isolates resistant to multiple antibiotics, including beta-lactam, quinolones, macrolides, aminoglycosides, sulphonamides, and amphenicols. It exhibits/indicates significant genetic diversity mainly due to the acquisition of antimicrobial-resistant (AMR) genes through horizontal gene transfer. In the current study, genotypic analysis of all the resistant isolates showed the presence of multiple antimicrobial resistance (AMR) genes (mphA, aac(3)-IIa, tetA, Sul1, gyrA, floR, qnrA, mcr-1) corresponding to the phenotypically observed resistance against commonly used antimicrobials.

Previous studies reported a high prevalence of SE isolates from broilers with notable resistance to commonly prescribed antibiotics (Castro-Vargas *et al.*, 2020; Bahramianfard *et al.*, 2021; Yu *et al.*, 2021; Nazari Moghadam *et al.*, 2023). The current molecular detection of AMR genes is in consistent with the previous studies conducted in Malaysia and Bangladesh where the molecular analysis of SE isolates from broilers revealed a strong correlation between phenotypic resistance and the presence of AMR genes. These studies also indicated the

significantly higher prevalence of AMR genes from cloacal swabs as compared to meat samples which are also aligned with the findings of our study (Alam *et al.*, 2020; Zakaria *et al.*, 2022). Similarly, a study conducted in Nigeria also reported the AMR genes from 18.8% positive SE isolates obtained from retail chicken (Igbinosa *et al.*, 2022). Recently, genomic characterization of SE (23.1%) isolated from the retail market of Saudi Arabia reported the presence of AMR genes responsible for phenotypic resistance to widely used antibiotic groups including the potentiated sulphonamides, macrolides, quinolones and tetracyclines (Alzahrani *et al.*, 2023). These findings validate the genetic foundation of phenotypically observed MDR that strengthens the need for continuous surveillance of AMR genes in pathogenic bacteria concerning irrational use of antimicrobials in poultry production.

The CRISPR-Cas system, which is known for its role in bacterial immunity, may also associated with the regulation of virulence and resistance gene expression indicated by some recent studies. In the current study, the molecular detection of *cas 1*, *cas 2*, and *cas 3* genes in SE isolates connects the complexity of understanding the AMR mechanisms. Our findings also demonstrated the upregulated expression of cas3 genes in MDR SE isolates treated with FF and NAC as compared to reference strain emphasizing the need for further investigation of specific mechanisms involved in antimicrobial resistance. This kind of differential expression is indicative of the possible role of the CRISPR-Cas system in the adaptive immune response of SE isolates under antimicrobial stress.

NAC has mucolytic, antibacterial properties and clinically used to treat acetaminophen intoxications as well (Galicia-Moreno *et al.*, 2024). Being a precursor of the antioxidant glutathione, it has been showed its protective effect against drug induced toxicities (Moon *et al.*, 2016; Shi and Puyo, 2020). Various studies also reported its antibiofilm potential against various bacterial infections (Dinicola *et al.*, 2014; Blasi *et al.*, 2016; Landini *et al.*, 2016; Pazarci *et al.*, 2024). It has been observed that NAC has a remarkable antibiofilm activity against different bacterial colonies when co-administered it with different antibiotics (Dinicola *et al.*, 2014; Moon *et al.*, 2016; Ciacci *et al.*, 2019; De Angelis *et al.*, 2022; Guerini *et al.*, 2022). The antibacterial mechanism of NAC is not fully understood yet; however, it is reported that the sulfhydryl group of this compound may interact with bacterial proteins and disturb the intracellular balance inside the bacterial cell (Tenório *et al.*, 2021; Abdulrab *et al.*, 2022). Although, Petkova *et al.* (2022) reported insignificant impact on PK parameters of doxycycline when administered with NAC in Mycoplasma infected commercial chickens.

The findings of various studies conducted globally are consistent with the results of the present study. The study conducted in Poland revealed the high antimicrobial resistance patterns with strong biofilm potential displayed by the SE strains isolated from poultry and human sources, underscoring the serious health concern. Similarly, a research study in Pakistan reported the identification of biofilm-forming isolates of SE from poultry, and its products exhibited significant antimicrobial resistance profiles. To the best of our knowledge, the current study is the first to report the synergistic and additive effects of FF

and its combination with NAC against MDR and XDR SE isolates, as indicated by FICI. These synergies suggest that NAC may enhance the efficacy of FF and could be a valuable candidate as an adjunct therapy to treat infections caused by MDR strains. Furthermore, evidence from several studies suggested the interference of NAC in biofilm formation may enhance the efficacy of traditional antibiotics.

The objective of this *in-vitro* study was to evaluate the synergy between FF and NAC for the first time in veterinary medicine by checkerboard method and validation of the results by time-kill analysis. In our study, FF, showed a synergistic effect with NAC. We further validated the synergistic effect between FF and NAC by time-kill analysis against the XDR field isolate of *Salmonella* Enteritidis. The isolates were tested for sensitivity to FF and NAC. *Salmonella* Enteritidis was sensitive to FF (MIC 64μg/ml) but these MIC values were much higher than that of the breakpoints described by CLSI (CLSI, 2016). In our study, the XDR *Salmonella* Enteritidis isolate was more susceptible to both drug combinations than a single drug. Both drugs showed synergy through the checkerboard method. The checkerboard analysis showed that the MIC of both drugs reduced to 1/8 and 1/4 by the actual value of MIC, which suggests a reasonable synergism between the two drugs. These results are aligned with the previous study (Ma *et al.*, 2024) which assessed the efficacy of FF in combination with tetracycline against *Salmonella typhimurium*. Noteworthy, we found that both drugs (FF+NAC) at the ratio between 2μg/ml+2mg/ml and 16μg/ml+8mg/ml resulted in significant *in-vitro* inhibitory action against *Salmonella enterica* subsp. *enterica* serovar Enteritidis. Ju *et al.* (2022) proposed the efficient ratio of a two-drug combination should relate to the maximal effective ratio of a single drug alone, showing growth reduction of bacterial isolates. In the present study, time-kill analysis validated the synergy through the checkerboard method.

FF possesses a concentration-dependent antibacterial property (Guo *et al.*, 2023). Numerous studies have demonstrated the *in-vitro* antibacterial and antibiofilm efficacy of NAC against both Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia* (Parry and Neu, 1977; Roberts and Cole, 1981; Alfredsson *et al.*, 1987; Blasi *et al.*, 2016; Pollini *et al.*, 2018; Aiyer *et al.*, 2021; Alarfaj *et al.*, 2022). Various mechanisms of action have been proposed. Noteworthy of which is the interaction with the thiol (-SH) functional group by disrupting the disulfide (S–S) bridges in bacterial proteins (Pedre *et al.*, 2021). This disruption of proteins leads to denaturation, thereby altering their structure and ultimately impairing their function (Costa *et al.*, 2017).

Conclusions: In conclusion, the synergistic combination of NAC and FF represents a promising approach to enhance treatment efficacy and combat antimicrobial resistance. Moreover, understanding the role of the CRISPR-Cas system in resistance mechanisms could unlock new avenues for therapeutic interventions. We believe that the findings of the current study will help to provide a novel solution to combat antimicrobial resistance in SE by combining NAC with FF.

Authors contribution: "Conceptualization, MA and MOO; methodology, MA and MABS; software, MA; validation, AS, MOO and ZI; formal analysis, ZI; investigation, MA; resources, AS and MABS; data curation, MA; writing-original draft preparation, MA; writing-review and editing, MA and MABS; visualization, ZI; supervision, MOO; project administration, MOO All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of University of Veterinary and Animal Sciences, Lahore vide approval No. DR/521 dated.11.12.2023.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations
The following abbreviations are used in this manuscript:

ABBREVIATION	FULL FORM
FF	Florfenicol
NAC	N-acetyl cysteine
BPW	Buffered Peptone Water
RVS	Rappaport-Vassiliadis
XLD	Xylose Lysine Deoxycholate
BGA	Brilliant Green agar
PBS	Phosphate Buffer Saline
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MPC	Mutant Prevention Concentration
PAE	Post Antibiotic Effect
BMD	Broth Microdilution
CFU	Colony Forming Unit
MAR	Multiple Antibiotic Resistance
FICI	Fractional Inhibitory Concentration Index
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CLSI	Clinical and Laboratory Standards Institute
FDA	Food and drug administration Authority
PCR	Polymerase chain reaction
SE	*Salmonella Enterica* Subsp. *Enterica* Serovar Enteritidis
In vitro	Experiments performed outside of living organism

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