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Contribution of Novel Substitutions in PmrAB to the Development of Resistance to Colistin in *mcr*-Negative *Salmonella* Isolates

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ABSTRACT: The emergence of colistin-resistant gram-negative bacteria poses a serious challenge for healthcare, and the two-component system PmrAB is closely associated with colistin resistance. This study aimed to characterize the colistin-resistant *Salmonella* isolates collected from 211 retail chicken meat samples between December 2020 and January 2022 in Shanghai, China. Overall, 90 *Salmonella* isolates (42.7%, 90/211) were identified, which were identified as 13 serotypes. Antimicrobial resistance profiling showed that 15.6% (14/90) of the isolates exhibited resistance to colistin. Among these, five isolates were found to carry the *mcr-1* gene, which could be horizontally transferred to other hosts. The mechanism of resistance in the remaining nine *mcr*-negative colistin-resistant isolates was further studied, and it was found that there were seven amino acid substitutions in PmrAB. Site-directed mutagenesis was used to construct mutants, demonstrating that three novel substitutions (L105P in PmrA, P94L, and L331R in PmrB) contributed to colistin resistance in *Salmonella* (MIC = 8 or 16 μg/mL). Quantitative PCR and lipid A analysis were then employed to explore the resistance regulatory pathway. It was found that these substitutions resulted in the production of L-Ara4N by upregulating the expression of the genes *udg* and *pmrK*, which in turn modified lipid A. Finally, the genes *udg* and *pmrK* in the mutants were knocked out to investigate whether these substitutions conferred colistin resistance through these genes. The colistin MIC of the *udg* or *pmrK* deletion in mutants was similar to that of the parental strains (MIC = 0.25 μg/mL), indicating that these substitutions might confer colistin resistance through the *pmrE* and *udg* pathways. These findings demonstrate that amino acid substitutions in PmrAB contribute to the development of colistin resistance in *Salmonella* by modifying lipid A through the genes *udg* and *pmrK* to produce L-Ara4N, providing insight into the mechanisms of colistin resistance in *Salmonella*.

Keywords: *Salmonella*, colistin resistance, PmrAB, *pmrK*, *udg*, *mcr-1*, retail chicken meat

1. Introduction

Salmonella is one of the most common foodborne pathogens, which leads to the highest incidence of diarrhea in children over five and adults across Africa, with an estimated rate of 122,090 cases per 100,000 individuals[1]. In China, it is reported that 70-80% of bacterial food poisoning incidents are caused by *Salmonella*[2]. Animal-derived foods, particularly chicken and pork meat, are recognized as the main carriers for spreading *Salmonella* and other pathogenic bacteria to humans[1,3].

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Antimicrobials are important means of treating *Salmonella* infections in human and animal husbandry. However, the number of antimicrobial-resistant *Salmonella* cases is increasing with the widespread use of antimicrobials[4,5]. Colistin is considered one of the last-resort antimicrobials for treating antimicrobial-resistant, especially carbapenem-resistant gram-negative bacteria[6]. Unfortunately, due to the extensive application of colistin in both human and veterinary medicine, epidemiological research have verified the emergence of colistin-resistant (COL^r) gram-negative bacteria in animals, humans and foods[7]. The colistin-resistant mechanism involves plasmid- or chromosome-mediated modifications of lipid A[8].

Plasmid-mediated mobile colistin resistance (*mcr*) gene was initially identified in *Escherichia coli* and now was prevalent in many *Enterobacteriaceae*[9,10]. The *mcr* gene encoded phosphoethanolamine (PEtN) to modify lipid A[8]. Meanwhile, the modification of lipid A in *Enterobacteriaceae* might also involve a chromosome-encoded mechanism linked to mutations in the regulatory genes of the PmrAB two-component system (TCS). The PmrAB two-component system plays a crucial role in regulating bacterial resistance, particularly against antimicrobial peptides and cationic antimicrobials. This system controls the expression of genes that modify the bacterial cell envelope, thereby enhancing resistance to these threats. Specifically, the response regulator PmrA activates genes such as *pmrHFIJKLM* and *udg*, which facilitate the addition of 4-amino-4-deoxy-l-arabinose (L-Ara4N) to the lipid A component of lipopolysaccharides. This modification neutralizes the positive charge of the membrane, reducing the binding affinity for cationic peptides[8,11,12]. Studies on chromosome-encoded colistin resistance mechanisms have been reported in *Escherichia coli*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*[11-13]. However, research on *Salmonella*, another significant pathogen in the *Enterobacteriaceae* family, has limited data on the chromosomal mechanisms of colistin resistance.

This study aimed to characterize the epidemiology and antimicrobial resistance profiles of *Salmonella* isolates in retail chicken meat in Shanghai, China. Furthermore, a focused analysis was performed on the colistin resistance mechanism of amino acid substitutions on the PmrAB in *Salmonella*.

2. Materials and methods

2.1. Samples collection and Salmonella isolation

From December 2020 to January 2022, 211 retail chicken meat samples were collected from 22 supermarkets and 5 retail markets in Shanghai, China. Among the 211 samples, 158 samples were unpackaged and 53 samples were packaged, as well as 114, 74 and 23 samples were stored under chilled, frozen and room temperature conditions, respectively. *Salmonella* isolates were collected from chicken meat samples following the International Organization for Standardization (ISO) 6579-1:2017 method[14]. 25 g meat sample was rinsed with 225 mL of buffered peptone water and incubated at (37 ± 1) ℃ with shaking at 160 r/min for 6 hours. Then, 1 mL of this culture was added to 9 mL of tetrathionate broth and incubated at 42 ℃. The enriched culture was then streaked onto xylose lysine tergitol 4 agar and incubated overnight at 37 ℃. Suspected *Salmonella* colonies were selected. The White-Kauffmann method was used to identify the

serotypes of *Salmonella*[15]. This method relies on the detection of specific O (somatic) and H (flagellar) antigens present on the surface of *Salmonella* bacteria.

2.2. Multi-locus sequence typing of Salmonella isolates

Seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*) of 90 *Salmonella* isolates were sequenced to identify the multi-locus sequence typing (ST)[16]. The GrapeTree software was used to construct a minimum spanning tree for the analysis of ST distribution[17].

2.3. Antimicrobial Susceptibility Testing of Salmonella isolates

The agar dilution method was used to determine the antimicrobial susceptibility of *Salmonella* isolates. A total of 18 antimicrobials belonging to 11 categories were tested, including nalidixic acid, ofloxacin, ciprofloxacin, ampicillin, cefotaxime, cefepime, meropenem, tetracycline, tigecycline, streptomycin, gentamicin, amikacin, kanamycin, colistin, chloramphenicol, azithromycin, fosfomycin and sulfisoxazole. The guideline of the Clinical and Laboratory Standards Institute (CLSI, 2020) was used to determine the minimum inhibitory concentration (MIC)[18]. Detailed information regarding the antimicrobials utilized, inoculum size, concentration ranges for each compound, and breakpoint criteria can be found in Table S3 of the supplementary materials. The *E. coli* ATCC 25922 was used as a quality control strain. The isolates resistant to at least three categories of antimicrobials were defined as multidrug-resistant (MDR).

2.4. Whole genome sequencing of COL^r Salmonella isolates

Whole genome sequencing (WGS) of COL^r *Salmonella* isolates was conducted on the Illumina HiSeq X10 platform. The obtained COL^r *Salmonella* isolates were tested for the genes *mcr-1* to *mcr-10*. Molecular analysis showed that only the gene *mcr-1* was identified. The PacBio RS II platform was used to sequence the *mcr-1*-positive isolate SJTUF15550 to get the complete circular maps. The ResFinder 4.1 (https://www.genomicepidemiology.org/) was used to identify the antimicrobial-resistant genes (ARGs). The oriTfinder (https://bioinfo-mml.sjtu.edu.cn/oriTfinder/) was used to predict the transfer ability of *mcr-1* gene. The sequence of genes *pmrA*, *pmrB*, *pmrD*, *phoP*, and *phoQ* in *mcr-1*-negative COL^r *Salmonella* were collected through WGS. Subsequently, the sequences were compared with those of *Salmonella* Typhimurium 14028 (St14) and *Salmonella* Enteritidis 13076 (Se13) to identify amino acid substitutions and eliminate potential synonymous polymorphisms.

2.5. Conjugation experiments of the mcr-1 gene

Conjugation experiments were conducted to verify whether the *mcr-1* gene could be transferred[19]. The *mcr-1*-positive *Salmonella* isolates served as donors, while the rifampicin-resistant *E. coli* J53 strain was utilized as the recipient. The conjugates were selectively cultured on Mueller-Hinton agar supplemented with colistin (2 μg/mL) and rifampicin (200 μg/mL). The *mcr-1* gene in the conjugates was identified by PCR.

2.6. Phylogenetic tree analysis of colistin-resistant Salmonella isolates and mcr-1-positive IncHI2 plasmids

The kSNP4.1 method was used to construct the phylogenetic tree of COL^r *Salmonella* isolates to explore their genetic relationships[20]. Additionally, to study the genetic relationships among different *mcr-1*-positive

IncHI2 plasmids, one plasmid from this study and 56 plasmids from the NCBI were used to construct the phylogenetic trees. The online tool iTOL v6 was used to visualize the phylogenetic trees (https://itol.embl.de).

2.7. Site-directed mutagenesis of PmrAB, and pmrK and udg deletion in Salmonella

The PmrAB of *Salmonella* is closely associated with the development of resistance to colistin. Amino acid substitutions in PmrAB of the COL^r isolates were identified by comparative genomics in section 2.4. To verify the role of these substitutions in the development of resistance to colistin, St14 was used as the parent strain, and the red homologous recombination method was used to construct the mutants with amino acid substitution in PmrAB on the chromosome. Furthermore, to determine whether these substitutions in PmrAB have similar functions in different *Salmonella* serotypes, Se13 was also used as the parent strain to construct the mutants. Subsequently, the colistin MIC and growth curves of these mutants were determined according to previous studies[21].

To study the roles of the genes *pmrK* and *udg* in the development of resistance to colistin in the constructed mutants, we also used the red homologous recombination method to knock out the genes *pmrK* and *udg* in the mutants to obtain the *pmrK/udg* deletion in mutants. In this experiment, plasmid pBAD33 was used to construct the *pmrK*/*udg* gene complemented strain. Detailed procedures for the construction of mutants, and s *pmrK* and *udg* deletion in *Salmonella* can be found in the supplementary materials.

2.8. Real-time RT-PCR of colistin-resistant isolates and mutants

To determine the expression levels of genes associated with lipid A modification, the expression of the genes *pmrC*, *pmrD, pmrK* and *udg* in *mcr*-negative COL^r isolates, parental strain St14 and mutants were evaluated. The gene *16S RNA* was used as the internal reference gene for expression level normalization. Real-time quantitative PCR was conducted utilizing the SYBR Green PCR Master Mix (Vazyme Biotech, China). The data are presented as the mean ± standard deviation of three independent experiments. The primers are listed in Table S1.

2.9. Analysis of lipid A by mass spectrometry

The lipid A of the parent strain St14, mutants, *pmrK* and *udg* deletion in mutants was extracted according to the previous methods[22]. The MALDI-7090 instrument was used to analyze the lipid A via electrospray ionization mass spectrometry in negative ion mode (Kratos Analytical Ltd, England). The obtained data was processed by the MassLynx V4.1 software (Waters Corp, USA).

2.10. Statistical analysis

A chi-square test was performed to assess the variations in the positive rates of *Salmonella* across different market types, packaging types and storage conditions. A significance level of $P < 0.05$ was used to determine statistical significance.

2.11 Data av.ailability

The genomic data of the COL^r *Salmonella* isolates can be accessed through Bio-Project accessions PRJNA1048682, PRJNA1048697, PRJNA1048699, PRJNA1048702, PRJNA1048705 and PRJNA1208761.

Accession numbers: JAYMGK000000000-JAYMGN000000000, JBKKQA000000000-JBKKQI000000000. Detailed information of the isolates collected from this study is in Table S2. Table S1 and Table S2 are provided in the Supplementary material.

3. Results

3.1. Prevalence characteristics of Salmonella isolates from chicken meat samples

Overall, it was shown in Table 1 that 90 *Salmonella* isolates were collected from 211 retail chicken meat samples between December 2020 and January 2022 in Shanghai, China, and the positive rate was 42.7% (90/211). In detail, the samples were collected from different markets, and the identification results showed that the positive rate of *Salmonella* in supermarkets (48.8%, 80/164) was significantly higher than in retail markets (21.3%, 10/47) ($P < 0.05$). The chicken meat samples were collected from different storage conditions, and the positive rate of *Salmonella* in chilled samples (45.7%, 86/188) was markedly higher than in fresh samples (17.4%, 4/23) ($P < 0.05$).

Table 1 Prevalence of *Salmonella* from retail chicken meat samples in Shanghai, China (2020–2022)

Variable	Category	Number of samples	Number of samples positive for *Salmonella*	Percentage of positive samples (%)
Market type	Supermarket	164	80	48.8[a]
	Retail markets	47	10	21.3[b]
Storage type	Room temperature	23	4	17.4[a]
	Chilled	114	56	49.1[b]
	Frozen	74	30	40.5
Package type	Packaged	53	23	43.4
	Unpackaged	158	67	42.4
Total		211	90	42.7

Note: Different lowercase letters indicate significant differences between the percentage of positive samples based on the Chi-square test. The room temperature is between 18℃ and 25℃.

According to the White-Kauffmann method, it was shown in Figure 1 and Table S2 that 13 different *Salmonella* serotypes were identified among the 90 isolates, of which *S.* Enteritidis (n=29) was the most common serotype, followed by *S.* Kentucky (n=15), *S.* Indiana (n=9), *S.* Typhimurium (n=9), *S.* Schwarzengrund (n=7), *S.* Rissen (n=7) and *S.* Thompson (n=5). It was shown that there was a high diversity and prevalence of *Salmonella* isolates in retail chicken meat through the Sankey plot.

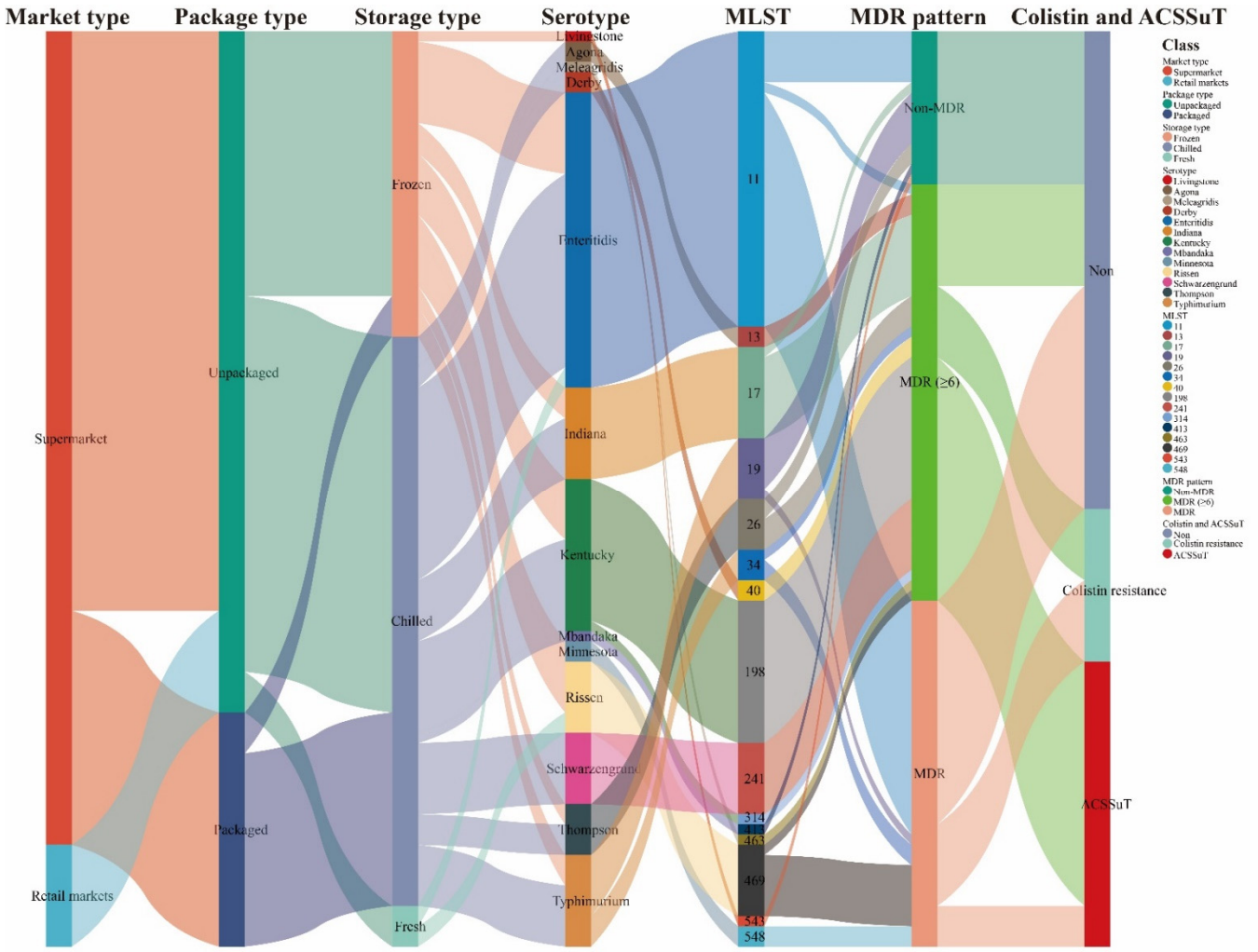


Figure 1. Characterization of *Salmonella* isolates (n=90) from retail pork: origin; storage conditions; serotyping; molecular typing and antimicrobial susceptibility patterns (Sankey plot). The number of *Salmonella* isolates is indicated by the height of the rectangle. The line indicates the distribution of the serotype in different market types, and storage conditions, and STs, MDR, colistin and ACSSuT phenotypes are colored by different serotypes.

The result of the minimum spanning tree was shown in Figure 2 that 15 STs were identified, of which ST11 (n=29) was the most prevalent, followed by ST198 (n=14), ST17 (n=9), ST241 (n=7), ST469 (n=7), ST19 (n=6), ST26 (n=5) and ST34 (n=3). It was found that a strong correlation between the *Salmonella* serotypes and ST types was determined, such as ST11 with *S.* Enteritidis, ST198 with *S.* Kentucky, ST17 with *S.* Indiana, ST241 with *S.* Schwarzengrund and ST19 with *S.* Typhimurium.

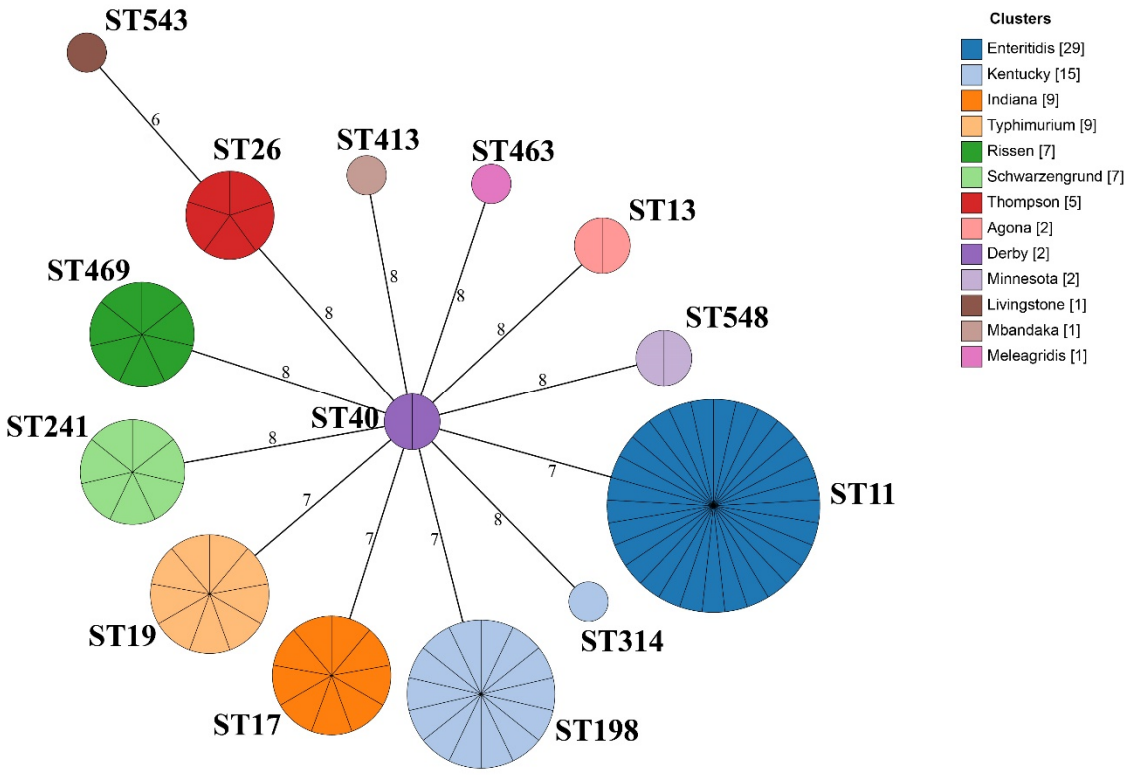


Figure 2. Minimum spanning tree of *Salmonella* isolates by multi-locus sequence typing. Each node in the tree corresponds to a unique sequence type (ST), with the size of the node reflecting the number of isolates associated with that ST. The branch length connecting nodes represents the genetic distance calculated from nucleotide variances in seven conserved housekeeping genes of *Salmonella*.

3.2. Antimicrobial susceptibility analysis of Salmonella isolates

It was shown in Figure 3 that all (100.0%, 90/90) *Salmonella* isolates were resistant to at least one category of antimicrobials, while 73 (81.1%, 73/90) isolates exhibited MDR and 29 isolates (32.2%, 29/90) showed ACSSuT pattern (defined as resistance to ampicillin, chloramphenicol, streptomycin, sulfisoxazole and tetracycline). The high resistance rates to sulfisoxazole (94.4%, 85/90), ampicillin (81.1%, 73/90), nalidixic acid (74.4%, 67/90), tetracycline (57.8%, 52/90), streptomycin (52.2%, 47/90), ciprofloxacin (42.2%, 38/90), cefotaxime (42.2%, 38/90), fosfomycin (36.7%, 33/90), azithromycin (24.4%, 22/90) and amikacin (21.1%, 19/90) were determined. Notably, 14 (15.6%, 14/90) *Salmonella* isolates were found to exhibit resistance to colistin. All isolates were susceptible to tigecycline and meropenem. Interestingly, it was found that different *Salmonella* serotypes exhibited different resistance patterns, with the MDR rates for *S.* Kentucky (100%, 15/15) and *S.* Indiana (88.9%, 8/9) being significantly higher than that of *S.* Typhimurium (44.4%, 4/9) ($P < 0.05$) (Figure S1).

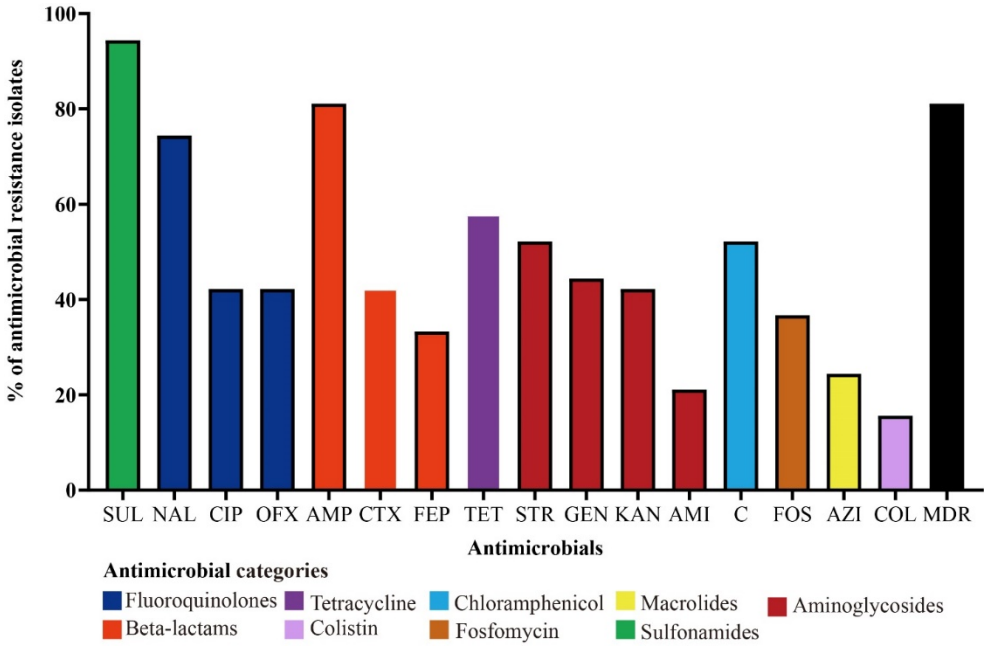


Figure 3. Antimicrobial susceptibility of *Salmonella* isolates (n=90). Percentage of *Salmonella* isolates resistant to different antimicrobials and multidrug resistance. NAL: nalidixic acid, OFX: ofloxacin, CIP: ciprofloxacin, AMP: ampicillin, CTX: cefotaxime, FEP: cefepime, TET: tetracycline, STR: streptomycin, GEN: gentamicin, AMI: amikacin, KAN: kanamycin, COL: colistin, C: chloramphenicol, AZI: azithromycin, FOS: fosfomycin, SUL: sulfisoxazole, MDR: multidrug resistance.

3.3. Genomic characteristics of COL^r Salmonella isolates

It was found in Table 2 that 5 of 14 COL^r *Salmonella* isolates were found to carry the *mcr-1* gene, of which four isolates were *S.* Schwarzengrund, and one was *S.* Agona. One complete chromosome and one plasmid pSJTUF15550 (249,103 bp, IncHI2) were identified from the *S.* Agona SJTUF15550, and there were 13 ARGs for seven categories of antimicrobials in the plasmid pSJTUF15550 (Figure S2). The *mcr-1* gene of five isolates was located on the IncHI2 plasmids and shared a common genetic structure *mcr-1*-pap2. The IncHI2 plasmid of *S.* Agona contained the *relaxase* and *T4CP* genes, which were essential for horizontal transfer, while these genes were absent in the IncHI2 plasmid of *S.* Schwarzengrund. This finding is consistent with the results of conjugation assays that the *mcr-1*-positive IncHI2 plasmid pSJTUF15550 from *S.* Agona was successfully transferred to *E. coli* J53 to get the conjugant. The conjugation frequencies ranged from 4.1×10^{-6} to 6.5×10^{-7} per recipient cell and the conjugant was found to exhibit resistance to multiple antimicrobials (including nalidixic acid, tetracycline, kanamycin, chloramphenicol and sulfisoxazole). In contrast, the *mcr-1* gene of the *S.* Schwarzengrund isolates could not be transferred.

Table 2. Characterizations and amino acid substitution in PmrAB of 14 colistin-resistant *Salmonella* isolates

Isolates	Collection date	Serotype	MLST	Colistin MIC (μg/ml)	*mcr-1* gene	Amino acid substitution in:	
						PmrA	PmrB
SJTUF15535	2021.04.19	Schwarzengrund	241	4	*mcr-1*		
SJTUF15536	2021.04.19	Schwarzengrund	241	4	*mcr-1*		
SJTUF15538	2021.11.26	Enteritidis	11	8			S280N
SJTUF15540	2021.12.24	Enteritidis	11	16			S280N, P94L
SJTUF15541	2021.12.24	Enteritidis	11	8		L105P	S280N
SJTUF15542	2021.12.24	Enteritidis	11	8			
SJTUF15543	2021.12.24	Enteritidis	11	8			

SJTUF15544	2021.12.24	Enteritidis	11	8		L331R
SJTUF15550	2021.03.01	Agona	13	4	*mcr-1*	
SJTUF15553	2021.04.19	Meleagridis	463	8		H274Y, D149Y
SJTUF15556	2021.04.19	Enteritidis	11	16		S124P
SJTUF15561	2021.04.19	Enteritidis	11	8		S280N
SJTUF15565	2021.01.13	Schwarzengrund	241	4	*mcr-1*	
SJTUF15566	2021.01.13	Schwarzengrund	241	4	*mcr-1*	

Note: Amino acid substitutions were found only in colistin-resistant *Salmonella* isolates after alignment with *Salmonella* Typhimurium 14028.

The one-letter designations for amino acids are used. R: Arginine, H: Histidine, Y: Tyrosine, D: Aspartic acid, S: Serine, N: Asparagine, L: Leucine, P: Proline

Except for the 5 *mcr-1*-positive isolates, the remaining 9 *mcr*-negative COL^r isolates were identified, of which 8 were *S.* Enteritidis, and 1 was *S.* Meleagridis. It was found that several nucleotide mutations resulted in 7 unique amino acid substitutions in PmrAB. Specifically, the COL^r isolate SJTUF15556, which had high colistin MIC (16 μg/mL), included a unique amino acid substitution S124P in PmrB. Another isolate SJTUF15540, which had a colistin MIC at 16 μg/mL, included two unique substitutions in PmrB (P94L and S280N). Meanwhile, the isolate SJTUF15541, which also had a colistin MIC at 8 μg/mL, included the unique substitutions L105P in PmrA and S280N in PmrB. The isolate SJTUF15553, which had a colistin MIC at 8 μg/mL, included two unique substitutions in PmrB (D149Y and H274Y). The isolates SJTUF15538 and SJTUF15561, both with a colistin MIC at 8 μg/mL, included a unique substitution S280N in PmrB. The isolate SJTUF15544, which had a colistin MIC at 8 μg/mL, included a unique substitution L331R in PmrB. No substitution in the PmrAB, PmrD and PhoPQ was determined in COL^r isolates SJTUF15542 and SJTUF15543. Overall, seven COL^r isolates were found to carry novel mutations, four of which were obtained from frozen samples, and the other three were obtained from chilled samples.

The MICs of antimicrobials other than colistin for the 14 colistin-resistant *Salmonella* isolates are presented in Table 3. The rates of resistance to other antimicrobials were as follows: 100% for nalidixic acid, 85.7% for ampicillin, 42.9% for ciprofloxacin, ofloxacin, gentamicin, and chloramphenicol, and 35.7% for cefotaxime and cefepime. The antimicrobial-resistant phenotype of *Salmonella* was closely associated with their ARGs. The genomic analysis found that 34 ARGs and 3 quinolone resistance-determining regions (QRDRs) mutations were identified in 14 COL^r isolates. These ARGs and QRDRs conferred resistance to 11 categories of antimicrobials, including aminoglycosides (n=12), folate pathway antagonists (n=5), fluoroquinolones (n=3), *β*-lactams (n=3), fosfomycins (n=2), colistin (n=1) and macrolides (n=1). The *β*-lactamase resistance genes *bla*$_{\text{TEM–1B}}$ and *bla*$_{\text{CTX-M}}$ (*bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-55}}$) were widely present in *Salmonella*, taking up 57.1% (8/14) and 35.7% (5/14) of the isolates, respectively. The isolates only carrying the *bla*$_{\text{TEM-1B}}$ were resistant to ampicillin, while the *bla*$_{\text{CTX-M}}$-positive isolates simultaneously exhibited resistance to ampicillin, cefotaxime and cefepime. The fluoroquinolones resistance genes *oqxAB*, *qnrB6* and *qnrS1* were identified in 7.1% (1/14), 7.1% (1/14) and 28.6% (4/14) of the isolates, respectively. ParC (T57S) and GyrA (D87Y; D87G) were found in 35.7% (5/14) and 57.1% (8/14) of the isolates, respectively. *Salmonella* isolates exhibited resistance to nalidixic acid when they only harbored the GyrA (D87Y or D87G) mutation. Isolates that carried the gene *qnrB6* showed low-level resistance to ciprofloxacin (1 μg/ml).

Additionally, resistance to ciprofloxacin was also observed in *Salmonella* isolates that had the ParC (T57S) mutation along with either the gene *qnrS1* or *oqxAB*. Furthermore, the genes *fosA* (*fosA3* and *fosA7*), *tet*(A), and *floR* conferred resistance to the corresponding fosfomycin, tetracycline and chloramphenicol, all accounting for 42.9% (6/14) of the isolates.

Table 3. MICs of antimicrobials against 14 colistin-resistant *Salmonella* isolates

Isolates	Antimicrobials MIC (μg/mL)														
	NAL	OFX	CIP	AMP	CTX	FEP	TET	GEN	STR	AMI	KAN	C	AZI	FOS	SUL
SJTUF15535	32	2	2	128	4	32	64	16	8	2	256	128	1	512	1024
SJTUF15536	128	8	8	128	16	64	64	64	16	1	256	128	2	512	1024
SJTUF15538	128	0.125	0.125	128	0.25	0.25	4	0.25	8	8	1	1	1	1	128
SJTUF15540	128	0.125	0.125	128	0.25	0.25	4	0.5	64	1	2	0.25	2	8	1024
SJTUF15541	128	0.125	0.125	128	0.25	0.25	4	1	64	2	8	1	1	16	512
SJTUF15542	128	0.125	0.125	2	0.25	0.25	4	4	8	1	1	1	0.5	32	64
SJTUF15543	128	0.125	0.125	128	0.25	0.25	4	1	256	0.5	2	2	2	16	1024
SJTUF15544	128	0.125	0.125	128	0.25	0.25	4	1	256	2	8	2	1	2	1024
SJTUF15550	128	2	1	128	0.25	0.25	32	16	4	8	256	128	1	256	1024
SJTUF15553	32	2	1	128	8	62	64	16	256	2	256	128	64	512	512
SJTUF15556	128	0.125	0.125	2	0.25	0.25	4	2	8	1	8	0.5	1	2	32
SJTUF15561	128	0.125	0.125	128	0.25	0.25	4	4	256	1	8	1	2	32	1024
SJTUF15565	128	8	2	128	16	32	64	32	8	64	256	128	2	512	1024
SJTUF15566	32	2	1	128	16	32	64	32	16	0.5	256	128	2	512	1024

Note. NAL: nalidixic acid, OFX: ofloxacin, CIP: ciprofloxacin, AMP: ampicillin, CTX: cefotaxime, FEP: cefepime, TET: tetracycline, STR: streptomycin, GEN: gentamicin, AMI: amikacin, KAN: kanamycin, C: chloramphenicol, AZI: azithromycin, FOS: fosfomycin, SUL: sulfisoxazole.

Plasmid replicon analysis revealed that 12 out of 14 *Salmonella* isolates (85.7%) carried at least one plasmid, encompassing 8 different known plasmid replicons (Table S2). The most prevalent plasmid was the IncFIB(S) plasmid, found in 6 isolates (42.9%, 6/14), followed by IncFII(S), IncHI2/IncHI2A and IncX1, each presented in 5 isolates (35.7%, 5/14).

3.4. Phylogenetic relationship of COL^r Salmonella isolates and mcr-1-positive IncHI2 plasmids

The phylogenetic tree was constructed for 14 COL^r *Salmonella* isolates to explore their genetic relationships. It was shown in Figure 4 that isolates with the same serotype or ST were more likely to cluster together. The isolates of *S.* Enteritidis (n=8) and *S.* Schwarzengrund (n=4), collected from different storage conditions, collection dates, and market types, showed minimal differences in their core genomes (SNP < 30), suggesting the possibility of clonal transmission of these two serotypes isolates in the retail chicken meat in Shanghai.

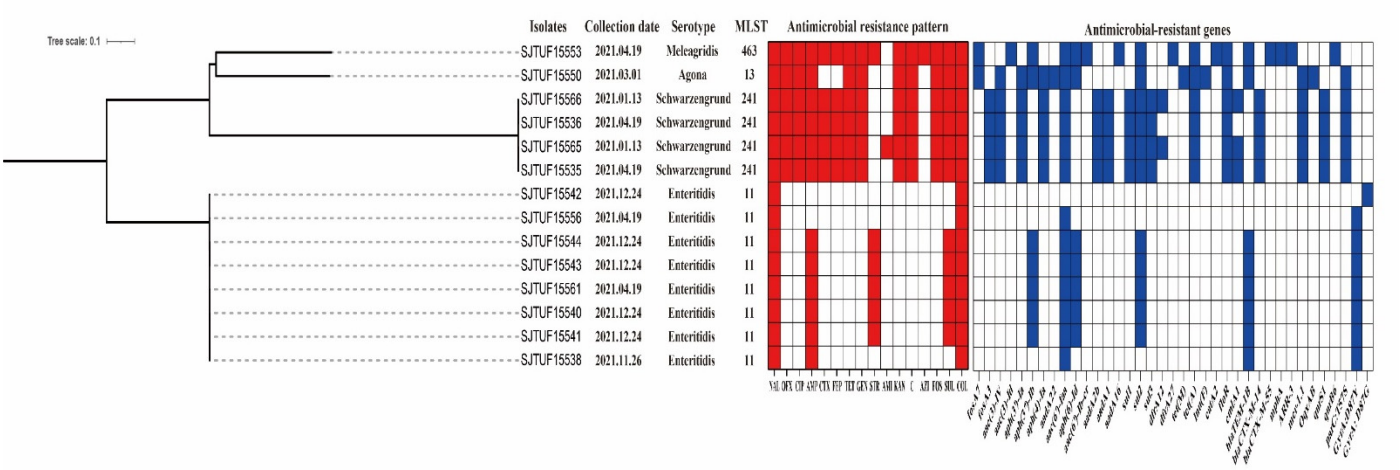


Figure 4. Phylogenetic analysis of 14 colistin-resistant *Salmonella* isolates. Note: Basic information for the isolates including collection date, serotype, MLST as well as the presence of antimicrobial resistance pattern (blue) and antimicrobial-resistant genes (blue). NAL: nalidixic acid, OFX: ofloxacin, CIP: ciprofloxacin, AMP: ampicillin, CTX: cefotaxime, FEP: cefepime, TET: tetracycline, STR: streptomycin, GEN: gentamicin, AMI: amikacin, KAN: kanamycin, COL: colistin, C: chloramphenicol, AZI: azithromycin, FOS: fosfomycin, SUL: sulfisoxazole.

The phylogenetic tree of 57 *mcr-1*-positive IncHI2 plasmids was shown in Figure S3 that plasmids collected from various sources (e.g., humans, food) in China showed diversity and multiple branches. In contrast, plasmids from other countries (e.g., Bangladesh, the United States, Egypt) clustered together to form a branch independent of those from China. Notably, KX023262 (237,939 bp), collected from chicken in China, clustered closely with plasmid pSJTUF15550. It was shown in Figure 5A that all resistance genes, except *mcr-1*, were located in the MDR region (position 10-70 kb). The *IS6* and *IS1* were adjacent to these resistance genes. Moreover, ten plasmids were found to lack the genes responsible for horizontal transfer (i.e., *relaxase* and *T4CP* genes). These non-transferrable plasmids were identified in various hosts and different branches of the phylogenetic tree, suggesting that the genes responsible for transfer might have been lost randomly.

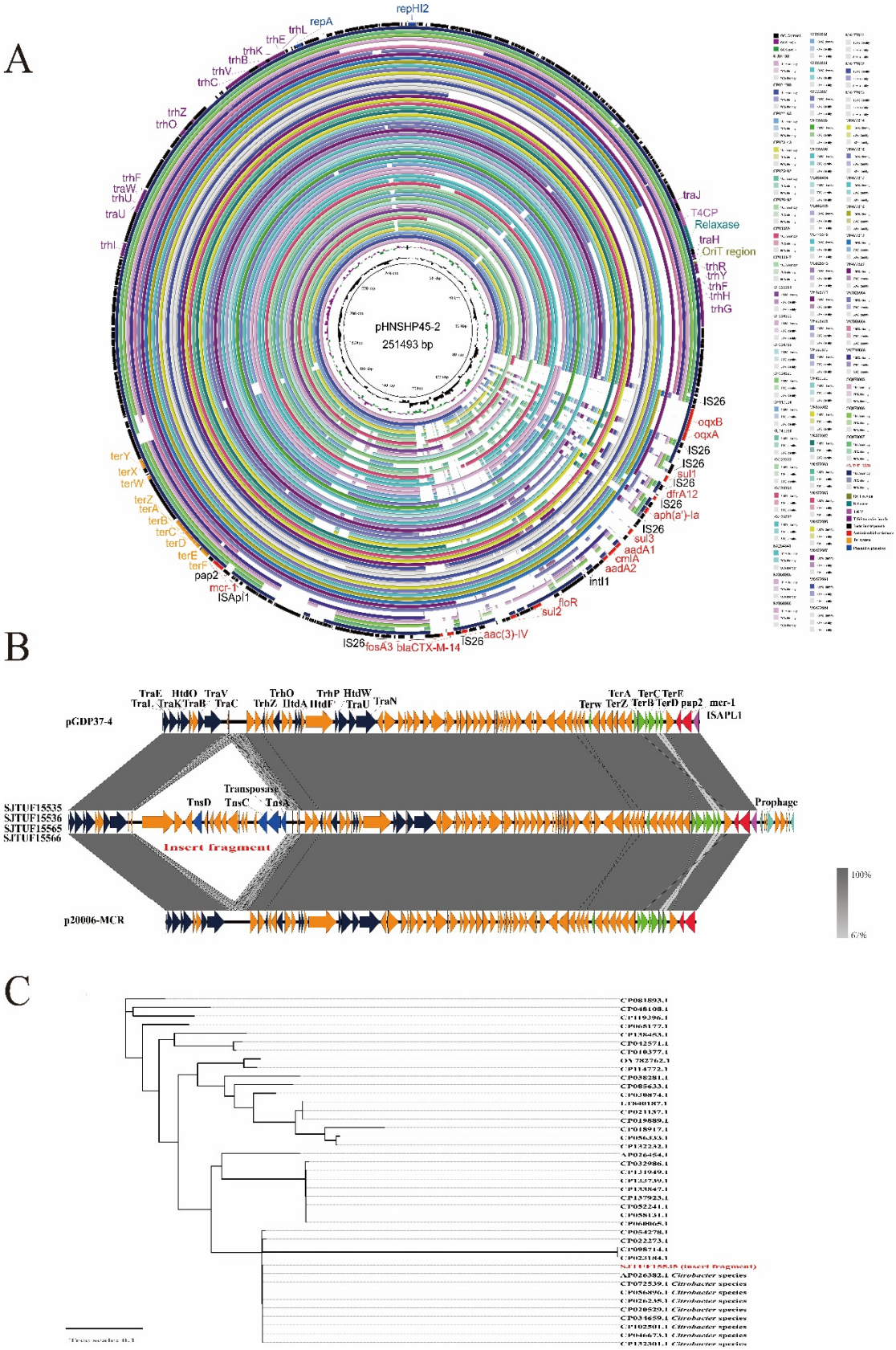


Figure 5. Comparative analysis of the complete IncHI2 plasmids carrying the *mcr-1* gene. A. Comparative analysis of 57 complete IncHI2 plasmids carrying the *mcr-1* gene using the BRIG tool. The outer ring represents the annotation of plasmid pHNSHP45-2. Genes are color-coded based on functional annotation. The genes responsible for horizontal transfer (*T4CP* and *relaxase* genes) and ARGs are marked in black and blue, respectively. B. Comparison of several plasmids shows that a 23,397 bp insertion fragment mediated by transposon Tn7 was found near the *mcr-1* gene in the IncHI2 plasmids of *S.* Schwarzengrund isolates. C. Phylogenetic analysis of 41 fragments. 40 fragments with close homology were downloaded from NCBI.

Furthermore, it was shown in Figure 5B that a 23,397 bp insertion fragment mediated by transposon Tn7 was found near the *mcr-1* gene in the IncHI2 plasmids of *S.* Schwarzengrund isolates. This insertion fragment comprised 16 genes, including TIR domains and patatin, which were associated with enhanced bacterial virulence[23]. The inserted fragments were subjected to BLAST alignment, and then 40 fragments with close homology were downloaded to construct a phylogenetic tree. The result was shown in Figure 5C that the insert fragment exhibited 100% homology to *Citrobacter* species, indicating a potential origin from this organism.

3.5. Role of amino acid substitutions in PmrAB in Salmonella

It was shown in Table 4 that 7 amino acid substitutions in PmrAB were identified in *mcr*-negative COL^r isolates in section 3.3. Fourteen mutants with substitutions in PmrAB were successfully constructed from parental strains Se13 and St14. Antimicrobial susceptibility testing showed that the colistin MIC for mutants PmrB^{P94L} (amino acid substitution P94L in PmrB) and PmrB^{S124P} (substitution S124P in PmrB) was 16 μg/mL, while for mutants PmrA^{L105P} (substitution L105P in PmrA) and PmrB^{L331R} (substitution L331R in PmrB) was 8 μg/mL. These results indicated that these four amino acid substitutions resulted in a 32- to 64-fold increase in colistin MIC compared to the parental strains Se13 and St14, which increased from 0.25 μg/mL to 8 or 16 μg/mL. The other three substitutions (D149Y, H274Y, and S280N in PmrB) did not apparently affect the colistin MIC at *Salmonella*, which was somewhat inconsistent with the results of a previous study that the substitution D149Y in PmrB of *S.* Typhimurium LT2 conferred resistance to colistin[24]. This discrepancy might be due to the differences of the parental strain. The growth curves showed that the mutants exhibited a similar growth pattern to the parental strains, suggesting that these substitutions in PmrAB did not apparently affect their growth (Figure S4).

Table 4. Colistin susceptibility in mutants and complemented isolates

Strain or mutants	Description	Colistin MIC (μg/mL)
St14	*Salmonella* Typhimurium 14028	0.25
Se13	*Salmonella* Enteritidis 13076	0.25
PmrA^{L105P}	Substitution L105P in PmrA of St14	8
PmrB^{P94L}	Substitution P94L in PmrB of St14	16
PmrB^{S124P}	Substitution S124P in PmrB of St14	16
PmrB^{D149Y}	Substitution D149Y in PmrB of St14	0.25
PmrB^{H274Y}	Substitution H274Y in PmrB of St14	0.25
PmrB^{S280N}	Substitution S280N in PmrB of St14	0.25
PmrB^{L331R}	Substitution L331R in PmrB of St14	8
Se13 PmrA^{L105P}	Substitution L105P in PmrA of Se13	8
Se13 PmrB^{P94L}	Substitution P94L in PmrB of Se13	16
Se13 PmrB^{S124P}	Substitution S124P in PmrB of Se13	16
Se13 PmrB^{D149Y}	Substitution D149Y in PmrB of Se13	0.25
Se13 PmrB^{H274Y}	Substitution H274Y in PmrB of Se13	0.25
Se13 PmrB^{S280N}	Substitution S280N in PmrB of Se13	0.25
Se13 PmrB^{L331R}	Substitution L331R in PmrB of Se13	8
St14_▲pmrK	Deletion of *pmrK* gene in St14	0.25
PmrA^{L105P}_▲pmrK	Deletion of *pmrK* gene in PmrA^{L105P}	0.25
PmrB^{P94L}_▲pmrK	Deletion of *pmrK* gene in PmrB^{P94L}	0.25
PmrB^{S124P}_▲pmrK	Deletion of *pmrK* gene in PmrB^{S124P}	0.25
PmrB^{L331R}_▲pmrK	Deletion of *pmrK* gene in PmrB^{L331R}	0.25
St14_▲udg	Deletion of *udg* gene in St14	0.25

$PmrA^{L105P}$_▲udg	Deletion of *udg* gene in $PmrA^{L105P}$	0.25
$PmrB^{P94L}$_▲udg	Deletion of *udg* gene in $PmrB^{P94L}$	0.25
$PmrB^{S124P}$_▲udg	Deletion of *udg* gene in $PmrB^{S124P}$	0.25
$PmrB^{L331R}$_▲udg	Deletion of *udg* gene in $PmrB^{L331R}$	0.25
St14_▲pmrK (pBAD_pmrK)	Complemented strain of *pmrK* gene	0.25
$PmrA^{L105P}$_▲pmrK (pBAD_pmrK)	Complemented strain of *pmrK* gene	8
$PmrB^{P94L}$_▲pmrK (pBAD_pmrK)	Complemented strain of *pmrK* gene	16
$PmrB^{S124P}$_▲pmrK (pBAD_pmrK)	Complemented strain of *pmrK* gene	16
$PmrB^{L331R}$_▲pmrK (pBAD_pmrK)	Complemented strain of *pmrK* gene	8
St14_▲udg (pBAD_udg)	Complemented strain of *udg* gene	0.25
$PmrA^{L105P}$_▲udg (pBAD_udg)	Complemented strain of *udg* gene	8
$PmrB^{P94L}$_▲udg (pBAD_udg)	Complemented strain of *udg* gene	16
$PmrB^{S124P}$_▲udg (pBAD_udg)	Complemented strain of *udg* gene	16
$PmrB^{L331R}$_▲udg (pBAD_udg)	Complemented strain of *udg* gene	8

Note: Se13 stands for *Salmonella* Enteritidis 13076, and St14 stands for *Salmonella* Typhimurium 14028. The one-letter designations for amino acids are used. R: Arginine, H: Histidine, Y: Tyrosine, D: Aspartic acid, S: Serine, N: Asparagine, L: Leucine, P: Proline.

3.6. The mRNA expression levels of pmrC, pmrD, pmrK and udg

In section 3.5, four amino acid substitutions in PmrAB were demonstrated to contribute to the development of resistance to colistin in *Salmonella*. The expression levels of the genes *pmrC*, *pmrD, pmrK* and *udg* in seven (except two isolates SJTUF15542 and SJTUF15543) *mcr*-negative COL^r isolates and these mutants ($PmrB^{P94L}$, $PmrB^{S124P}$, $PmrB^{L331R}$ and $PmrA^{L105P}$) were compared with those in St14. It was shown in Figure 6 that the expression levels of *pmrK* and *udg* genes in the *mcr*-negative COL^r isolates and the mutants $PmrB^{P94L}$, $PmrB^{S124P}$, $PmrB^{L331R}$, and $PmrA^{L105P}$ were significantly higher than those of the parental strain St14. The expression levels of the genes *pmrC* and *pmrD* was similar to those of the parental strain St14.

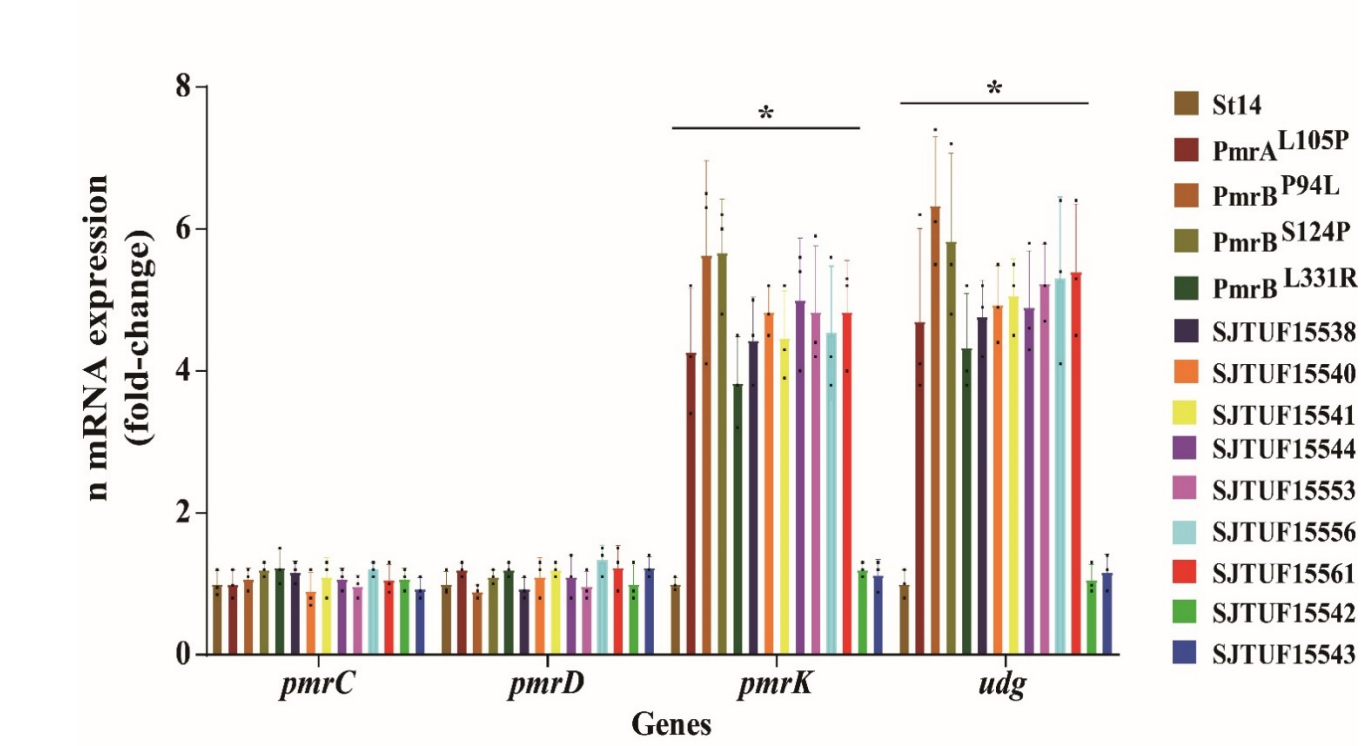
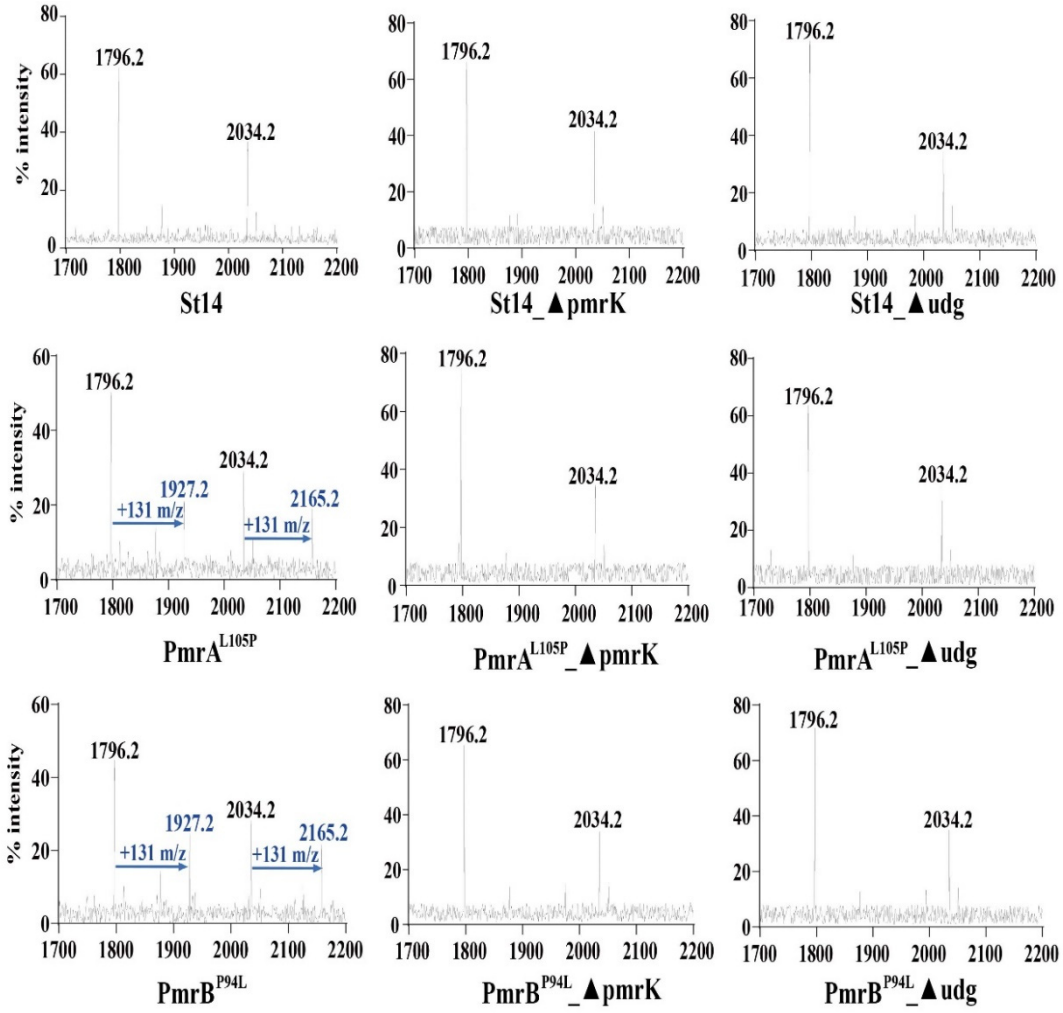


Figure 6. The mRNA expression levels of the genes *pmrC*, *pmrD*, *pmrE* and *pmrK* of the colistin-resistant isolates and mutants. The four mutants ($PmrA^{L105P}$, $PmrB^{P94L}$, $PmrB^{S124P}$ and $PmrB^{L331R}$) and nine colistin-resistant isolates versus the parental strain *S.* Typhimurium 14028 (St14) were conducted. The experiments were conducted in triplicate. * means $P < 0.05$.

3.7. Structural analysis of lipid A

The expression levels of *pmrK* and *udg* were linked to the production of L-Ara4N, which modified lipid A, and the modification was associated with the development of resistance to colistin in *Salmonella*[8]. The structures of lipid A in the 9 *mcr*-negative COL^{r} isolates and 4 mutants (PmrB^{P94L}, PmrB^{S124P}, PmrB^{L331R}, and PmrA^{L105P}) were determined by MALDI-TOF MS. It was shown in Figure 7 that the lipid A from St14 presented two major molecular ions at m/z 1796.2 and 2034.2, corresponding to monophosphate and diphosphate hexadecyl groups in lipid A, respectively (unmodified lipid A)[22]. Lipid A obtained from mutants PmrB^{P94L} and PmrB^{S124P} presented a major molecular ion at m/z 1927.2 (corresponding to L-Ara4N-modified diphosphoryl hexadecyl lipid A), alongside another major ion at m/z 1796.2, and two minor ions at m/z 2034.2 and m/z 2165.2 (attributed to L-Ara4N-modified monophosphoryl hexadecyl lipid A). Similarly, lipid A from mutant PmrA^{L105P} presented a major ion at m/z 1796.2, accompanied by three minor ions at m/z 2034.2, m/z 1927.2, and m/z 2165.2. Lipid A from mutant PmrB^{L331R} presented four major ions at m/z 1796.2, m/z 2034.2, m/z 1927.2, and m/z 2165.2. In Figure S5, lipid A from SJTUF15542 and SJTUF15543 presented two major molecular ions at m/z 1796.2 and 2034.2, whereas lipid A from the other seven *mcr*-negative COL^{r} isolates exhibited four major ions at m/z 1796.2, 2034.2, 1927.2, and 2165.2. These results showed that the lipid A of COL^{r} foodborne isolates (except SJTUF15542 and SJTUF15543) and the four mutants were modified by L-Ara4N.



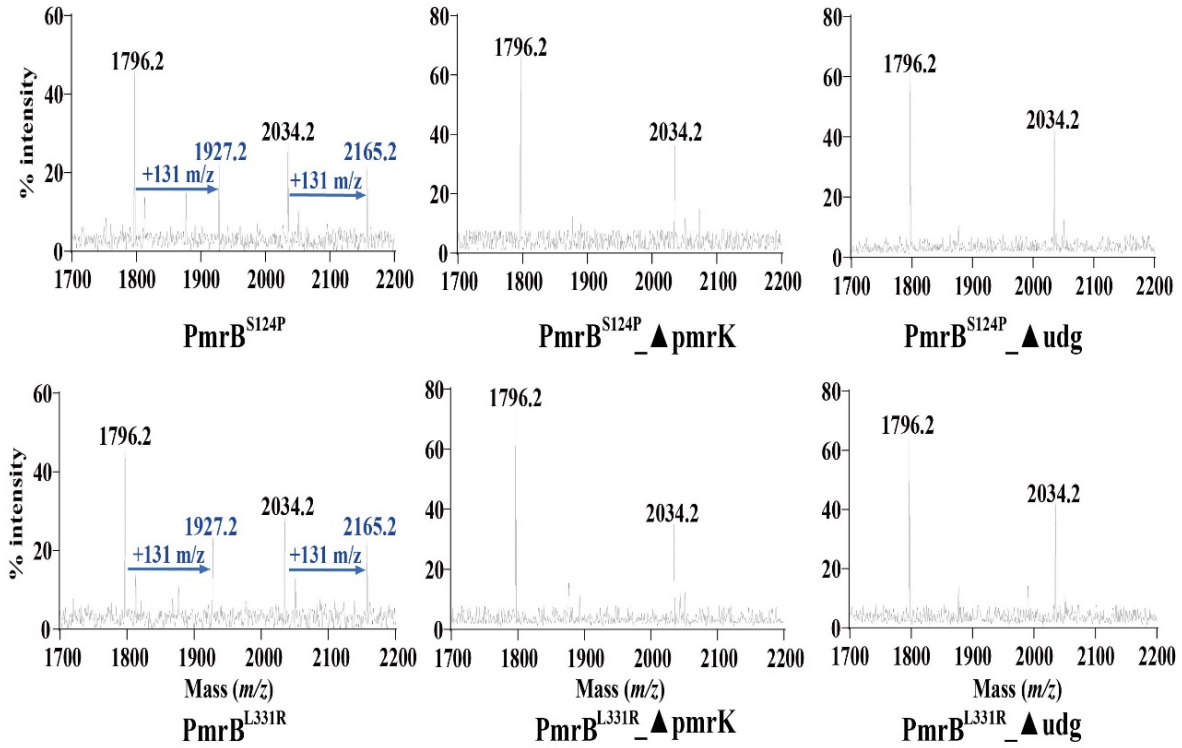


Figure 7. Structures of lipid A from *Salmonella* isolates determined by MALDI-TOF MS. Negative ion mode MALDI-TOF mass spectra of lipid A of *S.* Typhimurium 14028 and mutants PmrA^{L105P}, PmrB^{P94L}, PmrB^{S124P} and PmrB^{L331R}, as well as their corresponding *pmrK* and *udg* deletion isolates.

3.8. Role of the genes pmrK and udg in the development of resistance to colistin in mutants

In sections 3.5, 3.6 and 3.7, four amino acid substitutions in the TCS PmrAB (L105P in PmrA, P94L, S124P and L331R in PmrB) might lead to increased expression levels of the genes *udg* and *pmrK*, resulting in the production of L-Ara4N, which modified lipid A and ultimately caused the development of resistance to colistin. To verify the role of genes *pmrK* and *udg* in the development of resistance to colistin in the mutants, the genes *pmrK* and *udg* deletion and complementation strains were constructed in the parental strain St14 and four colistin-resistant mutants (PmrB^{P94L}, PmrB^{S124P}, PmrB^{L331R} and PmrA^{L105P}). As shown in Table 4, a total of 10 *pmrK*/*udg* deletion strains and 10 *pmrK*/*udg* complementation strains were constructed. Antimicrobial susceptibility testing showed that the *pmrK* or *udg* deletion in St14 did not apparently change its colistin MIC value. The *pmrk* or *udg* deletion in the four mutants reduced the colistin MIC of the mutants from 8 or 16 ug/mL to 0.25 ug/mL, which was consistent with the colistin MIC of the parental strain St14. While the colistin MIC value of the *pmrK*/*udg* gene complementation strains was restored to that of the mutants. Furthermore, the mass spectrometry results of lipid A from the 10 *pmrK* or *udg* deletion strains were similar to those of the parental strain St14, displaying two major molecular ions at m/z 1796.2 and 2034.2 (Figure 7). This indicated that there were no modifications in lipid A. These findings indicated that the genes *pmrK* and *udg* played an important role in the development of resistance to colistin in mutants. The four amino acid substitutions in the PmrAB led to the upregulation of *pmrK* and *udg* expression, resulting in the production of L-Ara4N, which modified lipid A and ultimately contributed to the development of resistance to colistin in *Salmonella*.

4. Discussion

In the present study, the positive rate of *Salmonella* in retail chicken meat in Shanghai was found to be 42.7% (90/211), which was higher than that reported in South Korea (21.4%)[25] and Jiangxi, China (11.9%)[26]. The varying positive rate of *Salmonella* across different regions could be attributed to the climate, geographic distribution and food sources[26,27]. The high positive rate of *Salmonella* found in this study demonstrated that retail chicken was an important vector for the spread of *Salmonella* and continuous monitoring of *Salmonella* contamination was essential to ensure food safety.

It was found that the MDR *S.* Kentucky ST198 and *S.* Indiana ST17 were the common serotypes in this study. This finding was consistent with global reports[5,28]. Extensive monitoring data indicated that MDR *S.* Kentucky and *S.* Indiana were rapidly emerging and becoming dominant in poultry and poultry products, which was likely driven by a combination of bacterial genetic factors, acquisition of ARGs and host-related factors[5,28,29]. Furthermore, the positive rate of COL^r *Salmonella* was up to 15.6% (14/90), which is higher than those reported in previous studies in China[19,30]. The high prevalence of MDR and COL^r *Salmonella* was likely due to the widespread use of antimicrobials in food animal production[31]. Therefore, it is imperative for governments to enhance regulatory measures and rigorously regulate antimicrobial use to safeguard the safety of meat products.

The observation that isolates carrying only the *bla*$_{\text{TEM-1B}}$ gene were resistant to ampicillin, while *bla*$_{\text{CTX-M}}$-positive isolates exhibited resistance to ampicillin, cefotaxime and cefepime in this study. This distinction underscores the different resistance mechanisms linked to these beta-lactamase genes. The presence of *bla*$_{\text{TEM-1B}}$ alone indicates a more limited resistance profile, primarily targeting ampicillin. This suggests that this gene may confer a basic level of resistance commonly found in *Salmonella*. In contrast, the simultaneous resistance of *bla*$_{\text{CTX-M}}$-positive isolates to multiple cephalosporins reflects a broader mechanism of resistance, often associated with the ability to hydrolyze a wider range of beta-lactam antibiotics. These findings are crucial for understanding the implications of beta-lactamase production in clinical settings, particularly regarding treatment options for infections caused by these resistant strains[32]. Furthermore, research revealed that Salmonella isolates showed resistance to nalidixic acid while remaining susceptible to ciprofloxacin when they possessed only the oqxAB genes or mutations in GyrA. The oqxAB genes code for efflux pumps that expel quinolones, whereas mutations in GyrA interfere with the binding of these drugs to the enzyme, resulting in quinolone resistance[33,34].

A research team in China was the first to discover the plasmid-mediated *mcr-1* gene in *E. coli* from food animals[10]. This finding highlights the potential for horizontal transfer of plasmids carrying *mcr-1* among bacterial populations. Further studies indicate that the primary carriers of the mcr gene belong to the *Enterobacteriaceae* family, which includes species such as *Enterobacter cloacae, Klebsiella pneumoniae* and *E. coli*[9]. In current study, the *mcr-1*-positive *S.* Schwarzengrund and *S.* Agona isolates were found, which was different from the commonly encountered *mcr-1*-positive *S.* Enteritidis and *S.* Typhimurium in food[35,36]. Although *mcr-1*-positive *S.* Schwarzengrund and *S.* Agona isolates have been found in humans and pig

samples in Italy, Brazil, the Netherlands and China[37-40], they have not been reported in retail chicken meat in China, which was the first to be reported in this study. Furthermore, the size of *mcr-1*-positive plasmid pSJTUF15550 (249 kb) of *S.* Agona was similar to the previously reported[19]. Phylogenetic analysis revealed that the plasmid pSJTUF15550 had a closely related to plasmid KX023262. Notably, there were multiple ARGs in the IncHI2 plasmid except the *mcr-1* gene, which means that due to the influence of other antimicrobials, the plasmid could persist for a long time even without colistin selection pressure. Therefore, it is necessary to reveal the impact of non-colistin antimicrobials on the development of resistance to colistin in *Salmonella* to prevent the further spread of the *mcr-1* gene.

All COL^r isolates (except SJTUF15542 and SJTUF15543) in this study exhibited higher mRNA expression levels of *pmrK* and *udg* compared to St14. This suggests that the increased expression of the *pmrHFIJKLM* operons and gene *udg*, regulated by the PmrAB system, leads to lipopolysaccharide (LPS) modification and contributes to colistin resistance in *Salmonella*. Additionally, none of the colistin-resistant isolates showed elevated *pmrD* expression levels relative to St14. These findings indicate that the colistin resistance mechanism in the studied *Salmonella* isolates is unlikely to result from *pmrD* overexpression, unlike what has been previously reported for *S.* Typhimurium[41].

The red homologous recombination was used to introduce seven amino acid substitutions in PmrAB on the chromosome, and *pmrK* or *udg* deletion in mutants to find out their role in the development of resistance to colistin. This approach was considered to be effective and could avoid the adverse effects associated with plasmid use described in previous studies[12,42]. It was demonstrated that substitutions L105P in PmrA and P94L, S124P, and L331R in PmrB resulted in the upregulation of *pmrK* and *udg* expression, leading to the production of L-Ara4N, which modified lipid A and ultimately contributed to the development of resistance to colistin in *Salmonella*. Furthermore, the mutants showed a similar colistin MIC to the corresponding foodborne isolates SJTUF15540, SJTUF15541, SJTUF15544 and SJTUF15556, which further demonstrated the important role of these substitutions in PmrAB. It was found in this study that there were 4 substitutions in PmrAB, among which substitution S124P in PmrB has been reported previously in laboratory strain[24], the other three substitutions have not been reported before, which are considered as novel substitutions. The substitution L105P was located within the CheY-homologous receptor domain of PmrA, whereas the substitutions P94L and L331R were situated within the HAMP and HATPase_c domain of PmrB, respectively. These novel substitutions in the PmrAB protein enhance both self-phosphorylation and PmrA activation through several mechanisms. First, these substitutions may induce conformational changes in the PmrAB structure that stabilize their active form. This stabilization can facilitate self-phosphorylation by promoting the correct alignment of key residues involved in the phosphotransfer reaction. Additionally, these substitutions may enhance the interaction between PmrB and its downstream target, PmrA. More efficient interactions could lead to increased phosphorylation of PmrA, thereby enhancing its ability to activate target genes associated with colistin resistance. This would result in a more robust expression of resistance-related genes, ultimately aiding bacterial survival in the presence of colistin. Moreover, these substitutions may affect

the overall dynamics of the PmrAB signaling pathway. By modulating the sensitivity of PmrB to environmental signals, these changes may lead to an increased response to stressors such as cationic antimicrobial peptides, further strengthening the bacterial resistance mechanism. In summary, these novel substitutions not only enhance the self-phosphorylation of PmrB but also amplify PmrA activity, providing a more effective response to antimicrobial challenges and contributing to a deeper understanding of colistin resistance at the molecular level[11,12,43,44].

In addition to the PmrA/PmrB substitutions found in SJTUF15540, SJTUF15541, SJTUF15544 and SJTUF15556, the other COL^r isolates exhibited several substitutions in PmrB (D149Y, H274Y, and S280N), some of which were predicted to be deleterious to protein function according to the silico software. However, site-directed mutagenesis experiments revealed that these three mutated PmrB proteins do not contribute to colistin resistance. One possibility is that these substitutions may influence the overall stability and functionality of the PmrAB signaling system, affecting how bacteria respond to environmental stressors. For instance, they might regulate interactions between PmrB and other regulatory proteins, thereby influencing the signaling cascade that activates resistance genes. Additionally, these mutations could play a role in how bacteria adapt to various antimicrobial agents or stress conditions, even if they do not directly alter colistin sensitivity. They may impact the expression levels of other resistance genes or pathways, providing a broader context for understanding resistance mechanisms. Future studies should investigate these substitutions in greater detail to uncover their specific contributions to resistance. Examining the molecular dynamics and interactions of these mutations within the PmrAB system could yield valuable insights into the complex network of mechanisms that bacteria employ to survive antimicrobial challenges[43,44].

5. Conclusions

It was found that the high prevalence of COL^r and MDR *Salmonella* in retail chicken meat in Shanghai posed a serious threat to food safety. Four *S.* Schwarzengrund and one *S.* Agona isolates from retail chicken meat were found to carry the *mcr-1* gene. Meanwhile, three novel amino acid substitutions (L105P in PmrA, P94L and L331R in PmrB) were demonstrated to contribute to the development of resistance to colistin in *Salmonella* by upregulating the expression levels of the genes *pmrK* and *udg*, leading to the production of L-Ara4N, which modifies lipid A. These findings enhanced the understanding of the development of resistance to colistin in *Salmonella* and offered important data for managing COL^r and MDR *Salmonella* in the food industry.

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Ethical approval

This article does not contain any studies related to human or animal subjects performed by any of the authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that

could have appeared to influence the work reported in this paper.

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