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Adaptation of foodborne *Salmonella* to sub-inhibitory levels of benzalkonium chloride induced resistance to tetracycline

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ABSTRACT

Benzalkonium chloride (BAC) is an effective disinfectant against *Salmonella* and widely used in the food industry. However, there are growing concerns about the risk of inducing *Salmonella* to produce cross-resistance to antibiotics resulting from the excessive use of BAC. In this study, 50 foodborne *Salmonella* isolates were exposed to sub-inhibitory concentrations of BAC. It was demonstrated that 7 isolates showed direct resistance to BAC, and 35 isolates showed cross-resistance to at least one of 10 tested antibiotics. Among these 35 isolates, 15 isolates exhibited an increase in minimum inhibitory concentration (MIC) for tetracycline, followed by 11 isolates for kanamycin and 9 isolates for ampicillin. *Salmonella* Enteritidis SJTUSM06 with the highest increase in MIC of tetracycline (from 4 to 32 μg/mL) was selected for further study. Tandem mass tag-labeled proteomics was used to determine key proteins involved in development of cross-resistance to tetracycline in BAC-adapted *S.* Enteritidis. It was identified that there were 146 differentially expressed proteins, including 95 up-regulated and 51 down-regulated proteins, most of which were involved in carbohydrate and lipid metabolism, ribosomes, transporters, virulence, motility, and stress response pathways. The efflux pump AcrB was significantly upregulated by 2.03-fold in BAC-adapted *S.* Enteritidis. It was also demonstrated that efflux pump inhibitor phenylalanine-arginine *β*-naphthylamide (PaβN) decreased the MIC of tetracycline in BAC-adapted *S.* Enteritidis from 32 to 8 μg/mL, indicating that active efflux pump played an important role in the development of resistance to tetracycline induced by BAC. Deletion of *acrB* resulted in a decrease in the resistance to tetracycline in BAC-adapted *S.* Enteritidis. Compared with the parent strain, MIC for tetracycline in Δ*acrB* mutant was 16 μg/mL with a 2-fold decrease. These results demonstrated that AcrB played a positive role in the development of cross-resistance to tetracycline after adaptation of *S.* Enteritidis to BAC.

1. Introduction

Salmonella is recognized as an important foodborne pathogen, causing approximately 59 000 deaths annually worldwide[1]. It can contaminate a variety of foods or spread through cross-contamination from production environments to food. Disinfectants in the food industry serve as effective measures to kill *Salmonella* and mitigate cross-contamination[2]. However, excessive usage of disinfectants may contribute to the emergence of resistant strains, accelerating the proliferation of antimicrobial resistance[3-4]. Especially, the increased dependence on quaternary ammonium compounds (QACs) has deepened this concern[5]. In the practical applications in food industry, pathogens are often exposed to sub-inhibitory concentrations of QACs due to their inappropriate use. Such stress may not only induce direct resistance but also contribute to cross-resistance to certain therapeutic antibiotics[6-8]. The increasing emergence of antibiotic-resistant *Salmonella* in food underscores a significant food safety hazard and public health concerns[9].

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Benzalkonium chloride (BAC), a widely used class of QACs, serves as a disinfectant in food production environments due to its stability and low cytotoxicity[5,10-11]. Nonetheless, sub-inhibitory concentrations of BAC can induce cross-resistance to various antibiotics in several bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Campylobacter jejuni*[12-16]. Sub-inhibitory exposure to QACs decrease the susceptibility of *E. coli* strains to ciprofloxacin in animal husbandry[12]. Exposure of *L. monocytogenes* to progressively increasing concentrations of BAC leads to the production of mutants with reduced susceptibility to gentamicin and kanamycin[14]. After treatment of sub-inhibitory concentrations of BAC, *C. jejuni* shows increased resistance to kanamycin and streptomycin[16]. The MICs of multiple antibiotics also increase after adaptation of *Salmonella* serovar Virchow to BAC, though the underlying mechanisms are not fully understood[17]. Moreover, cross-resistance between BAC and antibiotics has also been observed in microbial communities[18]. Therefore, evaluating the impact of BAC on antibiotic resistance and the involved mechanisms is essential.

Although the phenomena of cross-resistance induced by BAC have been described in bacteria, most studies only focus on phenotypes or biological features, and the underlying molecular mechanisms remain largely unknown[9]. It will be beneficial to understand the roles of important metabolic pathways or regulatory networks, and to identify key proteins involved in the development of cross-resistance to antibiotics. Recent advances in proteomics have provided deeper insights underlying cross-resistance to antibiotics induced by BAC. Proteomics analysis enables a comprehensive understanding of protein expression profiling in response to disinfectant and antibiotic challenges, revealing critical pathways involved in the development of resistance[19-22]. Machado et al.[23] utilize the proteomic approach to characterize the mechanism of resistance development in *P. aeruginosa* after adaptation to BAC, suggesting that different expressions of key proteins are associated with cross-resistance mechanisms between antimicrobial products and antibiotics. To our knowledge, no information is available regarding the characterization of the molecular mechanisms on the development of cross-resistance to antibiotics induced by BAC in *Salmonella* using Tandem mass tag-labeled proteomics.

Our study aimed to determine if there was any cross-resistance to 10 tested antibiotics induced by BAC in 50 foodborne *Salmonella* isolates, and to elucidate the underlying mechanisms on the development of cross-resistance to tetracycline after adaptation of *Salmonella* Enteritidis (*S.* Enteritidis) to BAC by proteomics and mutagenic analysis. Moreover, these results could provide a deeply understanding of the links between disinfectant failures and the emergence of cross-resistance to antibiotics, and offer valuable insights for the identification of new antibacterial targets.

2. Materials and methods

2.1 Isolates and their cultivation

All 50 tested *Salmonella* isolates were obtained from food samples in Shanghai markets between March 2016 and February 2017 and were characterized in the previous study from our laboratory and stored with 20% (*V/V*) glycerol at −80 °C[24]. Single colony of each culture was picked into 5 mL Luria-Bertani (LB) broth and incubated overnight at 37 °C at 180 r/min. Then, an optical density at 600 nm ($OD_{600\ nm}$) of each culture were adjusted to 0.5, the concentration of which was approximately 1×10^8 CFU/mL based on our previous experiment.

2.2 BAC and antibiotics

BAC (98.0%) purchased from J&K Scientific Ltd., Beijing, China, was prepared at 5 mg/mL in deionized water and filter sterilized (0.2 μm pore size) before use. All antibiotics for the test were purchased from Sigma–Aldrich (Shanghai, China).

2.3 Adaptation to BAC

The minimum inhibitory concentration (MIC) for BAC was determined by broth dilution method in LB broth for each tested isolate using 96-well microtitration plates. For adaptation to BAC, *Salmonella* isolates were exposed to gradually increased sub-inhibitory concentration of BAC in the LB broth[7]. Initially, the cells were adjusted to an $OD_{600\ nm}$ of 0.5, and then a 1:100 dilution with LB broth was performed to achieve a density of approximately 1×10^6 CFU/mL. Subsequently, the cultures were exposed to 0.25× MIC of BAC and incubation at 37 °C. After reaching an $OD_{600\ nm}$ of 0.5, the bacterial cultures were transferred at a 1:10 ratio into fresh LB broth containing 1.5 times the concentration of BAC compared to the previous one, and this process was repeated until no growth was found after incubation for 12 h at 37 °C. The cells survived in the last process were collected for subsequent tests.

2.4 Antibiotic susceptibility testing

The MICs for antibiotics were determined by the agar dilution method as mentioned by the Clinical and Laboratory Standards Institute (CLSI) standards[25]. Briefly, the bacterial cultures were spotted on Muller-Hinton agar (MHA) plates containing different concentrations of antibiotics and the MICs were determined as the minimum antibiotics concentration that led to no observed single colonies. The 10 tested antibiotics included ampicillin, imipenem, ciprofloxacin, nalidixic acid, streptomycin, kanamycin, gentamycin, tetracycline, chloramphenicol, and azithromycin. *E. coli* ATCC 25922 was selected as a quality-control strain for each antibiotic susceptibility test.

2.5 Protein extraction, quantification, and digestion

The BAC-adapted *S.* Enteritidis obtained from Section 2.3 and non-adapted *S.* Enteritidis were adjusted to $OD_{600\ nm}$ of 0.5, and diluted in 10 mL LB broth containing 2 μg/mL tetracycline and incubated for 6 h at 37 °C. Then, cells were washed 3 times with phosphate-buffered saline (PBS) and resuspended in 300 μL RIPA solution (250 mmol/L Tris-HCl, pH 7.6; 150 mmol/L NaCl; 10% Nonidet P-40; 10% sodium deoxycholate; 10% sodium dodecyl sulfate). After ultrasonicating for 2 min, the samples were centrifuged at 12 000 r/min for 10 min to obtain the supernatants. The protein concentrations in the supernatants were determined by bicinchoninic acid (BCA) Protein Assay Reagent (Beyotime Biotechnology, Shanghai, China).

A total of 100 μg of protein was taken from each sample and diluted to approximately 1 mg/mL. A total of 5 times the volume of acetone was added to the sample and then precipitated overnight at −20 °C. Afterward, the sample was centrifuged at 12 000 r/min for 10 min at 4 °C. The precipitation was carefully collected and washed twice with 200 μL of pre-chilled 80% acetone. Subsequently, 100 μL protein complex solution was added and subjected to ultrasonication for 5 min to dissolve the protein precipitate. Next, 5 mmol/L *DL*-dithiothreitol was introduced and allowed to incubate for 10 min to reduce disulfide bonds. The proteins were then alkylated with 10 mmol/L iodoacetic acid in darkness for 15 min. Finally, 0.5 μg/μL of trypsin was mixed with the samples and incubated overnight at 37 °C.

2.6 TMT labeling and high-pH pre-fractionation

TMT labeling was performed following the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of labeled samples from each group were mixed, and trifluoroacetic acid was added to precipitate sodium deoxycholate. After centrifugation at 12 000 r/min for 10 min, the supernatant was collected as labeled peptide samples. Subsequently, the samples were desalted using C_{18} cartridges and concentrated through vacuum drying overnight at 4 °C. Following lyophilization, the peptide samples were reconstituted to 50 μL with mobile phase A (10 mmol/L ammonium acetate aqueous solution, pH 10) and subjected to separation under alkaline conditions using reversed-phase ultra-performance liquid chromatography (RP-UPLC). The gradient elution was initiated with 5% mobile phase B (10 mmol/L ammonium acetate, 10% H_2O, 90% acetonitrile, pH 10) for 2 min, followed by 5%−30% mobile phase B for 40 min, 30%−40% mobile phase B for 10 min, 40%−90% mobile phase B for 4 min, 90% mobile phase B for 2 min, and 2% mobile phase B for 2 min. Fractions were collected every minute and then stored at −80 °C after vacuum drying.

2.7 Nano-liquid chromatography-mass spectrometry/mass spectrometry analysis

Approximately 1 μg of total peptides were separated by a nanoUPLC liquid system EASYnLC1200, coupled with a QExactive HFX mass spectrometer (Thermo Fisher Scientific) for data acquisition. A reversed-phase chromatographic column (100 μm × 15 cm, 1.9 μm, Reprosil Pur 120 C_{18}AQ, Dr. Math) was employed for chromatographic separation. After the chromatographic column was equilibrated with 100% phase A (0.1% formic acid-98% aqueous solution), the sample was directly loaded onto the chromatographic column *via* the autosampler and then separated by the chromatographic column gradient with 300 nL/min of flow rate and 90 min of gradient duration. The gradient elution was conducted with 2%−5% mobile phase B (0.1% formic acid-80% acetonitrile solution) for 2 min, followed by 5%−22% mobile phase B for 68 min, 22%−45% mobile phase B for 16 min, 45%−95% mobile phase B for 2 min, and concluded with 95% mobile phase B for 2 min.

Mass spectrometry (MS) analysis was conducted in data dependent acquisition (DDA) mode, utilizing positive ion detection, and the total analysis time was 90 min. For MS-1, the resolution was 120 000 and the *m/z* range was 350−1 600; for MS-2, the resolution was 45 000 and a fixed first mass was 110. The automatic gain control target for MS-1 was set to 3×10^6 with a maximum ion time of 30 ms, and 1×10^5 for MS-2 with max IT of 96 ms. The top 20 ions with the highest intensity were selected by the quadrupole and subjected to fragmentation *via* higher-energy collisional dissociation (HCD) with a normalized collision energy of 32% and an isolation window of *m/z* 0.7. The dynamic exclusion time was set to 45 s. Single-charge and > 6 valence ions were excluded from the DDA procedure.

2.8 Peptide analysis and protein identification

Raw MS processing and peptide identification were performed with Proteome Discoverer 2.4.0.305 and Mascot Server 2.6.1. MS data were searched against the *Salmonella* UniProt FASTA database. Precursor ion mass tolerance and fragment ion tolerance were set to 10×10^{-6} and 0.02 Da, respectively. Tryptic peptides with a maximum of 2 missed cleavages were allowed. The false discovery rate (FDR) was maintained at 1% for both peptide spectrum matches and peptide levels. Unique peptides and Razor peptides were employed for protein quantification, and the total peptide amount was used for normalization purposes.

2.9 Bioinformatics analysis

The Gene Ontology (GO) program was utilized to annotate differentially abundant proteins in terms of cell components, biological processes, and molecular function. Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted to assess the pathways associated with differentially abundant proteins.

2.10 Gene expression analysis

Differentially expressed proteins in TMT tests were validated at the mRNA level by RT-qPCR as described previously[26]. Total RNA was extracted from non-adapted or BAC-adapted *S.* Enteritidis treated with 2 μg/mL tetracycline using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. All primers used in this study were synthesized by the Sangon Biotech Co., Ltd. (Shanghai, China) and listed in Table 1.

2.11 Evaluation of efflux activity

The ethidium bromide (EtBr) agar method was performed to determine the changes of efflux activity in *S.* Enteritidis after adaptation to BAC[27]. Bacterial cultures were diluted to an $OD_{600\ nm}$ of 0.5 and swabbed onto duplicate LB agar containing an increasing concentration of EtBr from 0 to 3.2 μg/mL. These plates were incubated at 37 °C in aerobic conditions for 12 h. After incubation, the plates were observed under a UV transilluminator. Data were representative of 3 independent experiments.

2.12 Efflux pump inhibitors (EPIs) assay

To identify the positive role of efflux pumps in the development of cross-resistance to tetracycline in BAC-adapted

S. Enteritidis, EPIs were utilized[18]. The MICs for tetracycline in BAC-adapted *S*. Enteritidis were determined in the presence of PAβN and reserpine. The final concentrations of both PAβN and reserpine were 20 or 40 μg/mL. Experiments were repeated three times. Cells were cultivated only in the presence of EPIs to ensure there is no inhibitory effect.

Table 1
Genes and primers used for RT-qPCR analysis.

Gene	Primer (5′→3′)
pykA	F: TGTTGACTATCTGGCCGTCTCCT
	R: ATCGACTCCATCATTTGCGTTGC
rsmB	F: GCGAGATAGCTGGTTGGCTTTGC
	R: CGGCTTCGAGAATATGCGTGGTT
uspB	F: GCGCTATTTCTCATCACTACGCG
	R: AGGCTGACCACCACCAGACCACA
ugpA	F: CGACGCCTTCTGGACGACGATTA
	R: TGACGCAAATACCACCAGGAACAT
acrB	F: CATTGCTGGCGTAACGTTGACATCT
	R: CCGTCAGCAATGAAAGTACGACCAT
katG	F: GCTGCTTGAACCGATTGCTGATG
	R: TGGGCTTCCACTCGTAACGCATAT
mdh	F: TATGGACCGTTCCGACCTGTTTA
	R: ACTTCCGTTGGCAGCTTACCTTT
fadA	F: AAACGGAGATTATCCCGACTGGC
	R: GCACGACTTTCGCTCATTACCAG
sipA	F: CGAAAGTAAAGATGGAAAGGTGG
	R: CGTGTCTGATTGTAAGGTATCGGT
sipB	F: CAAGTTAATGACCCTACTGGGCGAT
	R: GCTTTGGCGTCTGTGCCTGCTTT
invE	F: GAGCAAACCGATCCGAAGACCCT
	R: AACGCTTTGGTAAACGAATACGA
invA	F: CGTTGATATTACTTGTGCCGAAGA
	R: CGTTACCCAGAAATACTGACTGCT
16S rRNA	F: CAGAAGAAGCACCGGCTA
	R: GACTCAAGCCTGCCAGTT

2.13 Construction of gene deletion mutant and complementation strain

Highly upregulated protein AcrB was selected to characterize its role in the development of cross-resistance to tetracycline in BAC-adapted *S.* Enteritidis. Deletion and complementation of *acrB* gene were performed as described previously[28]. Briefly, *acrB* gene was first replaced by an FRT site-flanked *hph* cassette, which was a hygromycin resistance gene used as the resistance selection marker to construct homologous recombinant fragments by conferring resistance to hygromycin of *acrB* deletion strain. For the construction of complementary strains, the full-length regulator protein gene was cloned into the pBAD33 vector with an arabinose-inducible promoter and then transformed into the corresponding mutant. The resulting strains were further confirmed by PCR and DNA sequencing. The plasmids and primers used were shown in Tables S1 and S2.

2.14 Determination of MIC for tetracycline

MICs for tetracycline were determined using the broth microdilution method recommended by CLSI[25]. BAC-adapted *S.* Enteritidis (BAC-SE), its *acrB* deletion mutant (Δ*acrB*), and the complemented strain (Δ*acrB*-C*)* were individually inoculated into 5 mL of LB broth and incubated overnight at 37 °C. Cultures were adjusted to an $OD_{600\ nm}$ of 0.5, then diluted in fresh LB broth (1:100) supplemented with the final concentration of tetracycline from 128 to 0.5 μg/mL, and incubated overnight at 37 °C. MIC was determined to be the lowest concentration of tetracycline that inhibited visible growth.

2.15 Statistical analysis

All the experiments were technically repeated at least 3 times with 3 biological replicates per assay. Statistical analysis was performed using the two-tailed unpaired *t*-test ($\alpha = 0.05$) by GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Adaptation of Salmonella to BAC induced its resistance to antibiotics

After adaptation to BAC, 7 of 50 isolates developed direct resistance to BAC with a 2-fold increase in MIC. Meanwhile, as shown in Table 2, 35 isolates showed cross-resistance to at least one of 10 tested antibiotics. Among these 35 isolates, 15 isolates exhibited an increase in MIC for tetracycline, followed by 11 isolates for kanamycin and 9 isolates for ampicillin. These results indicated that adaptation of *Salmonella* to BAC induced cross-resistance to certain antibiotics. Further analysis on the serotypes of BAC-adapted isolates with cross-resistance to tetracycline indicated that 8 isolates were *S.* Enteritidis, 5 isolates were *Salmonella* Typhimurium, 1 isolate was *Salmonella* Derby and 1 isolate was *Salmonella* Newport. *S.* Enteritidis SJTUSM06 with the highest increase in MIC for tetracycline from 4 to 32 μg/mL was selected for proteomics analysis to reveal the mechanisms on the development of cross-resistance. Our results demonstrated that adaptation of *Salmonella* to BAC induced its resistance to antibiotics. The emergence of antibiotic resistance is usually attributed to the misuse of antibiotics[29-30], the cross-resistance to antibiotics induced by BAC in *Salmonella* can provide new perspectives on this problem.

3.2 Overview of proteomics analysis in BAC-adapted S. Enteritidis

TMT-based proteomics analysis was performed to reveal the mechanisms on the development of cross-resistance to tetracycline in *S.* Enteritidis after adaptation to BAC. Furthermore, proteins with unique peptide ≥ 1, expression level ≥ 1.30-fold or ≤ 0.70-fold and *P*-value < 0.05 were considered as differentially expressed proteins. TMT-based proteomics analysis demonstrated that there were 146 significantly differentially expressed proteins, including 95 upregulated and 51 downregulated ones (Table S3). Transcriptional profiles of 12 proteins were validated by RT-qPCR, results of which showed that the relative expression levels were consistent with the trends in protein expression for all tested proteins, suggesting that the proteomics data in this study were reliable (Fig. 1).

3.3 Functional interpretation of differentially expressed proteins in S. Enteritidis isolate with resistance to tetracycline

GO and KEGG functional enrichment analysis of differentially expressed proteins (DEPs) of BAC-adapted *S.* Enteritidis were carried out in this study (Fig. 2). For molecular function, differentially

Table 2
MICs for antibiotics and serotypes before and after adaptation to BAC in 35 BAC-adapted *Salmonella* isolates.

Isolates	Serotypes	MIC for antibiotics (before/after, μg/mL)									
		AMP	MEM	CIP	NAL	STR	KAN	GEN	TET	CHL	AZI
SJTUSM01	Enteritidis	**8/16**	2/2	0.25/0.25	8/8	4/4	8/8	4/4	2/2	8/8	2/2
SJTUSM02	Enteritidis	**4/8**	1/1	0.5/0.5	**4/8**	16/16	4/8	2/2	0.5/0.5	16/16	4/4
SJTUSM03	Typhimurium	16/16	2/2	0.125/0.125	8/8	4/4	4/4	2/2	**2/4**	16/16	2/2
SJTUSM04	Enteritidis	8/8	1/2	**0.06/0.125**	128/128	16/16	8/8	2/2	**0.5/2**	32/32	8/8
SJTUSM05	Enteritidis	**8/16**	4/4	0.06/0.06	64/64	16/16	32/32	4/4	16/16	8/8	4/4
SJTUSM06	Enteritidis	8/8	2/2	1/1	16/16	8/8	16/16	4/4	**4/32**	4/4	4/4
SJTUSM07	Typhimurium	**8/16**	0.5/0.5	1/1	128/128	128/128	128/128	32/32	128/128	128/128	2/2
SJTUSM08	Enteritidis	128/128	2/2	0.25/0.25	128/128	128128	**16/32**	64/64	128/128	128/128	2/2
SJTUSM09	Typhimurium	128/128	0.5/0.5	0.03/0.03	128/128	128/128	4/4	**2/4**	4/4	**4/8**	2/2
SJTUSM10	Typhimurium	128/128	1/1	0.03/0.03	128/128	128/128	**8/16**	2/2	4/4	4/4	2/2
SJTUSM11	Enteritidis	**4/8**	1/1	**0.03/0.06**	32/32	4/4	4/4	2/2	4/4	4/4	2/2
SJTUSM12	Typhimurium	4/4	2/2	1/1	8/8	4/4	4/4	2/2	**1/4**	4/4	2/2
SJTUSM13	Enteritidis	8/8	2/2	1/1	8/8	**4/8**	**4/8**	2/2	**1/2**	4/4	2/2
SJTUSM14	Enteritidis	8/8	1/1	0.03/0.03	**2/4**	16/16	4/4	4/4	**4/8**	4/4	2/2
SJTUSM15	Newport	**4/8**	0.5/0.5	0.03/0.03	128/128	4/4	2/2	**2/4**	4/4	**2/4**	1/1
SJTUSM16	Enteritidis	**16/32**	1/1	0.06/0.06	128/128	128/128	4/4	2/2	4/4	4/4	2/2
SJTUSM17	Enteritidis	128/128	2/2	0.06/0.06	128/128	128/128	4/4	2/2	**4/8**	2/2	1/1
SJTUSM18	Enteritidis	4/4	1/1	0.125/0.125	4/4	4/4	4/4	4/4	**2/4**	2/2	1/1
SJTUSM19	Newport	16/16	1/1	0.5/0.5	128/128	16/16	4/4	2/2	**4/8**	2/2	1/1
SJTUSM20	Enteritidis	128/128	2/2	0.06/0.06	128/128	128/128	4/4	2/2	**4/8**	**2/4**	1/1
SJTUSM21	Typhimurium	128/128	0.5/0.5	1/1	128/128	128/128	32/32	128/128	**1/2**	4/4	2/2
SJTUSM22	Typhimurium	**16/32**	0.5/0.5	1/1	128/128	128/128	64/64	2/2	4/4	32/32	2/2
SJTUSM23	Typhimurium	128/128	0.5/0.5	1/1	128/128	64/64	8/16	16/16	16/16	32/32	2/2
SJTUSM24	Enteritidis	16/16	1/1	0.06/0.06	128/128	128/128	4/8	8/8	4/4	32/32	2/2
SJTUSM25	Typhimurium	128/128	0.5/0.5	1/1	64/64	32/32	8/16	16/16	16/16	64/64	2/2
SJTUSM26	Enteritidis	128/128	0.5/0.5	0.5/0.5	128/128	128/128	4/8	8/8	8/8	**4/8**	2/2
SJTUSM27	Enteritidis	**16/32**	1/1	0.03/0.03	128/128	128/128	4/4	2/2	8/8	4/4	2/2
SJTUSM28	Enteritidis	128/128	2/2	0.06/0.06	128/128	128/128	2/8	2/2	2/2	4/4	2/2
SJTUSM29	Typhimurium	64/64	1/1	0.06/0.06	128/128	128/128	4/4	2/2	**4/8**	2/2	2/2
SJTUSM30	Enteritidis	128/128	2/2	0.03/0.03	128/128	128/128	4/8	4/4	8/8	4/4	2/2
SJTUSM31	Enteritidis	128/128	1/1	0.25/0.25	128/128	128/128	8/8	2/2	**8/16**	4/4	2/2
SJTUSM32	Enteritidis	64/64	1/1	0.5/0.5	128/128	128/128	8/16	4/4	8/8	16/16	2/2
SJTUSM33	Derby	16/16	0.5/0.5	**0.06/0.125**	4/4	16/16	32/32	2/2	**4/8**	4/4	2/2
SJTUSM34	Typhimurium	128/128	0.5/0.5	0.5/0.5	128/128	128/128	128/128	16/16	**8/16**	2/2	2/2
SJTUSM35	Typhimurium	16/16	1/1	1/1	32/32	16/16	16/32	32/32	8/8	4/8	4/4

Note: The bold font indicates the MIC values which increased after adaptation to BAC. AMP, Ampicillin; MEM, Meropenem; CIP, Ciprofloxacin; NAL, Nalidixic acid; STR, Streptomycin; KAN, Kanamycin; GEN, Gentamicin; TET, Tetracycline; CHL, Chloramphenicol; AZI, Azithromycin.

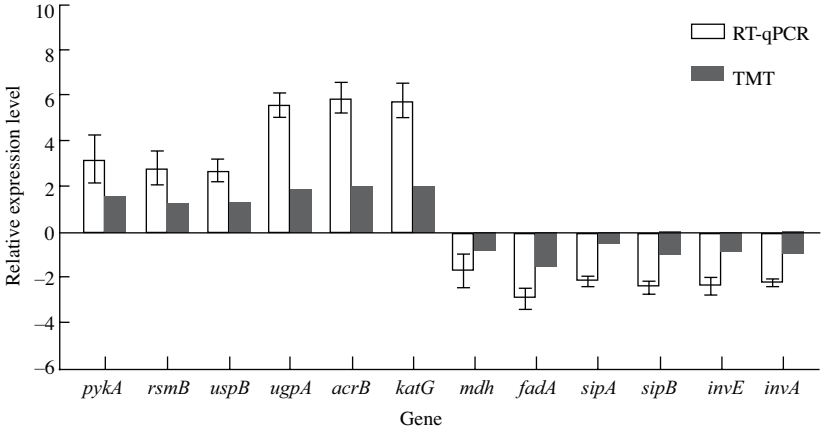

Fig. 1 Comparison between mRNA and protein levels of selected differentially expressed proteins revealed by RT-qPCR and TMT proteomics. Protein and mRNA levels were presented as fold change and $\log_2$ ratio, respectively. Error bars indicated the standard deviation of 3 technical replicates.

expressed proteins were enriched in catalytic activity, binding, ion binding, nucleotide binding, and transporter activity, etc. (Fig. 2A). In terms of biological process, differentially expressed proteins were more enriched in the cellular, cellular metabolic, small molecule and catabolic processes (Fig. 2B). Cellular component analysis revealed that differentially expressed proteins were primarily located in the cell, intracellular, cytosol, etc. (Fig. 2C). Moreover, KEGG analysis highlighted that differentially expressed proteins significant enrichment in the pathways related to carbohydrates and lipids metabolism, ribosome, biosynthesis of amino acids, transporters, flagellar assembly and stress response (Fig. 3).

To gain deeper and more comprehensive insights into the functional pathways contributing to the mechanism on the development of cross-resistance to tetracycline in BAC-adapted *S.* Enteritidis, the differentially expressed proteins in key pathways were mapped. A proposed model highlighting these proteomic changes was shown in Fig. 4, indicating the involvement of complex regulatory networks in the development of cross-resistance to tetracycline in BAC-adapted *S.* Enteritidis. Accordingly, detailed discussions of these functional pathways were undertaken to shed light on the potential mechanisms on the development of cross-resistance to tetracycline induced by BAC in *S.* Enteritidis.

3.3.1 Carbohydrate and lipid metabolism

It was demonstrated in Fig. 3A that there were a considerable number of differentially expressed proteins in BAC-adapted *S.* Enteritidis associated with carbohydrate and lipid metabolism. Proteins including acetyl-CoA synthetase (Acs), phosphoenolpyruvate kinase (PykA) and ribose 5-phosphate isomerase (RpiA) were upregulated. Interestingly, proteins involved in the tricarboxylic acid (TCA) cycle were downregulated. Additionally, FabH which is essential for fatty acid biosynthesis was upregulated, while proteins related to fatty acid oxidation, such as FadA and FadB, were downregulated in BAC-adapted *S.* Enteritidis. Although metabolic responses are complex, enzyme inactivation in central carbon metabolism can be a novel mechanism for resistance to antibiotics[31]. Microbial communities exposed to BAC show an increased abundance of functions related to carbohydrate and lipid metabolism[32]. Similarly, *Aeromonas hydrophila* INISA09 also exhibit significant protein function changes, particularly in carbohydrate and fatty acid metabolism, upon exposure to BAC[33]. These changes likely represent a strategic shift in metabolic pathways to cope with stress. Hence, it was suggested that BAC-adapted *S.* Enteritidis might regulate the activity of carbohydrate metabolism and fatty acid biosynthesis to enhance its survival ability.

3.3.2 Ribosome

Differentially expressed proteins were also enriched in those related to protein synthesis (Fig. 3B). For instance, higher levels of 50S ribosomal protein RpIL and RpIJ, as well as ribosomal methyltransferase RlmL were observed in BAC-adapted *S.* Enteritidis. Tetracycline primarily impairs bacterial growth and survival by inhibiting protein synthesis through binding to the ribosome. The upregulation of proteins integral to the biosynthetic machinery, particularly ribosomal protein subunits, has been previously identified as crucial for reducing susceptibility to tetracycline[34-35]. Therefore, modifications in the ribosome might contribute to the development of cross-resistance to tetracycline induced by BAC in *S.* Enteritidis.

3.3.3 Amino acid metabolism

Amino acids, including asparagine, valine, leucine, and isoleucine biosynthesis, were associated with the response of *S.* Enteritidis to BAC in the current work (Fig. 3C). Amino acids, as highly dynamic molecules, play a crucial role in the resistance and survival of *S.* Enteritidis under environmental stresses[36]. The multifaceted influence of amino acids on bacterial resistance is well-documented, specifically, certain amino acids have been shown to enhance metabolism and increase the activity of proton motive force (PMF)-dependent transport proteins[37-38]. Therefore, the regulation of amino acids in this study emerges as an important response to tetracycline in BAC-adapted *S.* Enteritidis.

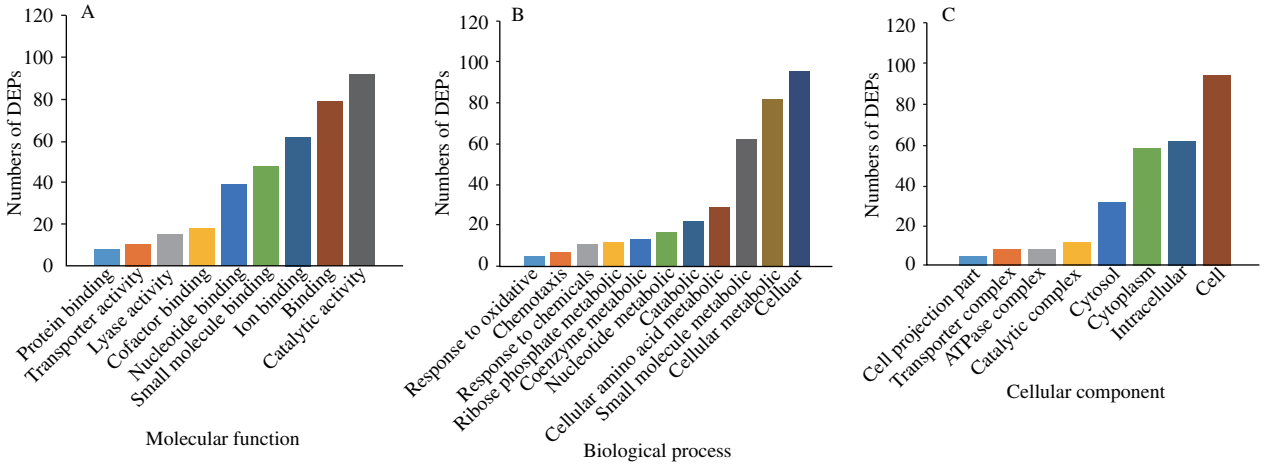

Fig. 2 Distribution of differentially expressed proteins in BAC-adapted *S.* Enteritidis after tetracycline treatment. (A) Molecular function. (B) Biological process. (C) Cellular component.

3.3.4 Transporters

ATP-binding cassette (ABC) transporter proteins, including the maltose transporter (MalEFGK), glycerol-3-phosphate transporter (UgpABDE) and amino acid transporters (LivFGK) were upregulated, while betaine transporters (ProVWX) were downregulated in BAC-adapted *S.* Enteritidis (Fig. 3D). These transporters play an important role in maintaining substrates balance between intra- and extra-cellular environments. Their widespread activity in modulating cellular interactions is a key aspect of microbial defense mechanisms[39]. The differential expression of ABC transporters certainly highlights their importance in the development of cross-resistance to tetracycline in *S.* Enteritidis.

In particular, the multidrug resistance efflux pump AcrB exhibited a significant upregulation of 2.03-fold in BAC-adapted *S.* Enteritidis. Efflux pumps like AcrB, part of the AcrAB-TolC multidrug efflux pump family, are vital for the extrusion of toxic substrates from cells, contributing to bacterial defense against antimicrobial agents. Both BAC and tetracycline are known substrates for such efflux pumps, and their upregulations have been documented as a resistance mechanism[40-41]. Therefore, the overexpression of AcrB could be crucial for the development of cross-resistant phenotype observed in this study. The important role of efflux pump and key protein AcrB in conferring resistance to tetracycline in BAC-adapted *S.* Enteritidis were further confirmed in subsequent studies.

3.3.5 Virulence and motility

Virulence-related proteins, particularly those associated with *Salmonella* pathogenicity island 1 (SPI-1) were downregulated in BAC-adapted *S.* Enteritidis (Fig. 3E). The expression of SPI-1 genes is regulated by a complex network comprising multiple regulators that respond to various environmental stimuli[42]. During the evolutionary dynamics of antimicrobial resistance, bacteria undergo the fitness cost that balances resistance and other survival functions, with reduced virulence being reported as one aspect[43]. In this study, proteins linked to flagellar assembly and motility, such as MotA and MotB were downregulated in BAC-adapted *S.* Enteritidis. The observations were similar with the previous work that *S.* Typhimurium diminishes motility and alters expression of several virulence proteins after exposure to QACs, alongside reduced susceptibility to antibiotics like ciprofloxacin, chloramphenicol, tetracycline, and ampicillin[44]. Our findings suggested that the decreased motility could be a common adaptive response to BAC treatment, potentially serving as an energy conservation strategy for developed cross-resistance to tetracycline in *S.* Enteritidis after adaptation to BAC.

3.3.6 Stress response

In this study, stress response-related proteins were regulated by tetracycline in BAC-adapted *S.* Enteritidis. These proteins were primarily associated with universal stress response, DNA repair, and redox processes (Fig. 3F). The stress response sigma factor RpoS and DNA mismatch repair protein MutL were upregulated in the BAC-adapted *S.* Enteritidis. Additionally, oxidative stress-related proteins catalase-peroxidase KatG and superoxide dismutase SodC were also upregulated. Given that tetracycline can induce oxidative cellular damage in *S.* Enteritidis[34], these results suggested that the development of cross-resistance to tetracycline might be attributed to an enhanced ability to cope with oxidative stress in BAC-adapted *S.* Enteritidis, which in turn reduced the damage by tetracycline.

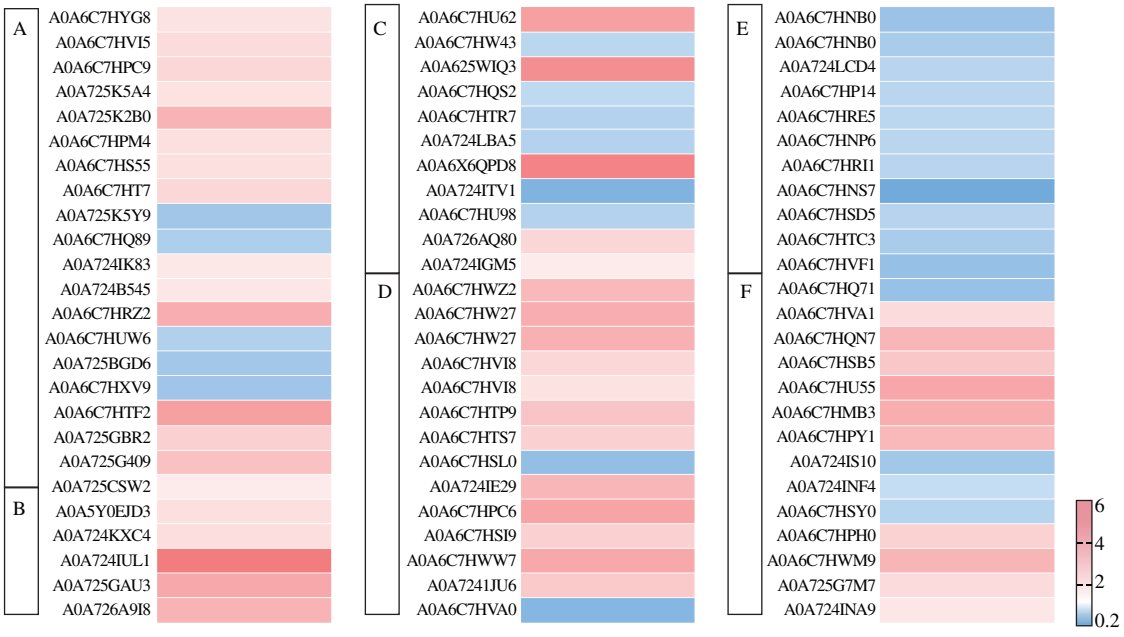

Fig. 3 Heat map of differentially expressed proteins involved in key functional pathways in BAC-adapted *S.* Enteritidis. (A) Carbohydrates and lipids metabolism. (B) Ribosome. (C) Amino acid metabolism. (D) Transporters. (E) Virulence and motility. (F) Stress response.

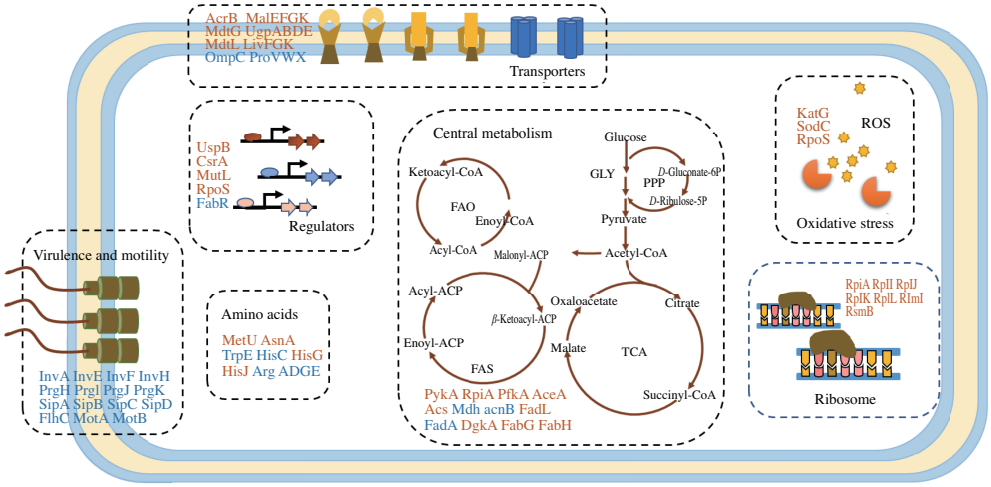

Fig. 4 Proposed response network for cross-resistance to tetracycline in *S.* Enteritidis after adaptation to BAC. The up- and down-regulated differentially expressed proteins in BAC-adapted *S.* Enteritidis are shown in red and blue, respectively.

3.4 Active efflux pump played an important role in the development of tetracycline resistance in BAC-adapted S. Enteritidis

It was demonstrated in Fig. 5 that fluorescence was observed at EtBr concentrations ranging from 0.4 to 3.2 μg/mL in non-adapted *S.* Enteritidis. However, there was no significant fluorescence observed even at the concentration of 3.2 μg/mL in BAC-adapted *S.* Enteritidis, indicating that the efflux system was highly active. The role of efflux pump was further investigated by evaluating the fold change in MICs for tetracycline in the presence of EPIs. The bacterial growth of *S.* Enteritidis in LB broth remained consistent with and without the addition of the 2 EPIs, indicating that neither PaβN nor reserpine had any impact on bacterial growth at the tested concentrations. The effects of 2 tested EPIs on MIC for tetracycline in BAC-adapted *S.* Enteritidis were presented in Table 3. PaβN at a concentration of 20 μg/mL reduced the MIC for tetracycline by 2-fold (from 32 to 16 μg/mL), and 40 μg/mL PaβN decreased the MIC by 4-fold (from 32 to 8 μg/mL) in BAC-adapted *S.* Enteritidis. However, reserpine did not affect the resistance of BAC-adapted *S.* Enteritidis to tetracycline under the tested concentrations.

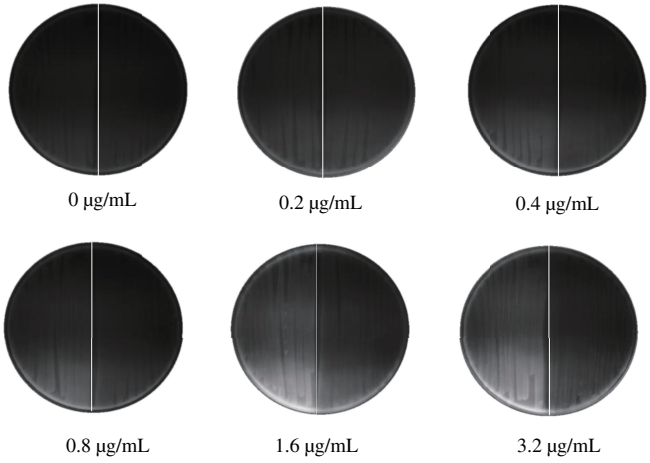

Fig. 5 Efflux pump activity in BAC-adapted *S.* Enteritidis and non-adapted *S.* Enteritidis. Fluorescence of non-adapted *S.* Enteritidis (left swabbed) and BAC-adapted *S.* Enteritidis (right swabbed) at increasing concentrations of ethidium bromide (0−3.2 μg/mL). The depicted plate was one representative of 3 repeats.

Table 3
Fold change in MICs for tetracycline in the presence of EPIs in BAC-adapted *S.* Enteritidis.

Inhibitors	Concentration (μg/mL)	Fold change in MIC
PaβN	20	2
	40	4
Reserpine	20	/
	40	/

Note: "/" indicates the MIC values for tetracycline did not change.

PaβN, a well-studied EPI, targets resistance-nodulation division (RND) family[45-46]. In this study, the presence of PaβN resulted in a decreased resistance to tetracycline in BAC-adapted *S.* Enteritidis, indicating that active efflux pump played an important role in conferring cross-resistance to tetracycline in BAC-adapted *S.* Enteritidis. The wide spectrum of substrates recognized by efflux systems has raised concern that exposure to one substrate may induce resistance to others. For example, exposure to BAC can trigger increased expression levels of the MdrL efflux pump in *L. monocytogenes*, leading to decreased susceptibility to ciprofloxacin[47]. Therefore, the significant upregulation of AcrB efflux pump could contributed to the development of resistance to tetracycline in BAC-adapted *S.* Enteritidis.

3.5 AcrB was crucial for the induction of cross-resistance to tetracycline in BAC-adapted S. Enteritidis

In this study, the gene *acrB* was deleted to characterize its role in the development of cross-resistance to tetracycline induced by BAC in *S.* Enteritidis. The MIC for tetracycline in Δ*acrB* mutant was 16 μg/mL, which was a 2-fold decrease compared to the parent strain. Meanwhile, there were no changes in MICs for tetracycline between the complemented strain and the parent strain. These results demonstrated that AcrB was crucial for the induction of cross-resistance to tetracycline in BAC-adapted *S.* Enteritidis.

AcrB, as a member of AcrAB-TolC tripartite complex, determines substrate specificity and export efficiency[48]. This efflux pump is important in conferring cross-protection against antibiotics. For instance, high salt concentrations have been reported to confer cross-protection

against antibiotic treatment in *E. coli*, which are attributed to the upregulation of the efflux pump AcrAB-TolC[49]. Additionally, cross-resistance to tetracycline induced by other antibiotics in *S.* Typhimurium is also associated with the efflux pump AcrAB-TolC[50]. In summary, our study demonstrated the positive role of the efflux pump AcrB in the development of cross-resistance to tetracycline induced by BAC in *S.* Enteritidis and suggested that the emergence of isolates with enhanced efflux pump may pose a potential risk for increased resistance to antibiotics.

Although increasing attention has been paid to cross-resistance against antibiotics induced by BAC in certain bacteria, further in-depth investigations remain essential. It is advisable to strictly utilize inhibitory concentration during disinfection with BAC, and cautiously evaluate the potential risks associated with cross-resistance to antibiotics. Moreover, efflux pump AcrB could be the link between adaptation to BAC and resistance to antibiotic in *S.* Enteritidis. Therefore, exploiting new inhibitors targeting AcrB may reduce the production and spread of antibiotic-resistant *Salmonella*. However, the resistance response to disinfectants and antibiotics involves complex mechanisms. Therefore, more comprehensive studies will be conducted in future research to explore whether different strains or disinfectants share similar mechanisms on the development of cross-resistance to antibiotics.

4. Conclusion

Adaptation of foodborne *Salmonella* to sub-inhibitory levels of BAC could induce cross-resistance to antibiotics. TMT-labeled proteomics analysis indicated that multiple pathways were coordinately involved in the development of cross-resistance to tetracycline in BAC-adapted *S.* Enteritidis. Efflux pump AcrB was demonstrated to be an essential protein contributing to the development of cross-resistance to tetracycline after adaptation of *S.* Enteritidis to BAC. Collectively, these results enriched the understanding of the mechanisms on the development of cross-resistance to antibiotics induced by BAC in *S.* Enteritidis, and highlighted the importance of effective use of BAC in the food industry.

Declarations of interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250409.

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