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Evolution of *Salmonella* Typhimurium under antibiotics from distinct classes reveals variant-dependent cross-protection against food preservation treatments

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ABSTRACT

The growing concern surrounding antimicrobial resistance (AMR) in human bacterial infections has prompted increased investigation into the role of the agri-food sector in AMR transmission and control. While contaminated food is a recognized route of transmission for AMR bacteria, the potential for AMR mechanisms to confer cross-protection and enhance bacterial tolerance to food-relevant preservation stressors such as heat, acidification, or antimicrobial compounds remains poorly understood.

In this study, nine resistant variants (RVs) of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 were isolated via adaptive laboratory evolution with increasing concentrations of antibiotics from distinct classes, including colistin (COL), amoxicillin (AMX), and erythromycin (ERY). These RVs displayed up to a 16-fold increase in minimum inhibitory concentrations relative to the parental strain. RVs frequently exhibited diverse cross-protection profiles against unrelated antibiotic families. Importantly, certain RVs also showed altered tolerance to food preservation stresses. One COL- and one ERY-RV showed a 10-fold increase in survival under heat treatment (54 °C, 30 min). All ERY-RVs exhibited marked cross-protection to carvacrol (200 μL/L, 30 min), with up to a 1000-fold increase in survival. One COL-RV showed a 100-fold increased tolerance to lactic acid (1% w/v, 40 min). Whole genome sequencing revealed mutations primarily in genes associated with several functional categories, including metabolism and signal processing. Among them, mutations in the efflux regulator *ramR* and in *rfbH*, involved in O-antigen synthesis, were identified as candidate determinants potentially linked to cross-protection.

The findings suggest that AMR-related mutations in foodborne pathogens such as *S.* Typhimurium can modify responses to food preservation stresses, highlighting the need to better understand how antibiotic adaptation may influence bacterial persistence under food-related control conditions.

1. Introduction

The extensive use of antibiotics in human and veterinary medicine has accelerated the emergence of antimicrobial resistance (AMR), which complicates infection control and increases healthcare costs (Davies and Davies, 2010; Ma et al., 2021). The economic burden of AMR is substantial, with projected costs reaching 1 trillion USD by 2050 if effective countermeasures are not implemented (World Bank, 2017; Dadgostar, 2019).

AMR develops through genetic mutations, which can be transmitted either horizontally via mobile genetic elements, such as plasmids carrying β-lactamases or carbapenemases, or vertically, from parent to progeny (Sun et al., 2019). While horizontal gene transfer is considered the primary concern in clinical settings due to its ability to rapidly spread resistance across diverse bacterial species without selective pressure, vertical gene transfer remains a crucial and often underappreciated mechanism (Sun et al., 2019). Mutations conferring resistance can occur spontaneously during replication or be selected under

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environmental stresses such as disinfectants, food preservatives, or other non-antibiotic agents (Foster, 2005; Wang et al., 2022). Understanding these mechanisms is essential to develop strategies to avoid and combat AMR. Adaptive laboratory evolution (ALE) is a valuable tool that mimics natural selection, favoring the emergence of metabolic adaptations and allowing researchers to investigate bacterial adaptation to toxic environments, including high concentrations of antimicrobials (Berdejo et al., 2021b; Santos-Lopez et al., 2021; Mavrommati et al., 2022; Wu et al., 2022; Campillo et al., 2025).

Bacteria evade antimicrobial action through various mechanisms classified into four main categories: (I) reduction of membrane permeability, (II) increase of efflux pump expression, (III) enzymatic degradation or modification of antimicrobial agents, and (IV) alteration of target structures to prevent antibiotic binding. Beyond these classical mechanisms, metabolic adaptations can significantly impact antibiotic susceptibility, yet they remain underdiagnosed in clinical settings (Munita and Arias, 2016; Lopatkin et al., 2021). These metabolic shifts enhance bacterial growth and survival under antibiotic stress and may contribute to cross-protection. In addition, subinhibitory concentrations of antibiotics can trigger regulatory and physiological responses beyond resistance itself. For example, exposure to low levels of tetracycline has been shown to enhance biofilm formation in *Salmonella* Typhimurium, promoting persistence and increasing tolerance to antimicrobial stress (Yuan et al., 2023a). Likewise, the coexistence of biofilm-forming ability, antibiotic resistance, and genomic determinants in *S.* Typhimurium highlights the relevance of persistence-associated traits in the ecology of AMR *Salmonella* (Yuan et al., 2023b).

Beyond antibiotics, bacteria can develop tolerance to other antimicrobials and food preservation treatments, such as essential oils, organic acids, and heat treatments (Chueca et al., 2016; Berdejo et al., 2022; Wang et al., 2023; Pagan et al., 2024). This phenomenon in which tolerance to one stressor confers tolerance to others is known as cross-protection. Many of these resistance mechanisms overlap with those conferring antibiotic resistance, resulting in cross-protection. Typically, cross-protection is observed among antibiotics within the same structural family due to shared biochemical targets. However, non-antibiotic antimicrobial agents and food-processing stressors may also select for bacterial strains with increased antibiotic resistance (Álvarez-Molina et al., 2020; Berdejo et al., 2021a, 2022; Pagan et al., 2024). Together. These findings support a bidirectional relationship between food-related stresses and AMR, suggesting that bacterial adaptation to antibiotics may also influence tolerance to food preservation methods. Although cross-protection to heat and carvacrol treatments has already been reported in ciprofloxacin and meropenem resistant bacteria (Campillo et al., 2025; Tavares da Silva et al., 2025), it remains unclear whether these observations are restricted to specific drugs or whether they extend to unrelated antibiotic classes with different targets and evolutionary routes, as well as to other food-relevant preservation interventions such as acidification. This is relevant for food safety because AMR-associated bacterial lineages may emerge or persist before entering the food chain and later encounter preservation hurdles during food processing and storage, potentially affecting the efficacy of microbial control strategies.

This study aims to address this gap by pursuing the following objectives: (a) to isolate *Salmonella enterica* serovar Typhimurium LT2 resistant variants using ALE with three unrelated antibiotics used in human and veterinary medicine (colistin, amoxicillin, and erythromycin); (b) to identify genetic mutations responsible for AMR; (c) to evaluate cross-protection to food microbial control treatments, including heat, carvacrol, and lactic acid; and (d) to determine the genetic basis of cross-tolerance mechanisms. The findings will enhance our understanding of AMR's impact on bacterial resilience in food environments and contribute to the development of improved food safety strategies.

2. Materials and methods

2.1. Microorganisms, growth conditions and reagents

Salmonella enterica subsp. *enterica* serovar Typhimurium strain LT2 (SeT) was obtained from the Spanish Type Culture Collection (CECT 722). *S.* Typhimurium LT2 was selected as a reference strain because its fully sequenced and extensively annotated genome (McClelland et al., 2001) makes it particularly suitable for adaptive evolution and genomic analysis. Throughout the study, all bacterial strains were stored at −80 °C in cryovials containing 20% (v/v) glycerol. Each week, fresh streak plates were prepared on tryptone soya agar supplemented with 0.6% (w/v) yeast extract (TSAYE; Oxoid, Basingstoke, United Kingdom) from cryopreserved cultures to maintain strain viability and ensure consistency across experiments.

For experimental use, a single bacterial colony was inoculated into 5 mL of cation-adjusted Mueller Hinton Broth (MHB; Sigma-Aldrich, Saint Louis, USA) and incubated overnight at 37 °C with orbital shaking at 130 rpm (Vibramax 100, Heidolph, Schwabach, Germany). This subculture was subsequently used to inoculate fresh MHB (10 mL), adjusting to an initial bacterial concentration of 10^6 colony-forming units per milliliter (CFU/mL). Cultures were incubated at 37 °C for 24 h under agitation at 130 rpm, reaching the stationary growth phase ($\sim 3 \times 10^9$ CFU/mL).

2.2. Minimum inhibitory concentration (MIC)

This study tested three structurally unrelated antibiotics classified as critically important by the WHO (2024): colistin sulfate (COL), amoxicillin (AMX), and erythromycin (ERY) (Sigma-Aldrich), the latter two being considered of highest priority. To capture a broader range of adaptive trajectories, these antibiotics were selected because they represent distinct mechanisms of action, namely polymyxins (cell membrane disruptors), β-lactams (inhibitors of cell wall synthesis), and macrolides (inhibitors of protein synthesis), respectively. Stock solutions of COL, AMX, and ERY were prepared in accordance with the guidelines established by the Clinical and Laboratory Standards Institute (CLSI, 2023).

MIC values were determined using the broth microdilution method following CLSI protocols (CLSI, 2023). Serial two-fold dilutions in MHB of each antibiotic were prepared in 96-well microplates (Thermo Fisher Scientific, Massachusetts, USA) with a final well volume of 100 µL. The concentration ranges tested were 0.25-512.00 µg/mL for COL, 0.06-128.00 µg/mL for AMX, and 2.00-4096.00 µg/mL for ERY. Wells were inoculated at an initial concentration of 10^5 CFU/mL in 100 µL of MHB containing the appropriate antibiotic concentration and incubated at 37 °C for 20 h.

Bacterial growth was assessed by measuring optical density at 595 nm (OD_{595}) using a spectrophotofluorometer (Genios, Tecan, Männedorf, Switzerland). MIC was defined as the lowest antibiotic concentration capable of achieving a ≥90% reduction in OD_{595} compared to the antibiotic-free control (Kohanski et al., 2010). The obtained MIC values were compared to clinical breakpoints from CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2023a) to classify isolates according to their resistance.

2.3. Adaptive laboratory evolution

Resistant variants (RVs) were generated by exposing SeT populations to increasing concentrations of COL, AMX, and ERY for 11 consecutive exposures over a 10-day adaptive evolution experiment. Five independent evolution assays (i.e. parallel lineages) were conducted per antibiotic to capture different evolutionary trajectories, following the approach adapted from Lopatkin et al. (2021).

This method was adapted from a previously described protocol developed by our group, which has been detailed elsewhere (Campillo

et al., 2025). A bacterial subculture prepared as described earlier was used to inoculate 50 mL of MHB at 10^6 CFU/mL. Cultures were incubated at 37 °C with shaking at 130 rpm for 3.5 h to reach exponential phase (~10^7 CFU/mL). Subsequently, five independent lineages were initiated for each antibiotic by inoculating bacterial suspensions into 5 mL of MHB containing the respective antibiotic at an initial concentration of 0.085 × MIC. Cultures were incubated at 37 °C for 24 h under agitation.

Each day, a 1:1000 dilution of the previous culture was transferred into fresh MHB containing the antibiotic at 1.85 × the previous day's concentration. This stepwise adaptation continued for 11 consecutive exposures. Cryopreserved samples (20% v/v glycerol) from each lineage were stored at −80 °C after each daily exposure to preserve evolutionary intermediates.

At the end of the evolution assays, bacterial isolates from the highest antibiotic concentration allowing bacterial growth were selected. Samples were diluted in phosphate-buffered saline (PBS; Oxoid) and spread-plated onto TSAYE without antibiotics. After 24 h of incubation at 37 °C, individual colonies were picked for further characterization.

To assess whether resistance acquisition was due to antibiotic exposure rather than adaptation to experimental conditions, five parallel control lineages were maintained in antibiotic-free MHB for 11 exposures. The control lineages used here were generated in an earlier study and utilized in the current work (Campillo et al., 2025). MIC values of all evolved isolates were determined as previously described to confirm resistance acquisition against the respective antibiotics. RVs were cryopreserved at −80 °C for subsequent analyses.

2.4. Whole-genome sequencing (WGS) and identification of genetic changes

Total genomic DNA from SeT and RVs was extracted using the DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. WGS of the parental and evolved strains was performed on the Illumina HiSeq 4000 platform using 150 bp paired-end reads (Azenta, Leipzig, Germany). Libraries were prepared using the NEBNext Ultra II DNA Library Preparation Kit. WGS generated between 6.82 and 12.2 million high-quality paired-end reads (150 bp) per sample, with 90.89–93.72% of bases achieving Q30 scores. Reads were aligned directly to *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 reference genome (NCBI accession: NC_003197.2) (McClelland et al., 2001), yielding high mapping rates of 99.67–99.88%, allowing for 150- to 350-fold coverage depth and yielding a sufficiently covered reference genome to precisely detect variations among the strains. No de novo assembly was performed; instead, all reads were directly aligned to the reference genome. WGS of SeT and the RVs analyzed in the present study was performed for this work. In contrast, sequencing data for the antibiotic-free control lineages (SeT_CTRL1–SeT_CTRL5) had been generated previously (Campillo et al., 2025) and were included here for comparison.

The accession numbers of the samples are listed below, grouped according to the datasets generated in the present study (RVs and parental strain) and those reused from the previous study (control lineages).

- BioProject PRJNA1236942 for evolved RVs: SeT (SAMN47409544), SeT_COL1 (SAMN47409545), SeT_COL3 (SAMN47409546), SeT_COL4 (SAMN47409547), SeT_COL5 (SAMN47409548), SeT_AMX5 (SAMN47409549) SeT_ERY1 (SAMN47409550), SeT_ERY3 (SAMN47409551), SeT_ERY4 (SAMN47409552), SeT_ERY5 (SAMN47409553).
- BioProject PRJNA1044031 for control lineages: SeT_CTRL1, (SAMN38365818), SeT_CTRL2 (SAMN38365819), SeT_CTRL3 (SAMN38365820), SeT_CTRL4 (SAMN38365821) and SeT_CTRL5 (SAMN38365822).

2.5. Cross-protection to antibiotics

The antimicrobial susceptibility of the RVs was assessed using the Vitek 2 Compact system (bioMérieux, Marcy l'Etoile, France) with the GN cassette AST-GN96 card, following the manufacturer's instructions. The system's specialized software automatically interpreted the results according to CLSI guidelines (CLSI, 2023), categorizing bacterial susceptibility as Resistant (R), Intermediate (I), or Susceptible (S). The antibiotics tested included 15 agents from different families, namely: Ampicillin, Amoxicillin/Clavulanate, Cefalotin, Cefoperazone, Ceftiofur, Cefquinome, Imipenem, Gentamicin, Neomycin, Flumequine, Enrofloxacin, Marbofloxacin, Tetracycline, Florfenicol, and Trimethoprim/Sulfamethoxazole.

2.6. Cross-protection to food preservation treatments

The tolerance of RVs to food-relevant preservation stressors was evaluated through exposure to heat (54 °C for 30 min), carvacrol (200 μL/L for 30 min), and lactic acid (1 % w/v for 40 min) treatments. Heat, carvacrol, and lactic acid were selected as food-relevant antimicrobial stressors with different modes of action. Carvacrol was included as a representative natural antimicrobial compound of interest in food preservation research, whereas lactic acid was selected as an acid-based antimicrobial relevant to food processing and decontamination settings. A single treatment condition was used for the initial screening of all RVs in order to compare strains under a standardized challenge. These conditions were selected based on preliminary experiments (data not shown) to cause substantial but incomplete inactivation of the parental strain, thereby allowing the detection of both cross-protection and cross-sensitization in the evolved variants. They were not intended to reproduce the full range of industrial processing conditions.

2.6.1. Cross-protection to heat treatments

Bacterial suspensions were prepared in McIlvaine citrate-phosphate buffer (pH 7.0) using disodium hydrogen phosphate and citric acid monohydrate (PanReac, AppliChem, Darmstadt, Germany). A standardized suspension (~3×10^9 CFU/mL) was centrifuged (10,000×*g* for 3 min), and the pellet was resuspended in citrate-phosphate buffer. A 60 μL aliquot was transferred into PCR tubes and subjected to heat treatment at 54 °C for 30 min using a thermal cycler (T100 Thermal Cycler, Bio-Rad, Madrid, Spain). Strains demonstrating increased heat tolerance were further tested at a lower concentration (~3×10^7 CFU/mL), with aliquots collected every 5 min to obtain survival curves and characterize inactivation kinetics. Samples were diluted in PBS, spread-plated on TSAYE, and incubated at 37 °C for 24 h before count.

2.6.2. Cross-protection to carvacrol treatments

Bacterial cultures (~10^7 CFU/mL) were exposed to 200 μL/L carvacrol in 10 mL of TSBYE medium (pH 7.3 ± 0.2) at 25 °C for 30 min. Strains exhibiting higher tolerance underwent further testing under identical conditions, with aliquots taken every 5 min to obtain survival curves and characterize inactivation kinetics. Samples were diluted in PBS, pour-plated on TSAYE, and incubated at 37 °C for 24 h.

2.6.3. Cross protection to lactic acid treatments

Bacterial suspensions (~10^7 CFU/mL) were exposed to 1% w/v lactic acid in 10 mL of TSBYE medium (pH 3.5 ± 0.1) at 25 °C for 40 min. Strains exhibiting higher tolerance underwent further testing under identical conditions, with aliquots taken every 5 min to obtain survival curves and characterize inactivation kinetics. Samples were diluted in PBS, pour-plated onto TSAYE, and incubated at 37 °C for 24 h.

2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7.00 (GraphPad Software, San Diego, CA, USA) and Microsoft

Excel 16 (Microsoft, Redmond, USA). Normality of the data was assessed using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Nonparametric data were analyzed using the Kruskal–Wallis test or *t*-tests considering differences statistically significant at $p < 0.05$. Mean values and standard deviations were calculated from a minimum of three biological replicates from independent cultures obtained on different days. Voronoi maps were designed using the Treemap application from Macrofocus GmbH (Macrofocus, Zürich, Switzerland). These maps were used as a descriptive visualization of the mutated genes and do not represent a statistical enrichment analysis. Functional categories and biological processes were assigned according to the KEGG Orthology (KO) and BRITE annotations associated with each protein.

3. Results

3.1. Isolation of S. Typhimurium LT2 RVs to colistin (COL), amoxicillin (AMX), and erythromycin (ERY)

To establish baseline susceptibility, the minimum inhibitory concentration (MIC) of the parental strain (SeT) was determined to COL, AMX, and ERY (Table 1). Five independent evolutionary lineages were conducted for each antibiotic. By the fifth exposure, all viable lineages exhibited growth at their corresponding MIC levels (Fig. 1). However, in each antibiotic group, one lineage failed to progress during the early stages of antibiotic exposure and did not show any increase in MIC relative to the parental strain. These lineages were therefore not included in subsequent stress assays or genomic analyses.

The terminal exposure days for the isolation of resistant variants (RVs) varied depending on the antibiotic and lineage. COL-RVs were isolated at the highest concentrations tested, followed by ERY and AMX. As a result, COL-RVs demonstrated the highest resistance levels, whereas AMX-RVs exhibited the lowest relative MIC increases. In total, 12 evolved strains were isolated from exposures ranging from 1.82 × to 21.62 × MIC. Additionally, five parallel control lineages (SeT_CTRL1-SeT_CTRL5) were evolved for 10 days in antibiotic-free conditions. No increases in MIC values against COL, AMX, or ERY were detected for any of the control lineages, so no acquisition of resistance could be attributed to the exposure to the growth medium (data not shown).

Strains exceeding resistance thresholds were retained for further analysis. MIC determinations were performed for each RV and compared to the parental strain (Table 1). All isolates, except SeT_AMX4, exhibited MIC increases relative to SeT. Increased resistance levels were observed in COL-RVs (SeT_COL1-SeT_COL5), with MIC values ranging from 4 × to 8 × those of SeT (32-64 μg/mL). AMX-RVs (SeT_AMX2-SeT_AMX5) exhibited the highest MIC increases of 8 × to 16 × that of SeT (8-16 μg/mL), with only SeT_AMX5 surpassing CLSI resistance breakpoints. ERY-RVs demonstrated increased resistance levels, with MIC values 4 × to 8 × higher than SeT (256-512 μg/mL). Since no established resistance breakpoints exist for ERY, all RVs were retained for further analysis.

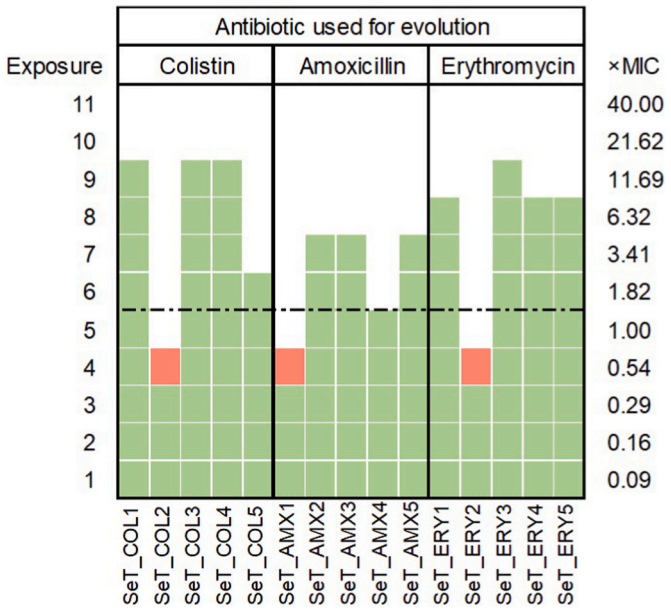

Fig. 1. Maximum number of days of exposure and isolation of each lineage of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 (SeT) studied with the respective increases in concentration of the antibiotics tested (colistin, amoxicillin, erythromycin). Green bars show the maximum exposure from which microorganisms could be isolated. The dotted line marks the exposure from which cryovials were stored for further evaluation. Red squares indicate the terminal points of lineages that failed to grow at their respective MIC.

3.2. Whole genome sequencing (WGS) of COL, AMX, and ERY-RVs

WGS of SeT (Table S1) revealed base mutations in genes with varied functions. Also, analysis of the domestication control strains (Table S2) revealed mutations in *rfbV* and *rfbI*, which emerged due to exposure to the growth medium rather than antibiotic selection. Consequently, as no MIC increases were observed in the control lineages, these mutations were not considered relevant to AMR.

WGS of the RVs against COL, AMX, and ERY (Table 2) identified a diverse range of mutations, including single nucleotide variations (SNVs) in *acrB, ramR, galE, lipA, phoP, spoT, basS, rfbD, yjjK, yfjG, rpoC,* and *rfbH*. Additionally, a stop-gain mutation was detected in *ramR*. Frameshift insertions were observed in *rfc* and *gppA*, while a large 4271 bp deletion affected *radA, nadR, yjjK,* and *slt*.

Despite being exposed to similar experimental conditions, both COL and ERY-RVs displayed distinct mutational profiles within their

Table 1
Minimum inhibitory concentrations (MICs) determined by broth microdilution for the parental *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 strain (SeT) and its RVs selected under colistin (SeT_COL1–SeT_COL5), amoxicillin (SeT_AMX1–SeT_AMX5), and erythromycin (SeT_ERY1–SeT_ERY5).

Colistin (μg/mL)			Amoxicillin (μg/mL)			Erythromycin (μg/mL)		
Strain	MIC	Int	Strain	MIC	Int	Strain	MIC	Int
SeT	8	**R**	**SeT**	1	**S**	**SeT**	64	*nc*
SeT_COL1	64	**R**	**SeT_AMX1**[a]	1	**S**	**SeT_ERY1**	256	*nc*
SeT_COL2[a]	8	**R**	**SeT_AMX2**	8	**S**	**SeT_ERY2**[a]	64	*nc*
SeT_COL3	64	**R**	**SeT_AMX3**	8	**S**	**SeT_ERY3**	256	*nc*
SeT_COL4	32	**R**	**SeT_AMX4**	1	**S**	**SeT_ERY4**	256	*nc*
SeT_COL5	32	**R**	**SeT_AMX5**	16	**I**	**SeT_ERY5**	512	*nc*

[Int: Interpretation of susceptibility: R: Resistant; I: Intermediate resistance; S: Susceptible; according to the standards established by the Clinical and Laboratory Standards Institute (CLSI, 2023). *Nc*: No criteria of resistance; MIC: Minimum inhibitory concentration].

[a] For SeT_COL2, SeT_AMX1 and SeT_ERY2, no increase in MIC relative to the parental strain was observed during ALE; therefore, the parental MIC value is shown. These lineages were not included in downstream stress assays or genomic analyses.

Table 2
Genetic variations identified by whole genome sequencing in colistin- (SeT_COL1, SeT_COL3, SeT_COL4 and SeT_COL5), amoxicillin- (SeT_AMX5), and erythromycin- (SeT_ERY1, SeT_ERY3, SeT_ERY4 and SeT_ERY5) RVs of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2, compared to the reference parental strain SeT (NCBI accession: NC_003197.2).

Strain	Genome position	Type of mutation	Change[a]	Locus tag	Gene	Description
SeT_AMX5	531423	SNV	C977T → Pro326Leu	STM0475	*acrB*	RND family, acridine efflux pump
	638679	SNV	A52C → Thr18Pro	STM0580	*ramR*	TetR/AcrR family transcriptional regulator
	841581	SNV	G20C → Gly7Ala	STM0776	*gaLE*	UDP-galactose 4-epimerase
SeT_COL1	695332	SNV	A499C → Thr167Pro	STM0633	*lipA*	lipoate synthase, an iron-sulfur enzyme
	1319324	SNV	T30G → Asn10Lys	STM1231	*phoP*	Response regulator in two- component regulatory system with PhoQ
	1410676	frameshift insertion	625dupA → Ile209fs	STM1332	*rfc*	O-antigen polymerase
	3934722	SNV	A641C → Gln214Pro	STM3742	*spoT*	(p)ppGpp synthetase II
	4122945	frameshift insertion	376_377insTCCGCTGGCTGTTCCGTCGTATG → Thr126fs	STM3913	*gppA*	exopolyphosphatase
	4533697	SNV	T29C → Leu10Pro	STM4291	*basS*	sensory kinase in two-component regulatory system with BasR
SeT_COL3	2176910	SNV	T583C → Cys195Arg	STM2096	*rfbD*	dTDP-4-dehydrorhamnose reductase
	3934461	SNV	T380A → Val127Glu	STM3742	*spoT*	(p)ppGpp synthetase II
	4533697	SNV	T29C → Leu10Pro	STM4291	*basS*	sensory kinase in two- component regulatory system with BasR
	4837755	SNV	A1090C → Thr364Pro	STM4581	*yjjK*	lipopolysaccharide biosynthesis protein
SeT_COL4	1319248	SNV	G106A → Glu36Lys	STM1231	*phoP*	Response regulator in two- component regulatory system with PhoQ
	4533697	SNV	T29C → Leu10Pro	STM4291	*basS*	sensory kinase in two-component regulatory system with BasR
SeT_COL5	2826072	SNV	T266A → Met89Lys	STM2687	*yfjG*	putative oligoketide cyclase/lipid transport protein
	4370815	SNV	G790T → Asp264Tyr	STM4154	*rpoC*	RNA polymerase, beta prime subunit
	4533697	SNV	T29C → Leu10Pro	STM4291	*basS*	sensory kinase in two-component regulatory system with BasR
SeT_ERY1	638684	SNV	C47A → Ala16Glu	STM0580	*ramR*	TetR/AcrR family transcriptional regulator
SeT_ERY3	638679	SNV	A52C → Thr18Pro	STM0580	*ramR*	TetR/AcrR family transcriptional regulator
	2174587	SNV	C523T → Gln175X	STM2090	*rfbH*	lipopolysaccharide biosynthesis protein
SeT_ERY4	2171386	SNV	A851T → His284Leu	STM2090	*rfbH*	lipopolysaccharide biosynthesis protein
SeT_ERY5	638676	stopgain	C55T → Gln19X	STM0580	*ramR*	TetR/AcrR family transcriptional regulator
	4835096	Deletion - 4271 pb	Deletion *radA nadR yjjK slt*	STM4579	*radA*	Putative ATP-dependent protease
				STM4580	*nadR*	Trifunctional NAD biosynthesis/regulator protein NadR
				STM4581	*yjjK*	Putative transport protein
				STM4582	*slt*	Soluble lytic murein transglycosylase

[SNV: Single nucleotide variation; Ins: insertion; Del: deletion; X: stopgain, fs: frameshift. Genetic variations between SeT and the reference genome are not shown. Mutations located in intergenic regions are indicated using the locus tag of the flanking regions.
[a] Position relative to the start codon of the affected coding sequence].

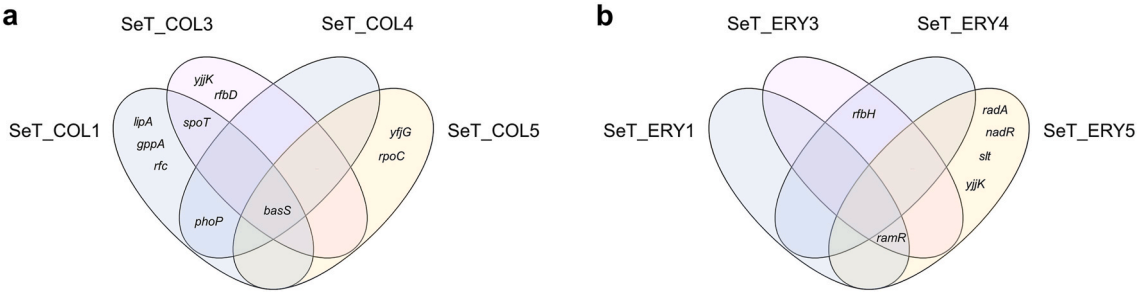

Fig. 2. Venn diagram showing the distribution of mutated genes in colistin- (A) and erythromycin- (B) RVs of *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2.

respective antibiotic selection pathways (Fig. 2). No profiles could be discerned for AMX since only one lineage was isolated for this antibiotic. Notably, all RVs from the same antibiotic shared at least one common mutated gene, indicating convergent evolutionary adaptations. Most mutations were antibiotic-specific; however, *ramR* and *yjjK* mutations emerged across antibiotic exposures. Specifically, *ramR* mutations were detected in four RVs evolved against AMX and ERY, while *yjjK* mutations appeared in two RVs selected against COL and ERY.

Several RVs within the same antibiotic exposure group exhibited overlapping mutational patterns, indicative of parallel evolutionary trajectories. Although COL-RVs displayed the highest degree of mutational heterogeneity (Fig. 2a), all four harbored mutations in *basS*, and two independently acquired mutations in *spoT* and *phoP*. Among ERY-RVs (Fig. 2b), *ramR* mutations were shared by SeT_ERY1, SeT_ERY3, and SeT_ERY5, while *rfbH* mutations were present in both SeT_ERY3 and SeT_ERY4.

To provide a descriptive overview of the functional distribution of the mutated genes, Voronoi diagrams were generated using KEGG Orthology and BRITE functional classifications (Fig. 3). While some affected genes remain unclassified, key functional categories included

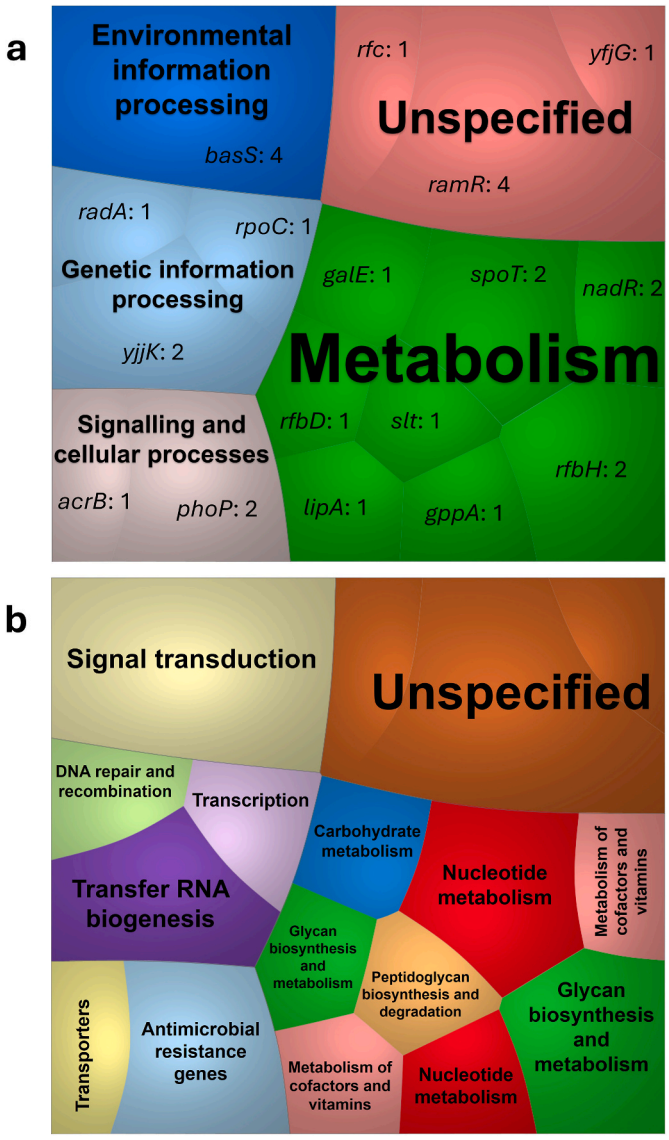

Fig. 3. Voronoi diagrams showing a descriptive functional classification of the genes mutated in the colistin-, amoxicillin-, and erythromycin-resistant variants (RVs) of *Salmonella enterica* subsp. *enterica* serovar Typhimurium. Functional categories were assigned according to KEGG Orthology (KO) and BRITE annotations based on the KO identifier associated with each protein. The diagrams are intended as a descriptive visualization and do not represent a statistical enrichment analysis. Each polygon represents one mutated gene, and its area reflects the frequency with which that gene was mutated among the RVs. Colors indicate grouping by functional category (A) and functional subcategory (B). Numbers indicate the frequency of mutation.

genetic information processing, signalling and cellular processing, environmental information processing, and metabolism.

3.3. Cross protection against antibiotics

Susceptibility testing (Table 3) revealed an expanded resistance profile in the AMX-RV, which exhibited increased resistance to ampicillin, cefalotin, ceftiofur, and florfenicol. Among the ERY-RVs, three distinct resistance patterns were observed despite their evolution under identical conditions. All ERY-RVs demonstrated a 2- to 4-fold increase in resistance to florfenicol compared to SeT. Notably, SeT_ERY3 and SeT_ERY5 shared a similar resistance profile, both displaying an 8-fold increase in MIC to tetracycline. SeT_ERY1 closely mirrored these strains but additionally exhibited a 16-fold increase in MIC to flumequine. In contrast, SeT_ERY4 showed a more selective resistance pattern, with a significant increase only against florfenicol. Minimal MIC increases were detected to other antibiotic classes in all ERY-RVs. For COL-RVs, no additional resistance was observed against any antibiotic class. Interestingly, the intrinsic intermediate resistance to florfenicol present in the parental strain was lost in all COL-RVs.

3.4. Cross-protection to food preservation treatments

All RVs were subjected to heat treatments at 54 °C for 30 min to assess thermal resistance (Fig. 4a). Most RVs showed similar or increased heat sensitivity compared to SeT; however, SeT_COL5 and SeT_ERY1 exhibited significantly lower inactivation (3.81 and 3.55 $\log_{10}$ cycles, respectively; $p < 0.05$) than SeT (4.62 $\log_{10}$; $p < 0.05$), while SeT_ERY5 showed increased heat sensitivity over limit of detection. Time-course assays revealed enhanced heat tolerance in SeT_ERY1 from 20 min onward and in SeT_COL5 from 25 min (Fig. 4b). Additional kinetic analysis based on Weibull modelling is provided in Table S4.

Cross-protection to carvacrol (200 µL/L, 30 min) varied among RVs (Fig. 4c). SeT_COL1 displayed increased sensitivity with an increase in its inactivation exceeding 0.63 $\log_{10}$ cycles, whereas all ERY-RVs showed significantly reduced inactivation (0.94–2.07 $\log_{10}$; $p < 0.05$) compared to SeT (>4 $\log_{10}$; $p < 0.05$). Survival curves (Fig. 4d) confirmed significant cross-protection in ERY-RVs after 10 min, with final inactivation reductions of 0.67–0.92 $\log_{10}$ cycles, except SeT_ERY4 that showed intermediate resistance with an inactivation of 1.82 $\log_{10}$; $p < 0.05$). These results correspond to 10^3–10^4-fold higher survival than SeT. Growth parameters under non-stress conditions are provided in Table S3. Notably, the ERY-RVs did not show a consistent reduction in growth rate relative to SeT, although SeT_ERY1 displayed an extended lag phase.

Regarding lactic acid treatments (1% w/v, 40 min) (Fig. 4e), SeT showed a 4.26 $\log_{10}$ ($p < 0.05$) reduction, while SeT_COL1 exhibited reduced inactivation (3.37 $\log_{10}$; $p < 0.05$). In contrast, SeT_COL2, SeT_COL4, SeT_COL5, SeT_ERY4, and SeT_ERY5 showed cross-sensitization, with increases in inactivation exceeding 0.74 $\log_{10}$ that of SeT. Time-course analysis of SeT_COL1 (Fig. 4f) revealed similar early inactivation to SeT, but increased tolerance from 15 min onward, reaching a ~2 $\log_{10}$ difference at 35 min, equivalent to a 100-fold higher survival risk. Additional kinetic analysis based on Weibull modelling is provided in Table S4.

4. Discussion

In this study, *S.* Typhimurium LT2 (SeT) was subjected to adaptive laboratory evolution (ALE) under increasing concentrations of colistin (COL), amoxicillin (AMX), and erythromycin (ERY) to generate resistant variants (RVs). Previous studies have reported that selection for ciprofloxacin and meropenem resistance (Campillo et al., 2025;Tavares da Silva et al., 2025) can be accompanied by increased tolerance to food-related stresses such as heat and natural antimicrobials. Here, we assess whether such cross-adaptive responses extend to RVs selected under unrelated antibiotic classes (COL, AMX and ERY) and whether they also change bacterial tolerance to acidification (lactic acid), a common food-processing hurdle. The stepwise exposure strategy (1.85 × MIC increase per day) enabled the selection of RVs carrying mutations consistent with antibiotic-driven selection, as described for comparable ALE schemes (Lopatkin et al., 2021; Campillo et al., 2025). None of the evolved lineages survived to the final exposure, and isolation occurred between exposures 5 (~1.0 × MIC) and 10 (~21.6 × MIC) (Fig. 1), resembling extinction dynamics reported under escalating antibiotic pressure in *E. coli* (Lopatkin et al., 2021). However, several AMX- and ERY-exposed lineages failed to persist beyond the MIC threshold, suggesting antibiotic- and lineage-dependent adaptive capacity in *S.* Typhimurium.

Resistance development was assessed by MIC determination

Table 3
Cross-protection to antibiotics in *Salmonella* RVs evolved under colistin (SeT_COL1–SeT_COL5), amoxicillin (SeT_AMX5), and erythromycin (SeT_ERY1, SeT_ERY3, SeT_ERY4, SeT_ERY5), as determined by VITEK 2.

Family	Antibiotic	Strain													
		SeT		SeT_AMX5		SeT_ERY1		SeT_ERY3 SeT_ERY5		SeT_ERY4		SeT_COL1		SeT_COL3 SeT_COL4 SeT_COL5	
		MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int
Beta-lactams	Ampicillin	≤2	S	**16**	**R**	≤2	S	≤2	S	**4**	**S**	≤2	S	≤2	S
	Amoxicillin-clavulanate	≤2	S	**8**	**S**	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S
Cephalosporins	Cefalotin	≤2	R	**16**	**R**	**4**	**R**	≤2	R	**4**	**R**	≤2	R	≤2	R
	Cefoperazone	≤4	S	≤4	S	≤4	S	≤4	S	≤4	S	≤4	S	≤4	S
	Ceftiofur	≤1	S	**4**	**I**	≤1	S	≤1	S	**2**	**S**	≤1	S	≤1	S
	Cefquinome	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S
Carbapenems	Imipenem	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S
Aminoglycosides	Gentamicin	≤1	R	≤1	R	≤1	R	≤1	R	≤1	R	≤1	R	≤1	R
	Neomycin	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S
Fluoroquinolones	Flumequine	≤1	S	≤1	S	**16**	**R**	**2**	**S**	**2**	**S**	≤1	S	≤1	S
	Enrofloxacin	≤0.12	S	≤0.12	S	**0.5**	**S**	≤0.12	S	≤0.12	S	≤0.12	S	≤0.12	S
	Marbofloxacin	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S
Tetracyclines	Tetracycline	≤1	S	≤1	S	8	I	8	I	4	S	≤1	S	≤1	S
Phenicols	Florfenicol	8	I	**≥ 32**	**R**	**≥ 32**	**R**	**≥ 32**	**R**	**16**	**R**	**≤ 1**	**S**	**4**	**S**
Diaminopyrimidines - Sulfonamide	Trimethoprim-Sulfamethoxazole	≤20	S	≤20	S	≤20	S	≤20	S	≤20	S	≤20	S	≤20	S

[Int: Interpretation of susceptibility: R: Resistant, I: Intermediate, S: Susceptible; according to the standards established by the Clinical and Laboratory Standards Institute (CLSI, 2023). MIC: Minimum inhibitory concentration (μg/mL). RVs that shared cross-protection patterns against antibiotics were grouped together. Data in **bold type** represents difference in relation to *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 parental strain (SeT)].

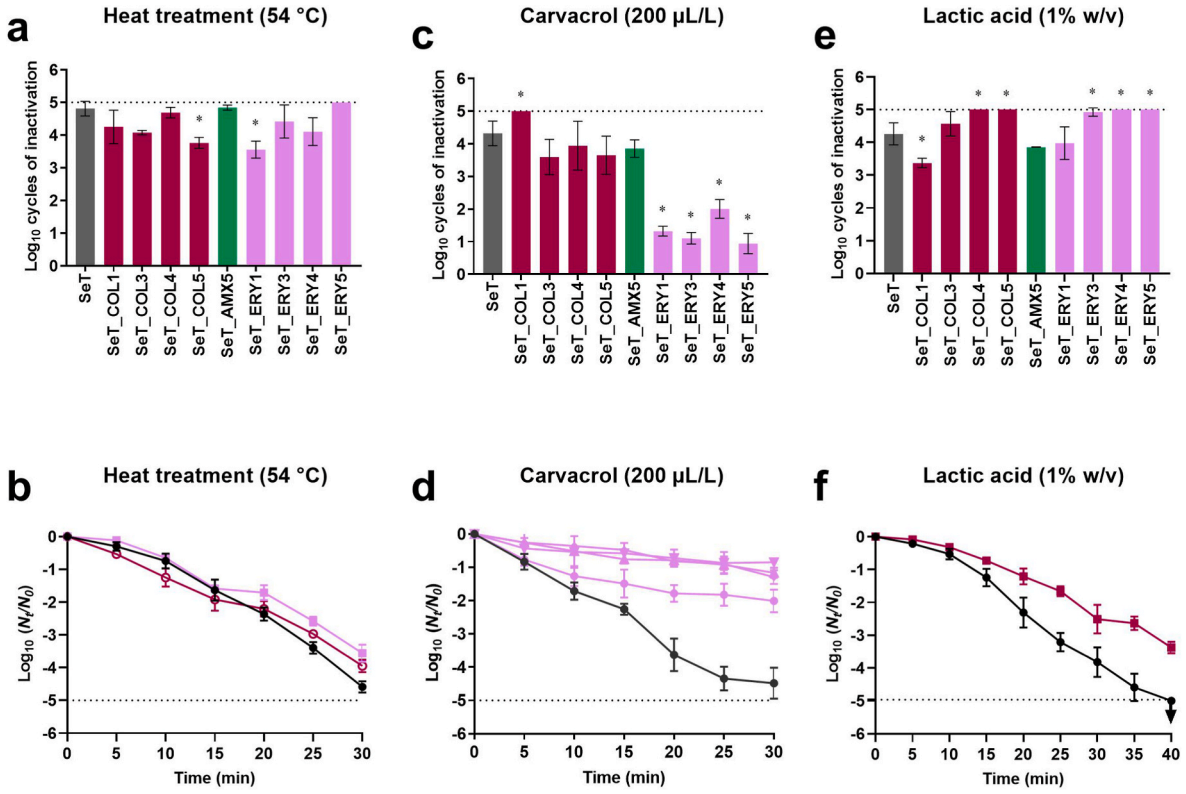

Fig. 4. A- Inactivation cycles of parental strain *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 (SeT) and its RVs to colistin (SeT_COL1, SeT_COL3, SeT_COL4, SeT_COL5), amoxicillin (SeT_AMX5) and erythromycin (SeT_ERY1, SeT_ERY3, SeT_ERY4, SeT_ERY5) after treatment at 54 °C, 30 min in McIlvaine buffer pH 7.0. **B-** Survival curves of SeT (●) and its colistin- (○SeT_COL5) and erythromycin- (■SeT_ERY1) RVs under the same heat-treatment conditions. **C-** Inactivation cycles of SeT and its RVs to colistin (SeT_COL1, SeT_COL3, SeT_COL4, SeT_COL5), amoxicillin (SeT_AMX5) and erythromycin (SeT_ERY1, SeT_ERY3, SeT_ERY4, SeT_ERY5) after carvacrol treatment at 200 μL/L, 25 °C, 30 min in TSB. **D-** Survival curves of SeT (●) and its erythromycin-RVs (▲SeT_ERY1, ▼SeT_ERY3, ●SeT_ERY4, ♦SeT_ERY5) under carvacrol treatment in the same conditions. **E-** Inactivation cycles of SeT and its RVs to colistin (SeT_COL1, SeT_COL3, SeT_COL4, SeT_COL5), amoxicillin (SeT_AMX5) and erythromycin (SeT_ERY1, SeT_ERY3, SeT_ERY4, SeT_ERY5) after treatment with 1% w/v lactic acid, 25 °C, 40 min in TSB. **F-** Survival curves of SeT (●) and its colistin-RV (■ SeT_COL1) under treatment with 1% w/v lactic acid in the same conditions. Horizontal dotted line represents the detection limit. Error bars indicate the standard deviation calculated from independent replicates. Asterisks represent statistical significance ($p < 0.05$). Points beyond the detection limit were indicated with a downward arrow.

(Table 1). SeT was intrinsically susceptible to AMX according to CLSI and EUCAST breakpoints (≤8 μg/mL) (CLSI, 2023; EUCAST, 2023a) and within reported MIC confidence intervals (EUCAST, 2023b), consistent with previous observations in *S. enterica* (Lindecrona et al., 2000; Abraham et al., 2022). In contrast, SeT exceeded the COL susceptibility threshold (≤2 μg/mL), compatible with reduced susceptibility described for some *Salmonella* spp. serotypes (Morales et al., 2012; Bertelloni et al., 2022; Fortini et al., 2022). For ERY, no CLSI or EUCAST criteria exist for *Enterobacteriaceae*, but our MICs align with prior reports (Carlson and Ferris, 2000; Gunell et al., 2010). RVs showed heterogeneous direct-resistance increases: COL-RVs reached up to 8-fold MIC increases (exceeding CLSI breakpoints), whereas AMX-RVs generally displayed smaller shifts, with only SeT_AMX5 reaching the CLSI resistance threshold. Resistance of ERY-RVs against ERY increased 4–8-fold relative to SeT, lower than increases reported for other Gram-negative bacteria under ALE (Marciano et al., 2022; Couto et al., 2024).

A control evolution without antibiotic exposure indicated that resistance was linked to antibiotic selection rather than medium adaptation. Domestication controls (SeT_CTRL1–SeT_CTRL5) showed no increases in MICs to the tested antibiotics, and only two minor mutations were detected (a *rfbI* deletion and a *rfbV* SNV), supporting that the RV phenotypes emerged primarily under antibiotic selection.

WGS (Table 2, Figs. 2 and 3) showed that several mutations were associated with genes annotated in metabolism- and signal processing-related categories, suggesting that global regulation and metabolic state may contribute to resistance and stress responses. However, the mutational profiles differed across replicate lineages, indicating that lineage-specific and potentially stochastic evolutionary trajectories also contributed to the observed phenotypes. In COL-RVs, recurrent mutations in *spoT*, *phoP*, and *basS* support envelope-centred routes involved in resistance. *SpoT* regulates (p)ppGpp homeostasis and coordinates metabolism with stress adaptation (Kim et al., 2018; Das and Bhadra, 2020). *PhoP* contributes to polymyxin resistance through lipopolysaccharide (LPS) remodelling and reduced membrane permeability (Yu et al., 2015; Moffatt et al., 2019), while *basS* regulates membrane integrity and stress-response functions (Ogasawara et al., 2012; Merino et al., 2023). Additional mutations (e.g., *lipA*, *gppA*, *yfjG*) are consistent with metabolic rewiring under antibiotic stress (Das and Bhadra, 2020; Lopatkin et al., 2021), and changes in O-antigen genes (*rfc*, *rfbD*) suggest that LPS architecture may also be a recurrent route of adaptation shaping susceptibility and stress phenotypes (Thomsen et al., 2003).

In the AMX-evolved SeT_AMX5, mutations in *acrB*, *ramR*, and *galE* point to efflux and envelope-associated contributions to increased resistance (Table 2). *AcrB* encodes a key component of an efflux pump (Wang-Kan et al., 2017), while *ramR* controls efflux regulation (Colclough et al., 2019). The *ramR* substitution at amino acid 18 (Thr→Pro) is in a region previously associated with increased efflux pump synthesis and multidrug resistance (Abouzeed et al., 2008). The *galE* mutation may further influence resistance via changes in LPS synthesis and membrane composition or metabolic activity (Fry et al., 2000; Lopatkin et al., 2021).

In ERY-RVs, *ramR* mutations in a similar region to SeT_AMX5 reinforce efflux-driven resistance as a recurrent route. Mutations in *rfbH* (two RVs) may also modulate envelope properties and susceptibility (Karash et al., 2022). Additional changes in *radA*, *nadR*, *slt*, and *yjjK* further point to DNA repair, NAD metabolism, and envelope remodelling as contributing processes in the overall adaptation to ERY (Koskiniemi and Andersson, 2009; Grose et al., 2005; Cestero et al., 2021). While not canonical determinants of stress tolerance, those changes can increase frequency of mutation or alter physiological state, facilitating multi-step resistance trajectories (Volkova et al., 2020; Németh and Szüts, 2024).

These genetic changes were consistent with cross-protection patterns across antibiotic families. COL-RVs lost intermediate florfenicol resistance, probably via membrane alterations (Yekani et al., 2023). SeT_AMX5 increased resistance to ampicillin and ceftiofur, and its florfenicol resistance is compatible with the upregulation of efflux pump activity linked to altered RamR control (Ricci et al., 2012; Roberts and Schwarz, 2016). ERY-RVs displayed increased resistance to florfenicol, tetracycline, and flumequine; the tetracycline phenotype may reflect shared ribosomal targeting and/or efflux contributions (Culebras et al., 2002; Ahmadpoor et al., 2021; Stefańska et al., 2022). More generally, resistance-associated metabolic shifts and fitness costs can reshape susceptibility profiles beyond the selecting drug (Lopatkin et al., 2021). In this context, growth parameters measured under non-stress conditions (Table S3) did not reveal a consistent fitness impairment among the ERY-RVs, suggesting that their marked cross-protection to carvacrol cannot be attributed simply to a general growth defect.

To test whether AMR-associated mutations modified resistance to food preservation stresses, RVs were exposed to heat and carvacrol, which have been previously associated with cross-protection against antibiotics (Campillo et al., 2025; Tavares da Silva et al., 2025), and to lactic acid, to investigate whether cross-protection induced by antibiotics occurs with this stressor. Prior work has reported MDR *S.* Typhimurium with increased heat tolerance (Walsh et al., 2005), and this association was later confirmed in ciprofloxacin-resistant variants (Campillo et al., 2025). SeT_COL5 and SeT_ERY1 showed increased heat tolerance, consistent with envelope-centred adaptations under COL selection and efflux/stress-regulatory changes under ERY selection (Fig. 4b). Weibull modelling showed that strain-dependent differences in heat tolerance became more evident as treatment time increased (Table S4), with limited differences at 30 min but greater predicted inactivation of the parental strain than of SeT_COL5 and SeT_ERY1 at 40 min. Given that colistin disrupts membrane permeability (Vaara, 2010), the *yfjG* mutation (potentially involved in lipid transfer) could contribute to thermal robustness (Tsui et al., 2019). In SeT_ERY1, *ramR* was the sole mutation detected, which is consistent with a contribution of altered RamR regulation to heat cross-protection (Campillo et al., 2025), in line with the role of RamR in controlling *ramA* and downstream efflux and stress responses (Ricci et al., 2012). Notably, SeT_ERY5 exhibited cross-sensitization, suggesting that additional mutations in *radA*, *nadR*, *slt*, and *yjjK* may influence heat sensitivity. For instance, *radA* is involved in DNA repair and genome stability, which are critical under heat stress conditions, while *nadR* plays a role in NAD metabolism and cellular redox balance (Koskiniemi and Andersson, 2009; Grose et al., 2005). Likewise, *slt*, encoding a lytic transglycosylase, may affect cell envelope remodelling, and *yjjK* has been associated with stress response regulation (Cestero et al., 2021). Alterations in these pathways could compromise the bacterial ability to cope with thermal stress, potentially explaining the observed phenotype (Huang et al., 2014). However, this cross-sensitization was not a predominant or consistent outcome across lineages, suggesting it represents a lineage-specific trade-off rather than a generalizable adaptive response. Therefore, while mechanistically relevant, its implications for food safety appear limited in the context of this study.

All ERY-RVs showed increased tolerance to carvacrol (Fig. 4d). Efflux activation has been involved in tolerance to plant-derived antimicrobials such as thymol and eugenol (Walsh et al., 2003), and the strongest carvacrol tolerance coincided with *ramR* mutations. SeT_ERY4 (without *ramR* mutation) showed lower tolerance, potentially reflecting alternative envelope routes such as *rfbH*, which has been linked to stress resistance and antibacterial defence in *Salmonella* spp. (Thomsen et al., 2003; Gantois et al., 2009). To further assess whether the identified mutations were associated with altered intracellular accumulation of compounds, an ethidium bromide (EtBr) accumulation assay was performed (Fig. S1). The results were consistent with reduced intracellular accumulation in most *ramR*-mutant RVs, supporting the involvement of efflux-related and/or permeability-related changes, although they do not by themselves demonstrate the underlying mechanism. Most RVs harboring mutations in *ramR* exhibited decreased EtBr uptake at higher concentrations, except for SeT_ERY5. This deviation suggests that the additional alterations in *radA*, *nadR*, *slt*, and *yjjK* may substantially

modify the physiological outcome expected from *ramR* alteration alone.

Interestingly, SeT_ERY4, which does not carry a mutation in *ramR*, displayed an intermediate level of EtBr accumulation. This observation may be associated with altered membrane permeability related to the mutation in *rfbH*, consistent with its intermediate resistance to carvacrol. This pattern is compatible with the idea that intracellular access contributes, at least in part, to carvacrol activity, as greater accumulation was associated with increased sensitivity in this variant. In contrast, SeT_ERY3, carrying mutations in both *ramR* and *rfbH*, showed the lowest EtBr accumulation among all RVs, which is consistent with a combined effect of these mutations on limiting intracellular accumulation and, consequently, reducing sensitivity to carvacrol.

Regarding lactic acid treatments, SeT_COL1 showed increased tolerance (Fig. 4f). Weibull modelling was consistent with this result, showing lower predicted inactivation of SeT_COL1 than of the parental strain at both 30 and 40 min (Table S4). The combination of *lipA* and *gppA* is consistent with altered *rpoS*-associated stress responses and (p)ppGpp signalling (Wang et al., 2007; Battesti et al., 2015; Kim et al., 2018; Das and Bhadra, 2020), while *phoP* and *rfc* may further influence envelope integrity and/or permeability during acid stress (Gunn, 2001; Simpson and Trent, 2019; Wang et al., 2021). Although cross-sensitization was not pursued further because increased inactivation is unlikely to compromise food safety, it may still provide mechanistic insight into adaptive trade-offs.

Overall, our results indicate that AMR evolution can reshape responses to non-antibiotic stressors, such as food preservation technologies, through a combination of efflux regulation, envelope remodelling, and metabolic reprogramming. This variability across antibiotics and mutational backgrounds suggests that resistance phenotype alone may not predict survival to food preservation treatments, supporting integrated genotypic and phenotypic assessment when considering the persistence of AMR lineages along the food chain. From an applied perspective, these findings should be interpreted within the context of industrial multi-hurdle preservation strategies, in which the antimicrobial effect often results from the combined and sometimes synergistic action of different preservation factors. While the cross-protection effects observed in vitro suggest a potential for altered stress tolerance, their impact in real food matrices is likely modulated by factors such as composition, water activity, and structural heterogeneity. In addition, because this study was performed in the laboratory reference strain *S.* Typhimurium LT2, the results should be regarded mainly as a proof of concept and will require validation in more representative foodborne, environmental, or clinical isolates. In addition, the stability of the evolved phenotypes was not assessed after propagation in the absence of antibiotic selective pressure, and this should be addressed in future work to determine the persistence and relevance of these adaptations. Therefore, although AMR-associated adaptations could influence the effectiveness of individual preservation hurdles, their overall impact under industrial conditions may be limited, and further validation in relevant food systems is warranted.

5. Conclusions

Stepwise selection of *S.* Typhimurium under three antibiotics from unrelated classes (COL, AMX and ERY) generated RVs showing antibiotic- and lineage-dependent cross-adaptive responses to food-relevant stresses (heat, carvacrol and lactic acid). Although all lineages went extinct before the final exposure day (exposure 11), isolates recovered at intermediate exposures exhibited measurable MIC increases, with the largest shifts observed under COL selection and more modest gains under AMX and ERY.

WGS analysis indicated that direct-resistance was largely associated with mutations affecting envelope regulation and permeability (e.g., *spoT*, *phoP*, *basS* and O-antigen related genes) and efflux regulation (notably *ramR*, together with *acrB* in the AMX-RV), alongside changes consistent with broader metabolic reprogramming. Importantly, these AMR-associated genotypes also modified tolerance to food preservation technologies in a variant-dependent manner: some RVs showed cross-protection to heat, carvacrol, or lactic acid; while others displayed cross-sensitization. Overall, these results support that AMR evolution can reshape non-antibiotic stress responses through combined effects on membrane architecture, efflux control, and physiology. Therefore, antibiotic resistance levels (such as MIC shifts) do not necessarily translate into higher or lower survival during heat, acid, or natural antimicrobial-treatments. Understanding these adaptive pathways and evaluating behaviour of RVs in real food matrices will be critical to improve pathogen control strategies.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 5.2 in order to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Ivo García-Penas: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Raúl Campillo:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Daniel Berdejo:** Writing – review & editing. **Laura Espina:** Writing – review & editing. **Pilar Conchello:** Writing – review & editing. **Rafael Pagán:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Diego García-Gonzalo:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2026.105138.

Data availability

Data will be made available on request.

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