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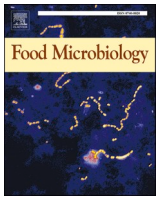

Meropenem-induced cross-protection in *Salmonella enterica* resistant variants: Insights from adaptive laboratory evolution and whole genome sequencing

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ABSTRACT

Adaptive laboratory evolution (ALE) assay provides a deep understanding of the genetic mechanisms and processes involved in bacterial responses. This study aimed to obtain resistant-variants (RVs) of *Salmonella enterica* subsp. *enterica* Typhimurium (SeT) and Enteritidis (SeE) after ALE assays with meropenem (MPM); to identify genetic modifications of RVs responsible for their increased resistance; and to evaluate their cross-protection against antibiotics and food preservatives (heat and carvacrol). Five MPM-RVs from SeT (SeT_M$_{1\text{-}5}$) and one from SeE (SeE_M$_5$) showed a 100 % increase in MPM minimum inhibitory concentrations (from 0.0312 to 0.0625 μg/mL). MPM-RVs had lower maximum growth rates and/or longer lag times, except in SeT_M$_5$ and SeE_M$_3$, which maintained or even improved growth fitness in the presence of MPM. Whole genome sequencing of RVs revealed single mutations in AMR-related and -unrelated genes in most MPM-RVs: *spoT* in SeT_M$_1$, *glnA* in SeT_M$_2$, *thrS* in SeT_M$_4$ and SeE_M$_2$, and *mrdA* in SeT_M$_5$, SeE_M$_3$ and SeE_M$_5$. Two mutations were identified in SeT_M$_3$ and SeE_M$_4$: *spoT* and *znuA* in SeT_M$_3$ and AWJ12_RS14025 and *rfbF* in SeE_M$_4$. The individual mutations resulted in cross-protection to at least one of the antibiotics tested and/or heat (reductions of up to 3.8–4.3 cycles of inactivation for MPM-RVs in contrast to 4.7 and 5.3 cycles for SeE and SeT, respectively) and/or carvacrol (3.4–3.7 cycles for MPM-RVs in contrast to 4.4 cycles for SeT). These findings highlight the importance of preventing the emergence of resistant bacterial variants, considering the cross-protection to other antibiotics and food preservatives, which plays an important role in spreading resistance in food chain.

1. Introduction

In 2022 salmonellosis was the second most reported foodborne illness in the European Union (EU), with 65,208 cases. It is estimated that more than 90,000 cases of salmonellosis are reported annually in the EU, which causes an economic burden of around €3 billion a year (European Food Safety Authority, 2023). The Center for Disease Control and Prevention (CDC) estimates *Salmonella* bacteria cause about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States every year (Centers for Disease Control and Prevention, 2024). Among the currently known *Salmonella* serovars, *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Enteritidis are predominant, causing serious public health morbidity and mortality (Nhung et al., 2024; Sandrasaigaran et al., 2023).

Over the past years, there has been a substantial increase in antimicrobial resistance (AMR) in *Salmonella* (Shang et al., 2019; Tang et al., 2022, 2023) followed by an increase in multidrug-resistant (MDR) in food related *Salmonella* strains worldwide (Qin et al., 2023; Xu et al., 2024). The increasing prevalence of MDR *Salmonella* is mostly attributed to the inconsiderate use of antimicrobial compounds for treating *Salmonella* infections (Molina et al., 2024; Shen et al., 2022). MDR represents a serious public health problem, as resistant infections can prolong

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the disease, increase its severity, and reduce the efficacy of treatment, increasing the risk of complications and deaths (Sodagari et al., 2023). Consequently, antibiotics have become less effective to treat severe salmonellosis (Bolkenov et al., 2024; Chaudhari et al., 2023; Gong et al., 2022), highlighting concerns about global spread of resistant *Salmonella* strains (Octavia et al., 2021; Umair et al., 2024).

Carbapenems, such as meropenem (MPM), are β-lactam antibiotics considered last-resort drugs for treating serious infections caused by multidrug-resistant Gram-negative bacteria (Huang et al., 2023; Wang et al., 2020). They act by inhibiting penicillin-binding proteins, disrupting the bacterial cell wall and causing osmotic lysis (Mohammadpour et al., 2025). Although carbapenems are intended exclusively for human use, MPM-resistant bacterial strains have been detected in food-producing animals and environmental samples, raising concerns about their emergence and dissemination via horizontal gene transfer from human-associated sources such as hospital effluents or unauthorized veterinary practices (El-Genedy et al., 2024; Tawyabur et al., 2020). In response to the increasing detection of *Salmonella enterica* strains resistant to MPM, it is unknown whether these resistant variants may exhibit cross-protection to preservation methods widely used in the food industry, thereby compromising microbiological control during food processing and increasing the risk of hard-to-treat human infections due to limited therapeutic options.

Essential oils have gained prominence as promising natural preservatives due to their broad-spectrum antibacterial, antiviral, and antiseptic properties (Rout et al., 2022). Among these compounds, carvacrol (4-isopropyl-2-methylphenol), a phenolic monoterpene derived from p-cymene, has stood out for its expressive biological potential. Carvacrol exerts its antibacterial effect primarily by altering membrane permeability and fluidity, and its incorporation into food products has been shown to reduce or inactivate foodborne pathogens, including *Salmonella* spp., *Escherichia coli*, and *Listeria monocytogenes* (Ashrafudoulla et al., 2024; Cid-Pérez et al., 2024).

However, exposure to antibiotics and to sublethal physical (e.g., cold, heat or radiation) or chemical (e.g., use of acids, salts and oxidants) conditions in food processing, can potentially promote the emergence of resistant variants (RVs). As a result, direct resistance to the selective agent or cross-protection to antibiotics or food preservation methods can be induced (He et al., 2023; Jiao et al., 2021; Rodríguez-Melcón et al., 2023). Furthermore, exposure to subinhibitory concentrations (sub--MICs) of antibiotics has been associated with the induction of biofilm formation in several microorganisms, including *Salmonella enterica* (Yuan et al., 2023a, 2023b).

Adaptive laboratory evolution (ALE), a technique used to study the evolution of organisms in response to selective pressures over time, may provide a deep understanding of the genetic mechanisms and processes involved in bacterial adaptation (Lopatkin et al., 2021; Ghoshal et al., 2023). Through ALE, bacteria are subjected to increasingly greater selective pressures over multiple exposures, allowing them to adapt and evolve in response to stress.

Resistant variants (RVs) of *S.* Typhimurium isolated from ALE with carvacrol (Berdejo et al., 2020), antibiotics (amoxicillin and colistin) (Merino et al., 2023) (ciprofloxacin) (Campillo et al., 2025), and plasma-activated water (Pagán et al., 2024a), as well as *S.* Enteritidis RVs isolated from ALE with acetic acid demonstrated increased resistance to the agent used in addition to presenting cross-protection to other antimicrobials and to different food preservation methods (Berdejo et al., 2021a). Therefore, ALE assays help to identify the potential likelihood of RVs emerging after exposure to stresses. Furthermore, associated to whole genome sequencing ALE is useful to understand the genetic mechanisms conferring resistance against antibiotics and/or food preservation methods (Ghoshal et al., 2024; Campillo et al., 2025).

Thus, the objectives of this study were (a) to isolate meropenem-resistant variants (MPM-RVs) of *Salmonella enterica* subsp. *enterica* Typhimurium and *Salmonella enterica* subsp. *enterica* Enteritidis; (b) to identify the genetic modifications associated with the increased resistance of these MPM-RVs; (c) to assess the cross-resistance of the isolated variants against other antibiotics; and (d) to assess the cross-protection against food preservative methods (heat and carvacrol).

2. Material and methods

2.1. Microorganism and growth conditions

Parental strains, *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (SeT) (Spanish Type Culture Collection 722) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 (SeE). Strains were maintained in cryovials at −80 °C with glycerol (20 % v/v), from which tryptone soy agar plates (TSA; Oxoid, Basingstoke, UK) with 0.6 % yeast extract (TSAYE; Oxoid) were prepared before beginning of the study. Subsequently, for working bacterial cultures, a colony was inoculated into 5 mL of Mueller Hinton broth (MHB; Sigma-Aldrich, Saint Louis, USA) and aerobically incubated for 14 h at 130 rpm (Heidolph Vibramax 100, Schwaback, Germany) at 37 °C (Incubig, Selecta, Barcelona, Spain). 10 μL of the subculture was inoculated in 10 mL of fresh MHB (initial concentration of 10^6 colonies forming units per mL (CFU/mL)) and incubated for 24 h at 37 °C and 130 rpm until reaching the stationary growth rate (10^9 CFU/mL approximately).

The same protocol was conducted to obtain the working bacterial cultures of the isolated strains derived from the evolution ALE assays with meropenem (MPM; Sigma-Aldrich, Steinheim, Westphalia, Germany).

2.2. Minimum inhibitory concentration (MIC)

MIC determination of MPM against SeT and SeE were determined according to CLSI standards (Clinical and Laboratory Standards Institute, 2020). MPM stock was serially diluted 2-fold in 96-well microtiter plates (BD Falcon, San Jose, CA, USA), containing fresh MHB (final volume of 100 μL per well), followed by inoculation with the test strains at 10^5 CFU/mL. The MPM concentrations tested ranged from 0.015 to 32 μg/mL. The microplates were incubated for 20 h at 37 °C. To determine bacterial growth, optical density was read at 595 nm (OD_{595}) using a microplate reader (Genios, Tecan, Mannedorf, Switzerland). MICs were stablished as the lowest concentrations of antibiotics capable of inhibiting growth. Clinical breakpoints established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were employed as criteria to determine whether increases in MIC could indicate a greater likelihood of treatment failure, resulting in the categorization of these variants as resistant (RVs) (European Committee on Antimicrobial Susceptibility Testing, 2023). As recommended by the CLSI guidelines, quality control testing has been performed using the reference strain *Escherichia coli* ATCC 25922.

2.3. Antibiotic laboratory evolution (ALE) assays

ALE assay was adapted from Berdejo et al. (2021b) and the classical approach from Lopatkin et al. (2021). To carry out the evolution assay, an overnight subculture was diluted 1:1000 in 50 mL of growth broth and incubated for 3.5 h at 37 °C and 130 rpm to obtain an exponential phase culture (10^7 CFU/mL). From that culture, 0.5 mL was inoculated into 4.5 mL of MHB, starting with the presence of 0.085 × MIC to MPM (day 1). This bacterial suspension was incubated 24 h/37 °C/130 rpm, and, once stationary phase was reached, OD_{595} was measured and the same procedure was repeated 10 times: the suspension resulting from the previous day was diluted 1:1000 in 5 mL of MHB with the corresponding dose of antibiotic and incubated (24 h/37 °C/130 rpm) applying an ascending gradient of MPM. The concentration was increased daily in fixed increments of 85 %, starting at 0.085 × the minimum inhibitory concentration on day 1, reaching 1 × MIC on day 5, and ending at 40 × MIC on day 11, consistent with previous reports

(Lopatkin et al., 2021; Campillo et al., 2025). The evolution assay was performed five times in parallel (5 lineages) to address different evolution outcomes. From the 5th day of exposure corresponding to the MIC, an aliquot of all strains was stored in cryovials at −80 °C with glycerol (20 % v/v). Strain isolation from the parental strains control lines were performed after 11th exposure to fresh MHB. Afterwards, the MIC for MPM of the selected evolved strains was evaluated and compared to those of SeT and SeE to verify any increase in direct resistance.

2.4. Growth curves of Salmonella Typhimurium and Salmonella Enteritidis meropenem-variants

SeT, SeE and their MPM-RVs were evaluated for growth in MHB and MHB with MPM (1/2 × MIC). Each strain was cultivated as described previously. 96-well microtiter plates (BD Falcon, San Jose, CA, USA) were inoculated at an initial concentration of 10^5 CFU/mL in MHB and, MHB with MPM. The plate was prepared with triplicate wells for each isolate and a medium without inoculum as a control (blank). Using a ClarioStar plate reader (BMG Labtech, Ortenberg, Germany), the plate was incubated at 37 °C with OD_{595} nm readings taken every 15 min. Each growth trial was performed in triplicate carried out on different working days. The values of OD_{595} at time zero were subtracted. Bacterial growth curves based on OD_{595} of SeT, SeE and their MPM-RVs were graphically displayed and modeled by modified Gompertz equation (Zwietering et al., 1990):

$$y = A\exp\{-exp[(\mu_m e/A)\ (\lambda - t) + 1] \quad (1)$$

where y: OD_{595}; t: time (h); A: maximum value reached (OD_{595} max); μ_m: maximum growth rate (h^{-1}); λ: lag time (h).

A least-squares adjustment was carried out to build the model and to obtain A, μ_m, and λ values using the GraphPrism® program (GraphPad Software Inc., San Diego, USA). The adjustment's goodness of fit was evaluated using standard error, R^2, and R^2 adjusted values, as well as the root mean square error (RMSE) (Table S1).

2.5. Whole genome sequencing (WGS) and identification of mutations

Total genomic DNA (gDNA) from the parental strains (SeT and SeE) and the MPM-variants (evolved strains) 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. Preliminary bioinformatic analyses were conducted by the sequencing company, including trimming adapters (Trimmomatic v0.36) and low-quality bases. The resulting delivered files had ≥80 % of bases with a Phred quality score of ≥ Q30. The sequencing company also provided variant calling results in VCF (Variant Call Format) files, generated using VarScan2 (Koboldt et al., 2012). The provided VCF files were used for variant annotation using SnpEff (Cingolani et al., 2012) via the UseGalaxy platform (The Galaxy Community, 2024), after creating customized databases with the reference genomes *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (National Center for Biotechnology Information; NCBI accession: NC_003197.2) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 (NCBI accession: GCF_001643395.1). Individual SNVs (Single Nucleotide Variations) and InDels (insertions and deletions ≤50 bp) were visually validated and coverage was further analyzed using the Integrative Genomics Viewer (IGV; Broad Institute, source: https://software.broadinstitute.org/software/igv/).

Tables S2 and S3 summarize the genetic divergences found between the parental strains (SeT and SeE, respectively) and their respective reference genomes. Genetic divergences were classified into types of SNV (Single Nucleotide Variations) and InDels (insertions and deletions ≤50 bp). Highlighting the importance of performing periodic resequencing of the genome of microbial strains stored and used in the laboratory, especially in experimental evolution studies, in order to monitor possible spontaneous mutations that may compromise the reproducibility and interpretation of the results. Table S4 lists the mutations detected in parental strain controls, after cyclic exposures to fresh MHB (SeT_CTRL$_{1\text{-}5}$ or SeE_CTRL$_{1\text{-}5}$) (Table S4). The resulting genome sequences were deposited in the Sequence Read Archive (SRA) of NCBI (Bioproject ID: PRJNA1199427 and PRJNA1199546 for *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 and *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, respectively). The accession numbers of the samples are SAMN45874018 (SeT), SAMN45874019 (SeT_M$_1$), SAMN45874020 (SeT_M$_2$), SAMN45874021 (SeT_M$_3$), SAMN45874022 (SeT_M$_4$), SAMN45874023 (SeT_M$_5$), SAMN45877886 (SeE), SAMN45877888 (SeE_M$_2$), SAMN45877889 (SeE_M$_3$), SAMN45877890 (SeE_M$_4$), and SAMN45877891 (SeE_M$_5$).

2.6. Assessment of cross-resistance to different antibiotics

The MPM-RVs of *Salmonella* Typhimurium str. LT2 and *S.* Enteritidis ATCC 13076 were tested for their susceptibility to antibiotics by Vitek 2 compact (bioMérieux, France) using a GN cassette AST-GN96 card according to the manufacturer's instructions. According to the Vitek 2 Compact system's specialized software, results were interpreted following the manufacturer's instructions. Following the CLSI criteria (CLSI, 2020), the susceptibility data were analyzed and categorized into Resistant (R), Intermediate resistant (I) and Susceptible (S). A total of 15 different antibiotics (ampicillin, amoxicillin/clavulanic acid, cephalothin, cefoperazone, ceftiofur, cefquinome, imipenem, gentamicin, neomycin, flumequine, enrofloxacin, marbofloxacin, tetracycline, florfenicol, and trimethoprim/sulfamethoxazole) were tested.

2.7. Assessment of cross-tolerance to heat and carvacrol

To apply heat treatments, citrate-phosphate buffer adjusted to pH 7.0 (McIlvaine buffer) was used as treatment medium. The buffer solution was elaborated from the combination of citric acid monohydrate (PanReac-AppliChem, Darmstadt, Germany) and disodium hydrogen phosphate (PanReac AppliChem). Initially, cells from stationary phase cultures were centrifuged (MiniSpin, Eppendorf, Hamburg, Germany) at 10000 RCF for 3 min, harvested and resuspended in McIlvaine buffer. 60 μL of the resulting resuspended cells (10^9 CFU/mL approximately) were transferred to sterile PCR tubes and exposed to heat in a thermocycler (T100 Thermal Cycler, Bio-rad, Spain) at 54 °C for 30 min (SeT and the evolved strains) or 20 min (SeE and the evolved strains). The heat treatment conditions were selected based on preliminary experiments (data not shown).

For carvacrol (CAR) (95 %; Sigma-Aldrich, Steinheim, Westphalia, Germany) exposure, tryptone soya broth (TSB; Oxoid, Basingstoke, UK) with 0.6 % yeast extract (TSBYE) was used such treatment medium. Briefly, cells were centrifuged as described above and resuspended in PBS. Then, cells were inoculated at initial concentration of 10^7 CFU/mL in 10 mL of TSBYE with 200 μL/L of CAR, which corresponds to its MIC (Pagán et al., 2024c). The bottles were mixed by vortexing and incubated at room temperature (25–26 °C) for 30 min. The bottles were mixed every 5 min to ensure uniformity of inactivation.

Before and after heat and CAR treatment, the samples were diluted in PBS, pour plated on TSAYE and incubated at 37 °C for 24 h. After CFUs were quantified by an automatic plate counter (Analytical Measuring Systems, Protos, Cambridge, United Kingdom).

2.8. Statistical analysis

All results were obtained from at least 3 independent experiments carried out on different working days with different bacterial cultures. Data were analyzed and submitted to comparison of averages using

analysis of variance (ANOVA), followed by Tukey's test (for comparing differences among all groups) or Dunnett's test (for comparing each group with the control, i.e., the parental strain) with Prism 8.0 software (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p \leq 0.05$.

2.9. Ethical statement

This study did not involve experiments with humans or animals, and was conducted exclusively with microorganisms on a laboratory scale.

3. Results

3.1. Isolation of meropenem-variants by ALE assays

All the evolution lines obtained after the ALE assays surpassed the MIC dose by the sixth day (5th exposure) (Fig. 1). In general, as exposure increases, microbial growth tends to decrease, due to the more intense selective pressures imposed by increasing doses of MPM. The last days for the isolation of stable MPM-RVs, one strain of each lineage, ranged from day 6 to day 8 of exposure.

The resistance of SeT_$M_{1\text{-}5}$ and SeE_$M_{1\text{-}5}$ against MPM was determined by assaying MIC values (Table 1). The results of the evolved strains were compared with those of SeT or SeE in order to assess increased direct-resistance to MPM.

All MPM-RVs strains from SeT (SeT_$M_{1\text{-}5}$) and one from SeE (SeE_M_5) showed an increase in the MIC to MPM, from 0.0312 μg/mL against SeT and SeE to 0.0625 μg/mL which corresponds to a 2-fold increase in resistance to MPM after ALE. Parental strains controls (SeT_$CTRL_{1\text{-}5}$ and SeE_$CTRL_{1\text{-}5}$), grown in MHB in the absence of MPM, showed no differences in their MIC (0.0312 μg/mL).

3.2. Further evaluation of increased resistance by modeling growth kinetics under meropenem stress

Growth kinetics curves in the absence and presence of MPM (0.015 μg/mL) were conducted and the growth parameters were compared with those of the parental strains (SeT or SeE) to characterize the growth fitness of MPM-RVs (SeT_$M_{1\text{-}5}$ and SeE_$M_{1\text{-}5}$).

Interestingly, SeT_M_2 did not show growth at the assayed conditions when performing the growth curves (Table 2). In the absence of MPM, SeT_$M_{1\text{-}4}$ showed reduced growth rates ($p < 0.0001$–0.0009), ranging from 0.028 to 0.046, and SeT_$M_{1\text{-}3}$ showed a prolonged ($p < 0.0001$) lag time, ranging from 7.124 to 7.979 h, when compared to SeT (3.998 h). In contrast, none of the MPM-RVs of SeE showed differences compared to the parental strain, with growth rates ranging from 0.082 to 0.091 ($p = 0.5395$->0.9999) and a lag time between 4.581 and 5.750 h ($p = 0.0924$–0.9984).

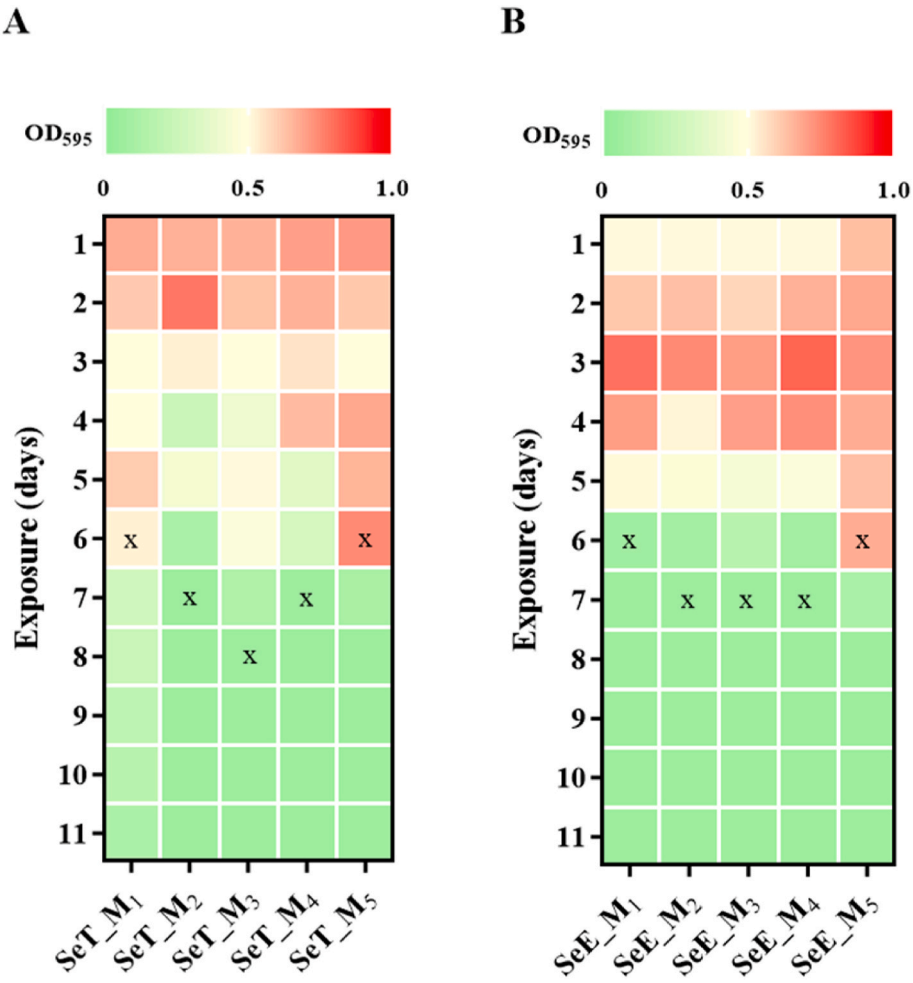

Fig. 1. Evolution assay diagram depicting the OD_{595} after every exposure (day 1–11) of the isolated meropenem (MPM) variants of (A) *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (SeT_M_1, SeT_M_2, SeT_M_3, SeT_M_4 and SeT_M_5) and (B) *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 (SeE_M_1, SeE_M_2, SeE_M_3, SeE_M_4 and SeE_M_5). Color gradient from red to green represents OD_{595} levels from high to low, respectively. Highlighted squares ("x") indicate the further exposition that permitted bacterial growth and isolation in fresh media. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Minimum inhibitory concentrations (μg/mL) of meropenem for *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 and *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 strains.

Strain	Meropenem MIC (μg/mL)
SeT	0.0312
SeT_M$_1$	0.0625
SeT_M$_2$	0.0625*
SeT_M$_3$	0.0625
SeT_M$_4$	0.0625
SeT_M$_5$	0.0625
SeE	0.0312
SeE_M$_1$	0.0312
SeE_M$_2$	0.0312
SeE_M$_3$	0.0312
SeE_M$_4$	0.0312
SeE_M$_5$	0.0625

[*Result obtained after 48 h of incubation, as this specific variant had a more expressive lag phase than the others. Results expressed as the mode of 3 independent replicates. *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 parental strain (SeT) and its variants derived from the evolution assays with meropenem: SeT_M$_1$, SeT_M$_2$, SeT_M$_3$, SeT_M$_4$ and SeT_M$_5$. *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 parental strain (SeE) and its variants derived from the evolution assays with meropenem: SeE_M$_1$, SeE_M$_2$, SeE_M$_3$, SeE_M$_4$ and SeE_M$_5$. MIC - Minimum inhibitory concentrations].

In the presence of 0.015 μg/mL of MPM (Table 2), SeT_M$_1$ and SeT_M$_3$ exhibited lower ($p < 0.0001$) maximum growth rates (0.026 and 0.011 OD_{595}/h, respectively) and shorter ($p < 0.0001$–0.0007) lag time (approximately 6.4 h) compared to SeT. SeT_M$_5$ did not differ ($p = 0.9999$) from SeT in terms of maximum growth rate, but it showed the shortest ($p < 0.0001$) lag time (4.008 h) among all strains, differing ($p < 0.0001$) from SeT.

Regarding MPM-RVs of SeE in the presence of 0.015 μg/mL of MPM, SeE_M$_3$ was the only one whose maximum growth rate differed ($p = 0.0114$) from SeE (0.136 instead of 0.076 OD_{595}/h). SeE_M$_2$ and SeE_M$_4$ showed longer lag time (6.198 and 5.739 h, respectively) compared to SeE (4.633 h) ($p = 0.0033$–0.0323) (Table 2).

3.3. Detection of genetic variations in evolved strains

In order to identify genetic variations associated with increased protection in evolved strains to MPM, as well as to other antibiotics, heat and carvacrol, WGS was performed on the parental strains (SeT and SeE), their respective MPM-RVs and parental strains controls (SeT_CTRL$_{1\text{-}5}$ and SeE_CTRL$_{1\text{-}5}$). The genetic variations between the reference genome and those of the parental strains were previously identified to be excluded as possible causes of resistance in MPM-RVs (Tables S2 and S3). Finally, the mutations present in the parental strain controls, after cyclic exposures to fresh MHB, were identified and discarded (Table S4). Among the parental strain controls in *S.* Typhimurium (SeT_CTRL$_{1\text{-}5}$), only two mutations were identified: a deletion in the *rfbI* gene and a single nucleotide mutation (SNV) in the *rfbV* gene, in SeT_CTRL$_1$ and SeT_CTRL$_5$, respectively. For the parental strain controls of *S.* Enteritidis, three mutations were detected by WGS: a deletion of a nucleotide at two points in the DNA chain (AWJ12_16805 and AWJ12_18500) in SeE_CTRL$_1$ and, a stop-gain SNV in the *rfbG* gene in SeE_CTRL$_5$ (Table S4). Consistent with standard methodological practices, these mutations were classified as not induced by antibiotic exposure (Campillo et al., 2025; Hernando-Amado et al., 2022).

Table 2
Growth parameters of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (SeT) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 (SeE) and their meropenem-variants. Growth curves were measured for 24 h at 37 °C in Mueller Hinton Broth (MHB) or Mueller Hinton Broth with ½ x MIC of meropenem (0.015 μg/mL). μ_m (maximum growth rate; OD_{600}/h) and λ (lag time; h) parameters of the modified Gompertz model.

Growth medium	Strain	Parameters	
		μ_m (OD_{595}/h)	λ (h)
MHB	SeT	0.056 ± 0.001^{a}	3.998 ± 0.260^{a}
	SeT_M$_1$	0.033 ± 0.002^{bA}	7.124 ± 0.476^{bA}
	SeT_M$_2$	#	#
	SeT_M$_3$	0.028 ± 0.000^{bA}	7.979 ± 0.848^{bA}
	SeT_M$_4$	0.046 ± 0.002^{bC}	5.005 ± 0.478^{aC}
	SeT_M$_5$	0.058 ± 0.005^{aD}	4.226 ± 0.436^{aC}
	SeE	0.091 ± 0.011^{a}	4.877 ± 0.519^{a}
	SeE_M$_1$	0.091 ± 0.010^{aA}	4.581 ± 0.311^{aA}
	SeE_M$_2$	0.082 ± 0.008^{aA}	5.750 ± 0.180^{aB}
	SeE_M$_3$	0.090 ± 0.011^{aA}	4.739 ± 0.504^{aAB}
	SeE_M$_4$	0.091 ± 0.003^{aA}	4.781 ± 0.485^{aAB}
	SeE_M$_5$	0.088 ± 0.004^{aA}	4.597 ± 0.413^{aA}
MHB with meropenem	SeT	0.062 ± 0.001^{a}	9.541 ± 0.715^{a}
	SeT_M$_1$	0.026 ± 0.006^{bA}	6.495 ± 0.780^{bA}
	SeT_M$_2$	#	#
	SeT_M$_3$	0.011 ± 0.002^{bB}	6.436 ± 0.939^{bA}
	SeT_M$_4$	0.068 ± 0.004^{aC}	8.230 ± 0.759^{aA}
	SeT_M$_5$	0.063 ± 0.008^{aC}	4.008 ± 0.567^{bB}
	SeE	0.076 ± 0.016^{a}	4.633 ± 0.412^{a}
	SeE_M$_1$	0.121 ± 0.025^{aA}	4.781 ± 0.340^{aA}
	SeE_M$_2$	0.101 ± 0.021^{aA}	6.198 ± 0.247^{bB}
	SeE_M$_3$	0.136 ± 0.020^{bA}	4.913 ± 0.556^{aA}
	SeE_M$_4$	0.123 ± 0.025^{aA}	5.739 ± 0.668^{bAB}
	SeE_M$_5$	0.109 ± 0.003^{aA}	5.118 ± 0.085^{aAB}

[#*Salmonella* Typhimurium v*ariant* derived from the evolution assays with meropenem no *showed growth.*
Salmonella enterica subsp. *enterica* serovar Typhimurium str. LT2 parental strain (SeT) and its variants derived from the evolution assays with meropenem: SeT_M$_1$, SeT_M$_2$, SeT_M$_3$, SeT_M$_4$ and SeT_M$_5$. *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 parental strain (SeE) and its variants derived from the evolution assays with meropenem: SeE_M$_1$, SeE_M$_2$, SeE_M$_3$, SeE_M$_4$ and SeE_M$_5$. MIC - Minimum inhibitory concentrations. Data is shown as mean ± standard deviations obtained from at least three independent experiments. Letters *a-b* show statistically similar and different values ($p \leq 0.05$) against SeT or SeE. Letter *A-D* show statistically similar and different values ($p \leq 0.05$) between meropenem-variants within the same serovar].

Then, we performed a comparative analysis of the genomes of the parental strains and their MPM-RVs (Fig. 2), aiming to identify genetic variations and thus determine the genes involved in resistance. Tables 3 and 4 present a comprehensive overview of all the genetic variations observed between parental strains and MPM-RVs, including alterations in genes previously implicated in antibacterial resistance and tolerance (e.g., *spoT*, *thrS*, *mrdA*). Additionally, we identified modifications in the *znuA* gene, whose association with antimicrobial resistance or tolerance in *Salmonella* still lacks complete elucidation, suggesting a potential new mechanism to be investigated.

Genomic comparison of the strains revealed at least one type of mutation among the SeT and MPM-RVs (Table 3). Among the common SNVs, the following changes were found: a common SNV (a valine by a glycine) was observed in *spoT* gene in SeT_M$_1$ and SeT_M$_3$; a replacement of tryptophan by an arginine in *glnA* gene in SeT_M$_2$; a substitution of tryptophan by cysteine in the *thrS* gene in SeT_M$_4$; and a replacement of lysine by glutamine in the *mrdA* gene in SeT_M$_5$ (Table 3). Moreover, a second mutation was observed in SeT_M$_3$: the insertion of one nucleotide in the *znuA* gene.

As detailed in Table 4, the MPM-RVs from SeE exhibited up to five SNVs and InDels in their genomes. WGS of SeE_M1 revealed no genetic alterations or evidence of resistance, leading to its exclusion from further analyses. On the other hand, a substitution of lysine by glutamic acid in the *thrS* gene was observed in SeE_M$_2$; a replacement of alanine by threonine in the *mrdA* gene was identified in SeE_M$_3$; two mutations in the same gene, *rfbF*, were detected in SeE_M$_4$: an insertion of one nucleotide and a substitution of glycine by aspartic acid; and finally, a glycine to aspartic acid substitution in the *mrdA* gene was also found in SeE_M$_5$.

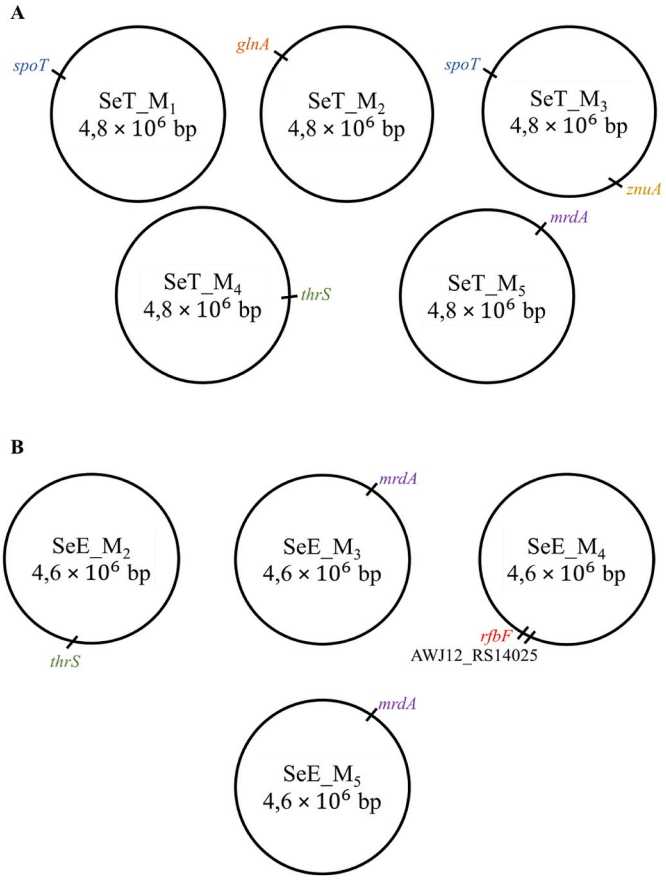

Fig. 2. Genomic maps (A) of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 meropenem-variants (SeT_M$_1$-SeT_M$_5$) or (B) of *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 meropenem-variants (SeE_M$_2$-SeE_M$_5$).

3.4. Cross-resistance to antibiotics

Table 5 presents the antibiotic susceptibility profile of MPM-RVs from SeT. Compared to the parental strain, the evolved strains show cross-protection to other beta-lactams: SeT_M$_3$ showed at least two-fold increase in resistance to cephalothin (MIC = 4 μg/mL), SeT_M$_1$, SeT_M$_3$ and SeT_M$_5$ exhibited at least a two-fold increase in MIC (MIC = 0.5 μg/mL), and SeT_M$_4$ a four-fold increase to imipenem (MIC = 1 μg/mL), compared to the MIC for this antibiotic for SeT (MIC ≤0.25 μg/mL). On the other hand, it is notable the increased resistance to florfenicol of SeT_M$_4$ and SeT_M$_5$ (MIC = 8 μg/mL) compared to SeT (MIC = 4 μg/mL), thus reaching an intermediate resistance level.

Table 6 presents the antibiotic susceptibility profile of MPM-RVs from *S.* Enteritidis. SeE_M$_3$ and SeE_M$_5$ exhibited a 2-fold increase in MIC for imipenem (MIC = 1 μg/mL) compared to SeE (MIC = 0.5 μg/mL). Unexpectedly, SeE_M$_2$ variant showed a 50 % reduction in MIC for cephalothin and imipenem compared to SeE. Similarly, a 50 % decrease in MIC for florfenicol was observed in this variant, which, unlike SeE, which showed intermediate resistance, was sensitive to this antibiotic, with an MIC of 4 μg/mL.

3.5. Cross-tolerance to heat and carvacrol

Cross-tolerance of MPM-RVs was evaluated against heat and carvacrol. Fig. 3 shows the $\log_{10}$ cycles of inactivation after heat exposure (54 °C/30 min for SeT or 54 °C/20 min for SeE) for the parental strains and their respective MPM-RVs in McIlvaine buffer at pH 7.0.

For SeT MPM-RVs (Fig. 3A), the SeT_M$_{1\text{-}4}$ showed higher thermotolerance, with reductions up to 4.3 cycles of initial population after exposure to heat at 54 °C for 30 min, differing (p = 0.0003–0.0050) from SeT (5.3 cycles of inactivation). The inactivation of initial populations of SeT_M$_5$ reached around 5.3 cycles, which did not differ (p = 0.9997) from SeT. In contrast, only SeE_M$_2$ and SeE_M$_4$ showed higher tolerance (p = 0.0033–0.0102) in comparison with SeE (Fig. 3B), with an inactivation of about 3.8 cycles of these MPM-RVs (Fig. 3B) in contrast to 4.7 cycles for SeE (Fig. 3B).

Fig. 4 shows the $\log_{10}$ cycles of inactivation ($\log_{10}$ N_t/N_0) after exposure to carvacrol (200 μL/L/30 min) for the parental strains (SeT and SeE) and their respective MPM-RVs in MHB. SeT_M$_1$, SeT_M$_2$ and SeT_M$_3$ showed higher tolerance (p = 0.0011–0.0162) to carvacrol (Fig. 4A): the inactivation of initial populations for these MPM-RVs were 3.4, 3.5 and 3.7 cycles, respectively, while for SeT the inactivation was up to 4.4 cycles of the initial population (Fig. 4A). On the other hand, although SeE_M$_2$ and SeE_M$_4$ showed higher resistance compared to heat, none of the MPM-RVs of SeE (SeE_M$_{2\text{-}5}$) showed tolerance (p = 0.1256–0.7798) to carvacrol (Fig. 4B).

4. Discussion

The exposure of microorganisms to selective pressures, such as the use of antibiotics, or the use of natural antimicrobials, such as essential oils, can favor the emergence of genetic variants with increased protection to these compounds (Berdejo et al., 2020; Merino et al., 2023; Pagán et al., 2024a). In this work, we describe for the first time the isolation of two *S. enterica* MPM -resistant variants, through MPM ALE assays. We assessed the increase in direct resistance in the isolated derived strains by determining MICs, comparing them with the values obtained for SeT and SeE (Table 1). The MICs obtained for SeT and SeE are in agreement with the international MIC distribution range for *Salmonella enterica*, as reported by EUCAST (0.03–0.06 μg/mL) (EUCAST, 2023). As a result, all SeT and one SeE evolved isolates showed a 100 % increase in resistance after MPM exposure (from 0.0312 to 0.0625 μg/mL). Although these strains are still classified as susceptible to meropenem according to CLSI (CLSI, 2020) criteria (MIC ≤1 μg/mL), the

Table 3
Genetic variations detected via whole-genome sequencing between the reference genome of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (NCBI accession: NC_003197.2) and the meropenem variants (SeT_M$_1$-SeT_M$_5$) derived from the evolution assays.

Strain	Genome position	Mutation type	Change*	Locus tag	Gene	Functional category**	Description
SeT_M$_1$	3,935,085	SNV	T1004G → Val335Gly	STM3742	*spoT*	Metabolism	(p)ppGpp synthetase II
SeT_M$_2$	4,216,547	SNV	T475A → Trp159Arg	STM4007	*glnA*	Metabolism	Glutamine synthetase
SeT_M$_3$	1,986,717	Frameshift Ins	CC 696	STM1891	*znuA*	Environmental information processing	High-affinity zinc uptake system protein
	3,935,085	SNV	T1004G → Val335Gly	STM3742	*spoT*	Metabolism	(p)ppGpp synthetase II
SeT_M$_4$	1,412,810	SNV	G618C → Trp206Cys	STM1333	*thrS*	Genetic information processing	Threonine–tRNA ligase
SeT_M$_5$	702,214	SNV	A1714C → Lys572Gln	STM0640	*mrdA*	Metabolism	Peptidoglycan D, D-transpeptidase

[Single nucleotide variation (SNV) and insertion (Ins). Genetic variations between the parental strain and the reference genome were omitted. *Position relative to the start of the coding region in the case of nucleotide changes and relative to the position in the protein in the case of amino acid changes]. **According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology.

Table 4
Genetic variations detected via whole-genome sequencing between the reference genome of *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 (NCBI accession: GCF_001643395.1) and the meropenem variants (SeE_M$_1$-SeE_M$_5$) derived from the evolution assays.

Strain	GenBank	Genome position	Mutation type	Change*	Locus tag	Gene	Functional category**	Description
SeE_M$_2$	LSHA01000031.1	1,019,961	SNV	A1741G → Lys581Glu	AWJ12_16800	*thrS*	Genetic information processing	Threonine–tRNA ligase
SeE_M$_3$	LSHA01000001.1	374,853	SNV	G1567A → Ala523Thr	AWJ12_01805	*mrdA*	Signalling and celular processes	Peptidoglycan D, D-transpeptidase
SeE_M$_4$	LSHA01000031.1	1,329,704	Frameshift Ins	CA 50	AWJ12_18490	AWJ12_RS14025	Metabolism	Rhamnosyltransferase
	LSHA01000031.1	1,342,693	SNV	G32A → Gly11Asp	AWJ12_18530	*rfbF*	Metabolism	Glucose-1-phosphate cytidylyltransferase
SeE_M$_5$	LSHA01000001.1	374,618	SNV	G1802A → Gly601Asp	AWJ12_01805	*mrdA*	Metabolism	Peptidoglycan D, D-transpeptidase

[Single nucleotide variation (SNV) and insertion (Ins). Genetic variations between the parental strain and the reference genome were omitted. *Position relative to the start of the coding region in the case of nucleotide changes and relative to the position in the protein in the case of amino acid changes]. **According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology.

Table 5
Minimum inhibitory concentrations (μg/mL) of antibiotics for *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 meropenem-variants.

Antibiotic	Strain											
	SeT		SeT_M$_1$		SeT_M$_2$		SeT_M$_3$		SeT_M$_4$		SeT_M$_5$	
	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int
Ampicillin	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S
Amoxicillin-clavulanic acid	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S
Cephalothin	≤2	R	2	R	2	R	**4**	**R**	≤2	R	≤2	R
Cefoperazone	≤4	S	≤4	S	≤4	S	≤4	S	≤4	S	≤4	S
Ceftiofur	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S
Cefquinome	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S
Imipenem	≤0.25	S	**0.5**	**S**	≤0.25	S	**0.5**	**S**	**1**	**S**	**0.5**	**S**
Gentamicin	≤1	R	≤1	R	≤1	R	≤1	R	≤1	R	≤1	R
Neomycin	≤2	S	≤2	S	≤2	S	TRM	TRM	≤2	S	≤2	S
Flumequine	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S
Enrofloxacin	≤0.12	S	≤0.12	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
Tetracycline	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S
Florfenicol	4	S	**2**	**S**	4	S	**2**	**S**	**8**	**I**	**8**	**I**
Trimethoprim-Sulfamethoxazole	≤20	S	≤20	S	≤20	S	≤20	S	≤20	S	≤20	S

[Data in **bold type** represents difference in relation to *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 parental strain (SeT). Variants derived from the evolution assays with meropenem: SeT_M$_1$, SeT_M$_2$, SeT_M$_3$, SeT_M$_4$ and SeT_M$_5$. TRM- Terminated results (Insufficient growth in the positive control well). MIC - Minimum inhibitory concentrations. Int - Interpretation of susceptibility (R - Resistant, I - Intermediate, S – Sensitive) according to the standards established by the Clinical and Laboratory Standards Institute].

observed increase may reflect an adaptive response of the bacteria to the induced stress. These findings are relevant because subclinical variations in MIC may precede the development of stable resistance and represent a warning of the risk of selection of less susceptible subpopulations in environments subjected to subinhibitory selective pressures. Merino et al. (2023) also observed an increase in the MIC value for *S*. Typhimurium RVs after 10 and 20 cycles of antibiotic exposure at constant concentration by ALE. RVs exposed to amoxicillin (1/2 MIC) increased their MIC by 100 % (from 1 μL/L to 2 μL/L), while RVs exposed to colistin (1/2 MIC) had a 200 % (from 2 μL/L to 8 μL/L) increase in MIC compared to parental strain. Research has also shown the emergence of *Salmonella* RVs to other antimicrobials such as essential oils and chemical agents. For instance, Berdejo et al. (2021a) reported, after the evolution assay, an increase of more than 100 % in *T. capitata*

Table 6
Minimum inhibitory concentrations (μg/mL) of antibiotics for *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 meropenem-variants.

Antibiotic	Strain									
	SeE		SeE_M$_2$		SeE_M$_3$		SeE_M$_4$		SeE_M$_5$	
	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int
Ampicillin	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S
Amoxicillin-clavulanic acid	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S
Cephalothin	4	R	**≤ 2**	**R**	4	R	4	R	4	R
Cefoperazone	≤4	S	≤4	S	≤4	S	≤4	S	≤4	S
Ceftiofur	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S
Cefquinome	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S
Imipenem	0.5	S	**≤ 0.25**	**S**	**1**	**S**	0.5	S	**1**	**S**
Gentamicin	≤1	R	≤1	R	≤1	R	≤1	R	≤1	R
Neomycin	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S
Flumequine	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S
Enrofloxacin	≤0.12	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
Tetracycline	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S
Florfenicol	8	I	**4**	**S**	8	I	8	I	8	I
Trimethoprim-Sulfamethoxazole	≤20	S	≤20	S	≤20	S	≤20	S	≤20	S

[Data in **bold type** represents difference in relation to *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 parental strain (SeE). Variants derived from the evolution assays with meropenem: SeE_M$_1$, SeE_M$_2$, SeE_M$_3$, SeE_M$_4$ and SeE_M$_5$. MIC - Minimum inhibitory concentrations. Int - Interpretation of susceptibility (R – Resistant, I - Intermediate, S – Sensitive) according to the standards established by the Clinical and Laboratory Standards Institute].

essential oil resistance in *S*. Typhimurium RVs (600 μL/L), while the parental strain was inhibited at 250 μL/L; similarly, Berdejo et al. (2020) and Pagán et al. (2024b) showed a 50–100 % increase in carvacrol resistance in *S*. Typhimurium RVs compared to the parental strain; and, Ghoshal et al. (2023) highlighted an increase of up to 30 % in acetic acid resistance in *S*. Enteritidis RVs in comparison with the parental strain.

In addition, MPM-RVs exhibited different growth characteristics; in general, the acquisition of MPM-resistance resulted in a decrease in the maximum growth rate and/or an increase in the lag time overall for MPM-RVs (Table 2). Antibiotic resistance mutations are widely studied and often associated with fitness costs, as they frequently affect critical cellular functions (Melnyk et al., 2015). However, the behavior of SeT_M$_5$ in the presence of MPM is remarkable, as it showed an improvement in growth fitness in the presence of MPM in comparison to the parental strain: while maintaining the maximum growth rate, the lag phase was reduced by more than half. These results show that this new RV might be more competitive than the parental strain when growing in the presence of MPM. Similarly, SeE_M$_3$ showed a better growth fitness in the presence of meropenem since the maximum growth rate was higher ($p \leq 0.05$) than that of the parental strain. Similar results have been previously observed by Pagán et al. (2024b) with a SeT carvacrol-RV, which showed better growth fitness than the parental strain when growing at refrigeration temperatures (8 °C) while retaining its virulence.

This study also describes for the first time that MPM-RVs evolved to a cross-protection against other antibiotics (Tables 5 and 6) and food preservatives (heat (Fig. 3) and carvacrol (Fig. 4)). These findings confirm the induction of cross-protection by exposure to antibiotics in *Salmonella*, enhancing the antibiotic resistance and tolerance to food preservation methods. Similar studies corroborate this observation, Pagán et al. (2024a) reported that *S*. Typhimurium RVs, isolated after lethal treatment with plasma-activated water, also showed cross-tolerance to one or more disinfectants widely used in the food industry, including a chlorine-based solution (sodium hypochlorite), an oxidative compound (peracetic acid), or an individual compound from essential oils (carvacrol). Similarly, Campillo et al. (2025) isolated *S*. Typhimurium RVs exposed to ciprofloxacin (CIP) and observed that two of them exhibited reduced thermal sensitivity in laboratory culture medium when compared to the parental strain.

To ascertain which genetic variations were associated with the evolved strains under the presence of MPM and were responsible for the cross-protection phenomena, we conducted WGS on SeT, SeE and MPM-RVs (Fig. 2). Our results indicated that, unlike previous studies (Berdejo et al., 2020; Pagán et al., 2024a, 2024b) which identified multiple mutations in the evolved strains, in this study only one mutation was observed in most of the evolved strains, with the exception of SeT_M$_3$ and SeE_M$_4$, which showed two different mutations in *spoT* and *znuA* gene and, rhamnosyltransferase (AWJ12_RS14025) and *rfbF* gene, respectively. These results suggest that, in most of our identified RVs, the increase in MPM resistance and the observed cross-protection may specifically be associated with a specific mutation.

SeT_M$_1$ harbors an interesting mutation in an AMR-related gene, *spoT* (Fig. 2A), which encodes an enzyme involved in the synthesis and degradation of the alarmone guanosine tetraphosphate (ppGpp). The stringent response signaled by this molecule is one of the most well-known molecular mechanisms involved in antibacterial tolerance and persistence (Pacios et al., 2020). Thus, this mutation points out the possible role of ppGpp in the cross-protection in SeT_M$_1$ against imipenem (Table 5), heat (Fig. 3A) and carvacrol (Fig. 4A). Given that carvacrol induces oxidative stress, mutations or alterations in *spoT* could enhance the bacterial ability to mitigate reactive oxygen species, thereby contributing to a more robust survival response. This suggests that *spoT*-mediated regulation may play a central role in coordinating the oxidative stress response during exposure to carvacrol, ultimately facilitating the development of tolerance or resistance to this antimicrobial compound (Chueca et al., 2014). Furthermore, the same SNV was observed in SeT_M$_3$, an isolate which also presented increased resistance to imipenem and cephalothin (Table 5), and tolerance to heat (Fig. 3A) and carvacrol (Fig. 4A), highlighting the relevance of this found mutation. A mutation in this gene has also been previously related to increased resistance to carbapenems in *Escherichia coli* and linked to the stringent response activation (Adler et al., 2016).

SeT_M$_2$ showed a very reduced growth rate (Table 2) and an increased tolerance against heat (Fig. 3A) and carvacrol (Fig. 4B), but without cross-protection against any antibiotic tested (Table 5). This isolate exhibits a mutation in the *glnA* gene (Fig. 2A), which has previously been found to cause the emergence of small colony variants with significantly reduced growth rates (Aurass et al., 2018), and our results are consistent with these findings.

SeT_M3 also displays a polymorphism in *znuA* (Fig. 2A), a gene involved in zinc uptake in *Salmonella* (Fitzsimmons et al., 2018). In this study, the *znuA* mutation appears to directly contribute to the altered resistance phenotype, rather than acting through co-selection mechanisms, which typically involve the physical linkage of metal transport and resistance genes on mobile genetic elements (Berthold-Pluta et al., 2024; Blencowe and Morby, 2003). In addition to the *znuA* mutation,

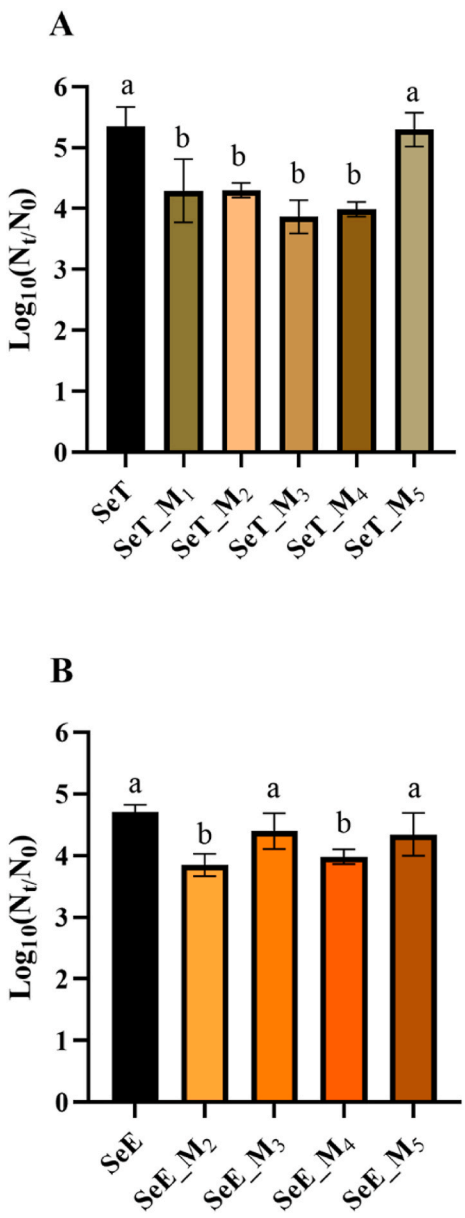

Fig. 3. Log_{10} cycles of inactivation (log_{10} N_t/N_0) after heat exposure (54 °C/30 min; A) *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 parental strain (SeT) and its variants derived from the evolution assays with meropenem: SeT_M_1, SeT_M_2, SeT_M_3, SeT_M_4 and SeT_M_5 or (54 °C/20 min; B) *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 parental strain (SeE) and its variants derived from the evolution assays with meropenem: SeE_M_2, SeE_M_3, SeE_M_4 and SeE_M_5. Data are means ± standard deviations (error bars) obtained from at least three independent experiments. Different lowercase letters in each graph indicate significant differences between mean values ($p \leq 0.05$) compared to parental strains (SeT or SeE, respectively).

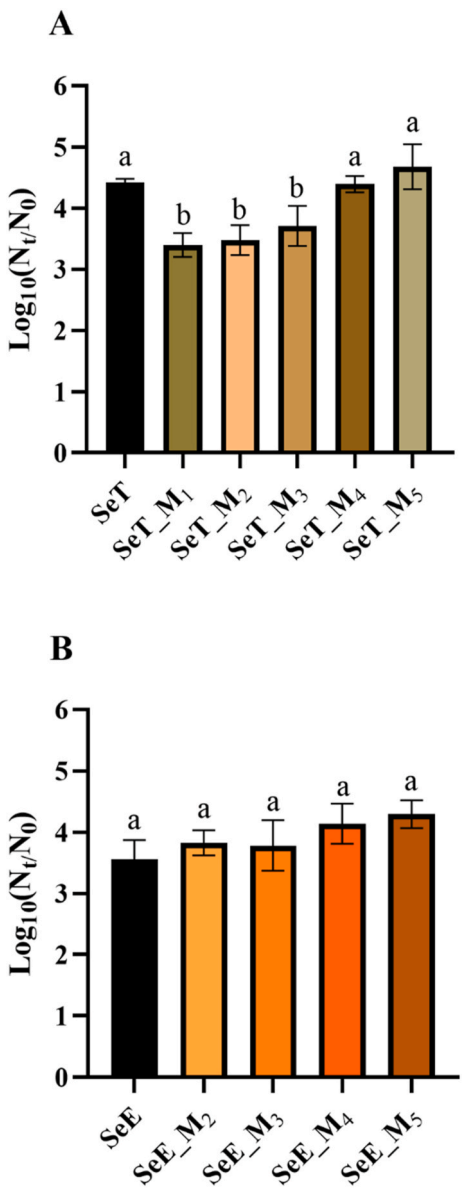

Fig. 4. Log_{10} cycles of inactivation (log_{10} N_t/N_0) after exposure to carvacrol (200 μL/L/30 min). (A) *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 parental strain (SeT) and its variants derived from the evolution assays with meropenem: SeT_M_1, SeT_M_2, SeT_M_3, SeT_M_4 and SeT_M_5. (B) *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 parental strain (SeE) and its variants derived from the evolution assays with meropenem: SeE_M_2, SeE_M_3, SeE_M_4 and SeE_M_5. Data are means ± standard deviations (error bars) obtained from at least three independent experiments. Different lowercase letters in each graph indicate significant differences between mean values ($p \leq 0.05$) compared to parental strains (SeT or SeE, respectively).

SeT_M3 also harbors a polymorphism in *spoT*, a gene implicated in the stringent response and antibiotic tolerance. While both mutations may independently influence resistance, we hypothesize that *znuA* might modulate or enhance the resistance effects conferred by the *spoT* mutation, possibly through changes in intracellular zinc homeostasis affecting stress response pathways. SeT_M_4, on the other hand, showed a mutation in a gene associated with protein synthesis (*thrS*) (Fig. 2A). The *thrS* gene encodes the enzyme threonyl-tRNA synthetase (ThrRS), a member of the aminoacyl-tRNA synthetase (aaRS) family, which is involved in protein synthesis (Caillet et al., 2003; Guo et al., 2020). Considering that this enzyme is important in the translation of the genetic code, changes in the *thrS* gene may affect protein synthesis and heat tolerance in various patterns. These mutations may indirectly affect the expression of heat shock proteins, which help maintain protein integrity and protect against extreme heat conditions. In addition, it may affect the response to antibiotics by impacting protein translation and the cellular stress response, thus explaining the increased tolerance of SeT_M_4 to heat (Fig. 3A). Furthermore, SeT_M_4 exhibited a notable increase in resistance to other carbapenem (imipenem), as well as resistance to florfenicol (Table 5). Like *spoT*, *thrS* has also been previously associated with the activation of the stringent response and resistance against carbapenems (Adler et al., 2016), although further studies would be needed to elucidate these relationships.

SeT_M$_5$ had a mutation in the *mrdA* gene (Fig. 2A). The *mrdA* gene encode the PBP2 (penicillin-binding protein 2) protein, one of the main penicillin-binding proteins. MPM exerts its action by irreversibly binding to the active site of PBPs, inhibiting the transpeptidase responsible for the formation of peptidoglycan cross-bridges, which leads to cell death. Structural alterations in PBP2, caused by mutations in the *mrdA* gene, may modify the antibiotic binding site, resulting in reduced affinity between the enzyme and the carbapenem. This reduced affinity may prevent MPM from binding effectively, conferring a resistance phenotype or increased tolerance to the antibiotic (Geng et al., 2022). Similarly, another study conducted by Yamachika et al. (2013) observed that the *mrdA* gene mutation confers resistance to meropenem, imipenem, and panipenem. MPM acts by irreversibly inhibiting penicillin-binding proteins responsible for bacterial cell wall biosynthesis, thus hindering cell wall formation and leading to cell death (Dabhi et al., 2024). However, small structural changes or mutations in penicillin-binding proteins result in significantly lower binding affinity of MPM to the protein, because of which the antibiotic cannot effectively inhibit cell wall synthesis. In addition to the increased resistance to imipenem, SeT_M$_5$ exhibited increased resistance to florfenicol (Table 5). These results are also in accordance with Zhou et al. (2019), who showed that resistance to certain antibiotics increased in *Salmonella* after induction with ceftriaxone. On the other hand, this mutation in *mrdA* does not seem to have any relationship with protection against heat or carvacrol, as shown in Figs. 3A and 4A.

SeE_M$_3$ and SeE_M$_5$ also harbor an interesting mutation in *mrdA* gene (Fig. 2B). Similar to SeT_M$_5$, the evolved strains SeE_M$_3$ and SeE_M$_5$ did not exhibit increased tolerance to heat (Fig. 3B) or carvacrol (Fig. 4B). However, all these RVs showed resistance to β-lactams, not only to MPM but also imipenem, in case of SeE_M$_5$. Thus, this mutation in *mrdA* gene may explain the enhanced protection in both serovars, since the different mutations in this gene have been associated with multi-drug resistance (Yamachika et al., 2013). Also, the improvement in the growth fitness of SeT_M$_5$ and SeE_M$_3$ in the presence of MPM may be related to the mutation in the *mrdA* gene (Tables 3 and 4), since this alteration might modify the function of the protein and can decrease susceptibility to MPM (Ranjitkar et al., 2019).

SeE_M$_2$ had a mutation in the *thrS* gene (Fig. 2B), in the same gene as in SeT_M$_4$, but being a different mutation. Thus, despite being the same gene, the phenotypic changes observed were slightly different. While SeE_M$_2$ also showed increased tolerance to heat (Fig. 3B) and slightly lower tolerance to carvacrol (Fig. 4B), no enhanced resistance to MPM or cross-resistance to other antibiotics was observed (Table 6). On the contrary, increased susceptibility to cephalothin, imipenem and florfenicol was seen. This different behavior might be explained by the different location of the mutation in the gene, as amino acid substitutions at specific sites can alter interactions or conformations, resulting in different phenotypes. The aforementioned possible variable derived from *thrS* mutations could also help explain the different phenotypic outcomes.

Finally, SeE_M$_4$ showed modifications related to the rhamnosyltransferase (AWJ12_RS14025) and in the gene encoding the glucose-1-phosphate cytidylyltransferase enzyme (*rfbF*) (Fig. 2B). Both enzymes are involved in the synthesis of complex sugars that compose the lipopolysaccharide (Correia et al., 2016), enhancing the structure and stability of the bacterial outer membrane. Modifications in the lipopolysaccharide structure result in a more rigid and less permeable membrane, which reduces the flow of water and ions across the membrane, thereby minimizing the destabilizing effects of heat. Therefore, these mutations, especially in *rfbF* gene, could be responsible for the increased tolerance by improving the response to heat (Fig. 3B).

In summary, mutations identified in SeT_M$_{1\text{-}5}$ and SeE_M$_{2\text{-}5}$ highlight the complexity of bacterial adaptation to different stressors. In this regard, Tables 3 and 4 include information about the biological processes outlined in the Gene Ontology of all the mutated proteins (Liebermeister et al., 2014). The analysis reveals that the genomic alterations observed in these MPM-RVs are closely associated with biological processes such as metabolism, including proteins involved in nucleotide metabolism (*spoT*), carbohydrate metabolism (*glnA*), as well as mutations involved in the processing of environmental information, more specifically in membrane transport (*znuA*). On the other hand, mutations exclusively linked to heat stress were observed in SeT_M$_4$ (*thrS*), SeE_M$_2$ (*thrS*) and SeE_M$_4$ (*rfbF*), correlating with process such as carbohydrate metabolism (*rfbF*), and genetic information processing, specifically translation (*thrS*). Although strains SeT_M$_5$, SeE_M$_3$ and SeE_M$_5$ did not exhibit mutations directly related to heat stress or carvacrol, the mutation in *mrdA* affected the response to MPM and promoted cross-protection to other classes of antimicrobials. Specifically, in SeT_M$_5$ and SeE_M$_3$, this mutation positively influenced cell growth in the presence of MPM, as evidenced by the evaluated growth curves. From the point of view of the biological processes involved, the mutation found in these RVs was associated with glycan biosynthesis and metabolism.

Both SeT and SeE variants exhibited modifications in important genes, some of which are widely described in the literature as classical molecular mechanisms involved in antibacterial tolerance and persistence, such as *spoT*, *thrS*, and *mrdA* (Caillet et al., 2003; Gong et al., 2022; Pacios et al., 2020). Other genes, such as *znuA*, are less explored, and there is still limited data on their association with these phenotypes. In general, we observed that some of the detected genetic alterations were associated with responses induced by exposure to MPM, while others appeared to involve the regulation of bacterial response mechanisms to heat and carvacrol. The differences in resistance behavior observed between serovars suggest the presence of intrinsic genetic and physiological factors that modulate their responses to selective pressure. Our findings align with previous studies that indicate a greater adaptive capacity of SeT strains when exposed to environmental changes and physiological challenges, compared to SeE. This adaptability is often associated with the ability to modulate gene expression and acquire mobile genetic elements, such as plasmids and pathogenicity islands, which are commonly linked to antimicrobial resistance (Wu et al., 2021; Peruzy et al., 2025).

5. Conclusions

This study demonstrated that sequential exposure to MPM can lead to the emergence of resistant bacterial variants. It was observed that most of the meropenem-resistant variants also showed cross-protection to other antibiotics and food preservation methods (heat and carvacrol). Furthermore, we provide valuable insights into the genetic adaptations that underlie the resistance and tolerance mechanisms of these variants to the adverse conditions analyzed. We have described how these mutations may alter protein functionality, leading to enhanced resistance to multiple stressors, such as heat, carvacrol or antibiotics.

These findings highlight the importance of preventing the emergence of resistant bacterial variants, considering that such variants can show cross-protection to various stresses encountered in the food chain. Further research may focus on identifying the specific genetic and molecular mechanisms responsible for the observed phenotypes, including the roles of individual mutations and their contribution to cross-resistance and stress response pathways. Experimental validation of the identified mutations will be essential to determine their direct involvement in these adaptive responses, especially in relation to oxidative stress mechanisms triggered by compounds such as carvacrol.

CRediT authorship contribution statement

Ruthchelly Tavares da Silva: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ivo García-Penas:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Laura Espina:** Writing – review & editing. **Diego García-Gonzalo:**

Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Marciane Magnani:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Rafael Pagán:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2025.104823.

Data availability

Data will be made available on request.

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