Applied Food Research 6 (2026) 101933

Contents lists available at ScienceDirect

Applied Food Research

journal homepage: www.elsevier.com/locate/afres

Ciprofloxacin-resistant variants of *Listeria monocytogenes* EGD-e show increased heat resistance in buffer and milk

María Mei Martínez de Zuazo, Jorge Andaluz-Arbe, Alberto Fau, Ivo García-Penas, Rafael Pagán, Diego García-Gonzalo, Natalia Merino *

Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA), 50013 Zaragoza, Spain

ARTICLE INFO

Keywords:
Adaptive evolution
Food safety
Whole-genome sequencing
Food preservation
Thermoresistance
Cross-Protection

ABSTRACT

The extensive use of antibiotics in primary production has promoted the emergence of resistant bacteria. Due to cross-protection phenomena, these antimicrobial resistant (AMR) bacteria may also withstand food preservation treatments applied in the food industry. This study aimed to evaluate the emergence of resistant variants (RVs) of *Listeria monocytogenes* EGD-e after prolonged exposure to antibiotics (amoxicillin, ciprofloxacin and oxytetracycline) based on adaptive laboratory evolution assays. RVs were selected by determining the minimum inhibitory concentration, then characterized phenotypically against heat treatments (58 °C/ 20 min) and genotypically to identify mutations responsible for changes in thermoresistance. Five ciprofloxacin RVs ($Lm_{Cip1–5}$) and one oxytetracycline RV (Lm_{Oxy}) were obtained. Several ciprofloxacin RVs showed greater thermoresistance in McIlvaine buffer (pH 7.0) than the parental strain, also observed in skimmed milk (pH 6.8). Mutations identified in *codY* (Lm_{Oxy}) and *fepR* and *parC* (ciprofloxacin RVs) are likely responsible for the antibiotic resistance. Moreover, mutations in genes linked to cell wall biosynthesis (*rml*), metabolism and RNA or energy processing (e.g., *cshA, atpA2, lmo2794*) may contribute to increased thermoresistance. These findings highlight the interaction between AMR and cross-protections mechanisms, and the potential risk posed by AMR bacteria in the food chain, which could compromise the traditional preservation methods.

1. Introduction

Foodborne diseases remain a major public health concern worldwide, affecting millions of people and causing significant health and economic burdens. Among the various agents involved, bacteria are the leading cause of these infections (Bintsis, 2017; World Health Organization [WHO], 2025). L. *monocytogenes*, a Gram-positive foodborne pathogen, is responsible for listeriosis, a severe zoonotic disease characterized by high hospitalization and fatality rates (Olaimat, 2018). According to the European Food Safety Authority (EFSA), listeriosis cases in 2024 reached their highest level since 2007 (EFSA, 2025).

Antibiotics are the primary treatment for foodborne infections. In livestock, tetracyclines and penicillins are among the most commonly used classes of antibiotics (European Medicines Agency [EMA], 2025a). In particular, aminopenicillins remain one of the main therapeutic options for treating L. *monocytogenes* infections (EMA, 2025b). Fluoroquinolones, although strictly regulated in food-producing animals, are also used under specific circumstances (EMA, 2025b). However, their widespread use in both human and veterinary medicine has contributed to the emergence and spread of antimicrobial resistance (AMR), which is currently considered a major public health threat (WHO, 2023). Due to their high therapeutic relevance and potential to select for resistant variants (RVs), many of these antibiotics are classified as critically important antimicrobials (CIAs) by the World Health Organization (WHO, 2024).

AMR can arise through spontaneous genetic mutations or via horizontal gene transfer (HGT) of resistance determinants, leading to functional and phenotypic changes that reduce the effectiveness of antimicrobials (Munita & Arias, 2016; Samtiya et al., 2022). This is particularly concerning when clinically relevant pathogens acquire resistance traits, as it may compromise treatment outcomes and increase the risk of severe infections in consumers (WHO, 2023).

Contamination of food with AMR bacteria can occur at any stage along the food chain, making food a potential vehicle for the

* Corresponding author.
E-mail address: merino@unizar.es (N. Merino).

https://doi.org/10.1016/j.afres.2026.101933
Received 8 January 2026; Received in revised form 23 March 2026; Accepted 25 March 2026
Available online 26 March 2026
2772-5022/© 2026 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

transmission of resistant pathogens (Samtiya et al., 2022). Indeed, recent studies have reported the presence of AMR *L. monocytogenes* strains isolated from food products and food processing environments, supporting the notion that the food chain can act as a reservoir and dissemination route for AMR bacteria (Escolar et al., 2017; Rippa et al., 2024; Silva et al., 2024). To reduce the risk of foodborne illnesses, the food industry relies on various microbial control strategies, with thermal processing being the most widely applied due to its proven efficacy in inactivating pathogens to microbiologically safe levels (Augusto et al., 2018; Lima et al., 2023).

The increasing prevalence of AMR has raised concerns beyond therapeutic failure, particularly regarding the phenomena of cross-protection. This refers to the ability of bacteria to develop resistance not only to the selecting agent (e.g., an antibiotic), but also to unrelated antimicrobials or food stressors, including heat. Such cross-protection is typically mediated by overlapping or convergent stress response mechanisms activated during adaptation (Bland et al., 2021; Liao et al., 2020). If present, this phenomena could undermine the effectiveness of food preservation treatments, complicating pathogen control and posing a serious threat to food safety.

Adaptive laboratory evolution (ALE) assays are a powerful approach for investigating the development of resistance in bacteria (Berdejo et al., 2019, 2022; Campillo et al., 2025). This methodology enables the selection of RVs: strains with increased resistance to a specific selective agent. Phenotypic and genotypic characterization of RVs facilitates the identification of resistance mechanisms, including cross-protection or cross-sensitization (Berdejo et al., 2022; Bland et al., 2021; Guérin et al., 2021; Lopatkin et al., 2021).

While several studies have investigated the relationship between antibiotic resistance acquired through ALE and cross-protection or cross-sensitization to food-associated stress (Byun et al., 2022; Campillo et al., 2025; Tavares da Silva et al., 2025; Walsh et al., 2001), only a limited number have focused on Gram-positive bacteria, and even fewer on the foodborne pathogen *L. monocytogenes* (Byun et al., 2022; Walsh et al., 2001). Consequently, the mechanisms underlying potential cross-protection or cross-sensitization to heat following antibiotic adaptation in *L. monocytogenes* remain poorly understood. The present study addresses this gap by investigating whether resistance to antibiotics acquired through ALE in *L. monocytogenes* can lead to cross-protection or cross-sensitization to heat in a food matrix.

The aims of this study were to (a) generate RVs of *L. monocytogenes* EGD-e through in vitro ALE assays based on continuous exposure to increasing concentrations of amoxicillin, oxytetracycline, and ciprofloxacin (five independent lineages per antibiotic); (b) assess the level of resistance acquired by the selected RVs to each antibiotic; (c) identify genetic mutations potentially responsible for the observed resistance phenotypes; and (d) evaluate whether the RVs display cross-protection to heat treatments, both in laboratory media and in a food matrix (skimmed milk).

2. Materials and methods

2.1. Microorganism and growth conditions

L. monocytogenes EGD-e was provided by Prof. Chakraborty (Institute for Medical Microbiology, Giessen, Germany). Throughout this investigation, the strain was maintained at −80 °C in cryovials containing glycerol (20 % *v/v*) from which isolated colonies were obtained on tryptone soy agar (Oxoid, Hampshire, UK) with 0.6 % (*w/v*) yeast extract (Oxoid) (TSAYE) plates by streak plating and incubated at 37 °C for 48 h.

The medium used for bacterial growth was Mueller Hinton broth (Millipore, Saint Louis, USA) supplemented with 5 % of 50 % horse lysate blood, prepared according to Clinical and Laboratory Standards Institute (CLSI, 2017) guidelines (MHBLHB). This medium is recommended for antimicrobial sensitivity assays because of its ability to provide optimal conditions for the growth of *L. monocytogenes* EGD-e without affecting the antimicrobial activity of the compound, encouraging the development of RVs. Subcultures were obtained by inoculating one colony in a test tube containing 5 mL of MHBLHB and then incubated in an orbital shaker (Heidolph Instruments, Schwabach, Germany) for 12 h at 37 °C and 140 rpm. Subsequently, to obtain the working bacterial cultures, flasks containing 10 mL of MHBLHB were inoculated with the subculture at a concentration of 10^6 colony-forming units per mL (CFU/mL) and were incubated for 24 h at 37 °C and 140 rpm to obtain a stationary growth phase bacterial suspension (2.4×10^8 CFU/mL). The same protocol was followed to obtain the bacterial cultures of the isolated strains obtained from the evolution assays with antibiotics. In this case, MHBLHB was replaced with tryptone soy broth (Oxoid) with 0.6 % (*w/v*) yeast extract (TSBYE). The stationary growth phase of these bacterial suspensions ranged between 1.2×10^9 and 2.2×10^9 CFU/mL.

2.2. Minimum inhibitory concentration (MIC)

MIC is defined as the lowest concentration of an antimicrobial that is capable of inhibiting bacterial growth (CLSI, 2017). Antibiotics evaluated in this study included three structurally unrelated antibiotics classified as CIAs by the World Health Organization (WHO, 2024): amoxicillin, oxytetracycline, and ciprofloxacin (Sigma-Aldrich, St. Louis, USA). These compounds belong to three major antibiotic classes: β-lactams (cell wall synthesis inhibitors), tetracyclines (protein synthesis inhibitors), and fluoroquinolones (DNA gyrase and topoisomerase IV inhibitors), respectively. Their selection enables the evaluation of adaptive responses to antibiotics with distinct cellular targets and the potential cross-protection mechanisms.

Stock solutions of amoxicillin, oxytetracycline and ciprofloxacin were prepared following the guidelines of the CLSI (2017). Minimum inhibitory concentration of the antibiotics studied in this investigation was determined according to broth microdilution method (CLSI, 2017) in a 96-well microtiter plate (Thermo Fisher Scientific, Rochester, USA).

First, antibiotics were added, creating a decreasing concentration gradient. The maximum concentration tested was applied in the first column, from which serial two-fold (1:2) dilutions were performed across subsequent columns, progressively reducing the antibiotic concentration relative to the previous column. The antibiotic concentration ranged from 64.00 to 0.03 μg/mL for amoxicillin and oxytetracycline, and from 4.00 to 0.002 μg/mL for ciprofloxacin. Microtiter plates containing oxytetracycline were protected from light to prevent compound degradation. Subsequently, *L. monocytogenes* EGD-e was inoculated at a final concentration of 10^5 CFU/mL in each well. Each experiment included positive control wells (inoculated with microorganism 10^5 CFU/mL in the absence of antibiotics) and negative control wells for each antibiotic (inoculated with the maximum antibiotic concentration tested in the absence of microorganism). The final volume in each well was 100 μL. After the incubation of microtiter plates (37 °C/ 24 h/ 140 rpm), MIC was determined. As the growth of *L. monocytogenes* EGD-e in MHBLHB medium is characterized by the formation of aggregates, MIC reading was performed by naked eye, using a light beam to facilitate its determination (CLSI, 2017).

2.3. Adaptive laboratory evolution assays (ALE)

The parental strain *L. monocytogenes* EGD-e (Lm_{Wt}) was subjected to an ALE assay based on a prolonged antibiotic exposure protocol described by Lopatkin et al. (2021) to obtain *L. monocytogenes* EGD-e RVs. Five independent evolution lineages were performed for each antibiotic. The initial antibiotic concentration (0.085 × MIC) was increased daily by 85 % (× 1.85-fold). The duration of exposure to each antibiotic was determined by the ability of the strain to grow under increasing concentrations. The maximum estimated exposure concentration was 40 times the MIC, reached on day 10. Initially, a single colony was inoculated into 5 mL of MHBLHB and incubated for 12 h at

37 °C and 140 rpm. From this subculture, a bacterial concentration of 10^6 CFU/mL was inoculated into 10 mL of MHBLHB and incubated for 3.5 h at 37 °C and 140 rpm to obtain an exponential phase culture with a concentration of approximately 10^7 CFU/mL Subsequently, 500 µL of the culture were transferred to test tubes containing 4.5 mL of MHBLHB with antibiotic (amoxicillin, oxytetracycline or ciprofloxacin) at an initial concentration of 0.085 × MIC and incubated for 24 h at 37 °C and 140 rpm. Tubes containing oxytetracycline were protected from light to prevent compound degradation. After reaching the stationary phase, the same step was repeated: 500 µL of an intermediate 1:100 dilution were inoculated into test tubes containing 4.5 mL MHBLHB with antibiotic at a concentration increased by 85 % (× 1.85-fold) and then incubated (37 °C/ 24 h/140 rpm). This procedure was repeated 10 more times. Aliquots from all lineages that exhibited growth after reaching the MIC on the fourth day were stored in cryovials containing 20 % glycerol at − 20 °C for subsequent assays. This procedure was designed to continue until day 10; however, once the microorganism was unable to grow, the experimental lineage was terminated. Lineages were considered resistant when sustained growth was observed for at least day 4 at which the MIC of Lm_{Wt} was reached. Consequently, any lineage failing to grow by day 4 was excluded from further analysis.

Upon completion of the ALE assays, from each resistant evolution lineage, RVs were isolated from the last exposure concentration that showed growth. Isolation was carried out on Mueller–Hinton agar (Sigma Aldrich) plates supplemented with the highest antibiotic concentration at which *L. monocytogenes* EGD-e was able to grow. After incubation at 37 °C for a minimum of 48 h (in the case of oxytetracycline plates, incubation could exceed 72 h due to the slow growth of RV colonies), three colonies were randomly selected to determine their MIC. In case the three selected colonies showed the same increase in MIC, one colony was randomly chosen for genotypic and phenotypic characterisation; while in case they showed differences in MIC, the one showing the highest increase in MIC was selected.

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

DNA extraction of Lm_{Wt}, Lm_{Cip} and Lm_{Oxy} strains was performed using the DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany), following the Gram-positive bacteria DNA extraction protocol. The elution step was performed in duplicate using 25 µL of elution buffer (AE buffer) in order to obtain a higher DNA concentration in the sample. DNA was quantified via fluorescence using QubitTM 4 Fluorometer (Invitrogen, Thermo Fisher Scientific, Singapore), ensuring a minimum concentration of 20 ng/µL. WGS was performed by Azenta Life Sciences (GENEWIZ Germany GmbH). Samples were standardized to 20 ng/mL and then the sequencing process was conducted using Illumina technology (NovaSeq 2 × 150), which enabled the generation of paired-end reads producing fragments of 150 base pairs per sample (2 × 150 bp) generating ~1 Gb of data output (per sample). Following genome sequencing, bioinformatic analysis was performed by Azenta Life. The sequencing results were provided in FASTQ format, which included both the DNA sequences and the quality scores of each read. Once the results were obtained, single nucleotide polymorphisms (SNPs), insertions (Ins), and deletions (Del) were detected and verified using the Integrative Genomics Viewer (IGV) software (University of San Diego, San Diego, California, USA) by comparison with the reference genome of the *L. monocytogenes* EGD-e strain obtained from the National Center for Biotechnology Information (NCBI) database (NCBI: NC_003210.1). It should be noted that mutations shared between the Lm_{Wt} strain and the RVs were excluded. Then, SnpEff tool, available on the USEGALAXY platform, was used to determine the mutation type (e.g., single nucleotide variant, frameshift insertion. non-frameshift insertion, frameshift deletion. non-frameshift deletion, premature stop), to predict their functional impact based on the resulting changes in the amino acid sequence.

The resulting genome sequences were deposited in the Sequence Read Archive (SRA) of NCBI (BioProject ID: PRJNA1355191). The accession numbers of the samples are SAMN53033343 (Lm_{Wt}), SAMN53033344 (Lm_{Cip1}), SAMN53033345 (Lm_{Cip2}), SAMN53033346 (Lm_{Cip3}), SAMN53033347 (Lm_{Cip4}), SAMN53033348 (Lm_{Cip5}), SAMN53033349 (Lm_{Oxy1}), and SAMN53033350 (Lm_{Oxy2}).

2.5. Cross-protection to heat treatments

In order to observe differences in thermoresistance, Lm_{Wt} and the RVs were subjected to heat treatments. The selected conditions (time, temperature, and pH) were based on previous studies (Berdejo et al., 2022) in order to achieve approximately five $\log_{10}$ cycles reduction of *L. monocytogenes* EGD-e (data not shown).

2.5.1. In laboratory media

Heat treatment was performed in McIlvaine buffer (citrate-phosphate buffer) adjusted to pH 7.0 and pH 4.0 (simulating conditions in various food matrices) prepared using citric acid monohydrate (PanReac-AppliChem, Darmstadt, Germany) and disodium hydrogen phosphate (PanReac). For each treatment, 1 mL of culture (obtained as described in Section 2.1) was centrifuged (MiniSpin, Eppendorf, Hamburg, Germany) at 12 000 g for 3 min. After the centrifugation, the supernatant was discarded and the pellet was resuspended in 1 mL of McIlvaine buffer (pH 4.0 or 7.0), obtaining a final concentration of approximately 10^9 CFU/mL Subsequently, 60 µL from each resuspended strain (both Lm_{Wt} and RV strains) were subjected to heat treatments in a thermal cycler (T100 Thermal Cycler, Bio-Rad, California, USA) under the following conditions: 15 min at 58 °C (pH 7.0) and 15 min at 54 °C (pH 4.0). After heat treatment, aliquots were serially diluted in PBS, plated on TSAYE, and incubated at 37 °C for 48 h. For RVs that showed increased resistance compared to Lm_{Wt} strain, survival curves were obtained taking counts every 2.5 min and extending heat treatment time to 20 min following the same protocol described previously.

2.5.2. In skimmed milk

To assess cross-protection to heat in food matrices, survival curves were obtained for RVs that showed increased thermoresistance compared to Lm_{Wt}. As heat cross-protection was only observed at pH 7.0, heat treatment was conducted in skimmed milk (La Asturiana Desnatada UHT, pH 6.8) at 58 °C for 20 min, following the previously described protocol for McIlvaine buffer (pH 7.0). This food matrix was selected due to its pH close to 7.0 and its association with *L. monocytogenes* foodborne outbreaks. Furthermore, *Listeria* monitoring programmes also include products derived from raw milk, pasteurised milk and milk subjected to low-temperature treatments, such as cheeses, highlighting the importance of this food matrix (EFSA, 2025).

2.6. Statistical analysis

Phenotypic characterization results from MIC determinations, heat treatments and survival curves presented in this study were obtained from at least three replicates. All experiments were carried out on independent days and using different bacterial cultures obtained under the same working conditions. Prism® 8 software (GraphPad Inc, San Diego, USA) was used to determine the means and standard deviations, graphical representation and statistical analysis. Specifically, data were analysed and submitted to comparison of averages using one-way ANOVA analysis, followed by pot-hoc Dunnett's multiple comparison test (heat treatments) and by post-hoc Tukey test (survival curves). Statistically significant differences were considered at a 95 % confidence level for $p < 0.05$.

Table 1
Minimum inhibitory concentration (MIC, μg/mL) of *Listeria monocytogenes* EGD-e parental strain (Lm_{Wt}) against the following antibiotics: amoxicillin, oxytetracycline and ciprofloxacin.

Antibiotic	MIC (μg/mL)
Amoxicillin	0.25
Oxytetracycline	2.00
Ciprofloxacin	1.00

3. Results and discussion

3.1. MIC of L. monocytogenes EGD-e for selected antibiotics

The MIC value of Lm_{Wt} was 0.25 μg/mL for amoxicillin, 2.00 μg/mL for oxytetracycline, and 1.00 μg/mL for ciprofloxacin, as shown in Table 1. MIC values were interpreted according to EUCAST MIC distribution data (2025). EUCAST provides MIC distribution ranges for oxytetracycline and ciprofloxacin in *L. monocytogenes*, and for amoxicillin in *S. aureus*, which was used as a reference organism. The MIC value obtained for amoxicillin is within the range reported by EUCAST (0.25 μg/mL; EUCAST range: 0.06–0.25 μg/mL), as are the values obtained for ciprofloxacin (1 μg/mL; EUCAST range: 0.5–2 μg/mL) and oxytetracycline (2 μg/mL; EUCAST range: 1–4 μg/mL).

3.2. ALEs with antibiotics enabled the isolation of L. monocytogenes RVs

Fig. 1 represents the ALE assays, showing the number of days of sustained growth for each *L. monocytogenes* EGD-e lineage exposed to increasing concentrations of antibiotics. As shown in Fig. 1, amoxicillin-exposed lineages (AMX1–AMX5) did not grow after day 3, and consequently no RVs were obtained under the tested conditions. Although amoxicillin-RVs have been successfully generated through ALE in other bacteria such as *Staphylococcus epidermidis* and *Escherichia coli* (Merino et al., 2023; Mira et al., 2022), the absence of RVs in this study suggests that the selective pressure applied was insufficient to induce adaptive mutations in this bacterium. However, it is possible that amoxicillin RVs could develop under other ALE conditions (e.g., lethal treatments or constant subinhibitory concentrations). In contrast, oxytetracycline -exposed lineages OXY2, OXY3 and OXY4 growth at the MIC level, whereas all ciprofloxacin-exposed lineages (CIP1 to CIP5) showed sustained growth at concentrations ranging from 6.3- to 11.7-fold the MIC. From each resistant lineage, three colonies were selected from the last time point at which visible growth occurred, resulting a total of 24 isolates: 9 from oxytetracycline-exposed lineages (day 4) and 15 from ciprofloxacin-exposed lineages (days 6–7). To confirm the acquisition of antibiotic resistance, MIC values of the isolates obtained from each resistant lineage were determined and compared with the Lm_{Wt} (Table 2). In most cases, the three colonies isolated from each lineage displayed identical MIC values, with an exception in lineage CIP3, in which one colony displayed a lower MIC (16.00 μg/mL) compared to the other two (32.00 μg/mL), showing intra-lineage heterogeneity. OXY3 was the only lineage in which none of the isolated colonies showed an increased MIC compared to the Lm_{Wt}, being excluded from further phenotypic and genotypic characterization. Overall, oxytetracycline -adapted lineages exhibited a 2-fold increase in MIC, whereas ciprofloxacin-adapted lineages showed a 16- to 32-fold increase. These values exceed the 2- to 16-fold increase previously reported for ciprofloxacin-resistant *L. monocytogenes* variants obtained through ALE (Jiang et al., 2018).

When all three colonies from a lineage showed the same MIC, one was randomly selected for further analysis. In the case of CIP3, one of the two colonies with the highest MIC (32.00 μg/mL) was selected. A total of seven RVs were selected for detailed characterization: two from the oxytetracycline-adapted lineages OXY2 and OXY4 (designated as Lm_{Oxy1} and Lm_{Oxy2}, respectively), and five from the ciprofloxacin-

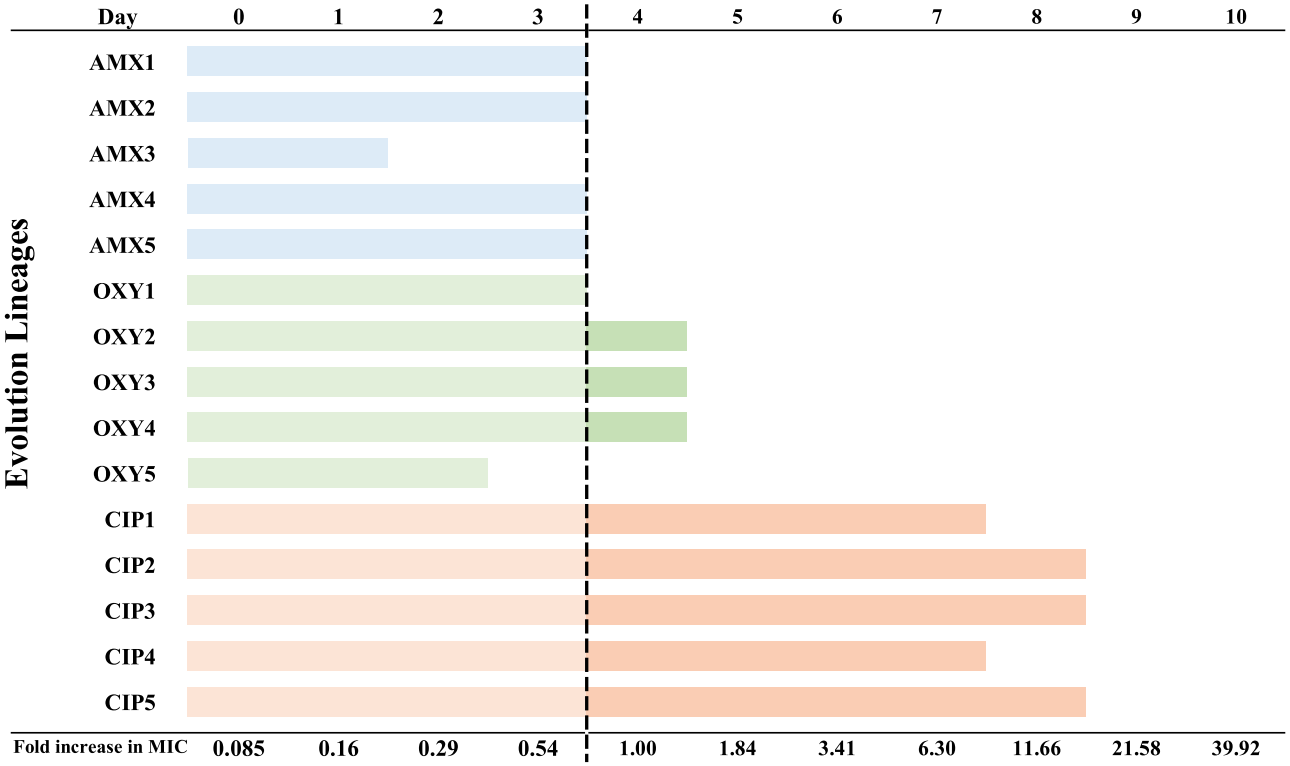

Fig. 1. Evolution assay diagram depicting the number of days of sustained growth for each *Listeria monocytogenes* EGD-e lineage (five per antibiotic; 1–5) after each antibiotic exposure (from 0.085 to 39.92-fold increase in MIC). AMX- amoxicillin exposed lineages, OXY- oxytetracycline exposed lineages, CIP- ciprofloxacin exposed lineages. The dotted line indicates the day on which the MIC value is reached. Darker coloured bars indicate growth at or above the MIC.

Table 2
Minimum inhibitory concentration (MIC, μg/mL) of *Listeria monocytogenes* EGD-e parental strain (Lm_{Wt}) and *Listeria monocytogenes* EGD-e derived lineages (OXY- oxytetracycline lineages, CIP- ciprofloxacin lineages). MIC values for each lineage were determined from three isolated colonies. When all three colonies from a lineage exhibited the same MIC, a single representative value is shown. Values in bold indicate an increase in MIC relative to the Lm_{Wt}.

Oxytetracycline MIC (μg/mL)		Ciprofloxacin MIC (μg/mL)	
Lm_{Wt}	2.00	Lm_{Wt}	1.00
OXY2	**4.00**	CIP1	**16.00**
OXY3	2.00	CIP2	**32.00**
OXY4	**4.00**	CIP3	**16.00–32.00**
		CIP4	**16.00**
		CIP5	**32.00**

adapted lineages (Lm_{Cip1} to Lm_{Cip5}). These strains, together with Lm_{Wt}, were subjected to phenotypic and genotypic characterisation.

3.3. RVs displayed cross-protection to heat treatments in laboratory media and skimmed milk

To determine whether the acquisition of AMR conferred cross-protection against heat treatments, the thermoresistance of the RVs was evaluated. Fig. 2 presents the $\log_{10}$ reductions in viable counts observed after heat exposure in McIlvaine buffer at pH 4.0 (54 °C for 15 min; Fig. 2.a) and at pH 7.0 (58 °C for 15 min; Fig. 2.b).

At pH 4.0 (Fig. 2a), none of the RVs exhibited enhanced thermoresistance compared to Lm_{Wt}. On the contrary, several strains displayed increased heat sensitivity. These included Lm_{Oxy1}, Lm_{Oxy2}, Lm_{Cip1}, Lm_{Cip4}, and Lm_{Cip5}, which displayed inactivation levels ranging from 5.1 to 6.5 $\log_{10}$ cycles, compared to 3.4 $\log_{10}$ units observed for Lm_{Wt}. Only Lm_{Cip2} and Lm_{Cip3} displayed inactivation values similar to those of Lm_{Wt}, indicating the absence of either cross-protection or sensitization. In contrast, distinct responses were observed at pH 7.0 (Fig. 2b). Lm_{Oxy1}, Lm_{Oxy2}, and Lm_{Cip5} showed no statistically significant differences ($p > 0.05$) compared to Lm_{Wt}, indicating that the acquisition of AMR in these RVs did not alter their thermoresistance under neutral conditions. Lm_{Cip1} exhibited cross-sensitization, with a significantly higher inactivation (7.0 $\log_{10}$) than Lm_{Wt} (6.0 $\log_{10}$; $p < 0.05$). Notably, Lm_{Cip2}, Lm_{Cip3}, and Lm_{Cip4} demonstrated clear cross-protection, showing lower inactivation levels ranging from 3.5 to 4.8 $\log_{10}$ reductions.

Heterogeneous responses in thermoresistance among Gram-positive RVs have been reported previously. For instance, *L. monocytogenes* variants from ALE assays based on prolonged sublethal exposure to carvacrol did not exhibit increased thermoresistance at pH 4.0 (Berdejo et al., 2022). Similarly, Walsh et al. (2001) observed no differences in thermoresistance between streptomycin-resistant and wild-type *L. monocytogenes* strains after heat treatment at 55 °C. In contrast, other studies have reported an increased thermoresistance. A carvacrol-resistant *L. monocytogenes* strain from short-term lethal treatments ALE showed enhanced thermoresistance (Berdejo et al., 2022). This cross-protection was also demonstrated in carvacrol-limonene oxide *Staphylococcus aureus* RVs (Berdejo et al., 2019), as well as in antibiotic-resistant *L. monocytogenes* RVs (Byun et al., 2022). These heterogeneous responses among RVs highlight variability in adaptive outcomes, suggesting that the relationship between AMR and thermoresistance depends on specific genetic changes and stress adaptation pathways.

To further characterize the observed cross-protection, survival curves were generated for Lm_{Cip2}, Lm_{Cip3}, and Lm_{Cip4} at 58 °C for 20 min in McIlvaine buffer at pH 7.0 (Fig. 3a). Significant differences ($p < 0.05$) between RVs and Lm_{Wt} were detected after 5 min for Lm_{Cip2} and Lm_{Cip3}, and after 7.5 min for Lm_{Cip4}. After 20 min of treatment, Lm_{Wt} showed a 6.3 $\log_{10}$ reduction, whereas Lm_{Cip2}, Lm_{Cip3}, and Lm_{Cip4} exhibited reductions of 4.6, 3.0, and 5.2 $\log_{10}$ cycles, respectively. Although these results indicate that all three RVs displayed cross-protection to heat, the increased resistance differed among them. The highest protection was observed in Lm_{Cip3}, which showed an approximately 1000-fold increase in survival, followed by Lm_{Cip2} and Lm_{Cip4}, with more than a 10-fold increase compared to the parental strain.

Unlike in heat treatments performed in buffer at pH 7.0, Lm_{Cip4} did not show significant differences ($p > 0.05$) relative to Lm_{Wt} in skimmed milk, indicating a loss of cross-protection in this food matrix. In contrast, significant differences ($p < 0.05$) relative to Lm_{Wt} were observed from 5 min for Lm_{Cip3} and from 10 min for Lm_{Cip2}. After 20 min, Lm_{Wt} exhibited a 6.0 $\log_{10}$ reduction, while Lm_{Cip2} and Lm_{Cip3} showed reductions of 4.9, 2.5 $\log_{10}$ cycles, respectively, highlighting Lm_{Cip3} as the most thermoresistant RV obtained, followed by Lm_{Cip2}. These findings indicate that the effect of antibiotic resistance on thermoresistance is not only strain-specific but also shaped by environmental conditions.

Although only a few studies have investigated cross-protection to heat in AMR bacteria in real food matrices, as observed in laboratory media, different responses to heat treatments have been reported. For example, Walsh et al. (2001) reported no differences in thermoresistance for streptomycin RVs *L. monocytogenes* isolated after antibiotic exposure in minced meat and potatoes. In contrast, other RVs from *Salmonella typhimurium* from ALE assays with ciprofloxacin have shown enhanced thermoresistance in liquid foods, such as orange juice, milk, and in liquid-whole egg (Campillo et al., 2025).

Overall, differences in heat resistance were observed among the RVs both in laboratory media and in the food matrix, with distinct responses depending on the environment. These variations may reflect different mutations acquired during ALE assays, underscoring the importance of subsequent genotypic characterization to understand the mechanisms underlying thermoresistance.

3.4. WGS revealed mutations in RVs

WGS was performed on Lm_{Wt} and the selected RVs to identify genetic alterations potentially responsible for their increased resistance to antibiotics and heat. Mutations shared by Lm_{Wt} and the RVs, after comparison with the reference genome (*L. monocytogenes* EGD-e; NCBI accession number: NC_003210.1), were excluded from the analysis (data not shown).

3.4.1. Mutations consistent with oxytetracycline resistance

Table 3 summarizes the genetic alterations identified in the RVs derived from oxytetracycline exposure. Since both isolates Lm_{Oxy1} and Lm_{Oxy2} from lineages OXY2 and OXY4 respectively shared the same mutation, they were treated as a single strain, hereafter referred to as Lm_{Oxy}.

Lm_{Oxy} carried a three-nucleotide deletion (359_361delGTG) in the *codY* gene, resulting in the loss of a glycine (Gly120del) and affecting a region in the GAF domain of the protein (position 1–155). *codY* encodes a global transcriptional regulator involved in the control of metabolic and virulence gene expression in Gram-positive bacteria, including *L. monocytogenes* (Biswas et al., 2020; Lobel & Herskovits, 2016). Notably, *codY* acts as a repressor of sigma factor B (σ^B), which is a key regulator of the stress response (Guerreiro et al., 2020; Lobel & Herskovits, 2016; Zhou et al., 2012). Furthermore, it has been demonstrated that σ^B enhances the resistance of *L. monocytogenes* EGD-e to antibiotics that inhibit protein synthesis, such as tetracycline (Zhou et al., 2012). Previous studies have linked mutations in *codY* to antimicrobial resistance in *S. aureus* (Martini et al., 2025). Consequently, the observed deletion in *codY* in Lm_{Oxy} may reduce its repressor activity, leading to the partial activation of stress-related genes. This regulatory shift may explain the observed increase in oxytetracycline MIC in the Lm_{Oxy} strain. However, despite this alteration in global regulation, Lm_{Oxy} did not exhibit enhanced thermoresistance under either acidic or neutral conditions. This suggests that *codY* disruption alone is insufficient to confer cross-protection to heat.

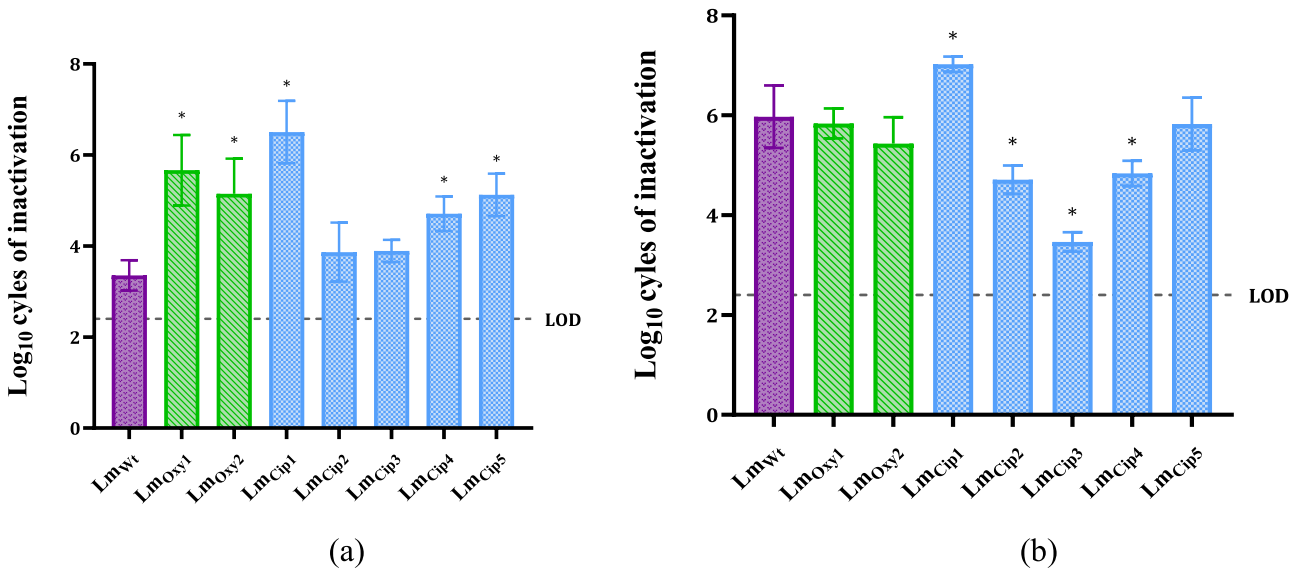

Fig. 2. Log_{10} cycles of inactivation for parental strain of *Listeria monocytogenes* EGD-e (Lm_{Wt}) and resistant variants (RVs) to oxytetracycline (Lm_{Oxy1} and Lm_{Oxy2}) and ciprofloxacin (Lm_{Cip1}-Lm_{Cip5}) after heat treatment in McIlvaine buffer at pH 4.0 and 54 °C for 15 min **(A)**, and at pH 7.0 and 58 °C for 15 min **(B)**. Error bars indicate standard deviation. Asterisks (*) denote statistically significant differences ($p \leq 0.05$) compared to Lm_{Wt}. LOD: limit of detection.

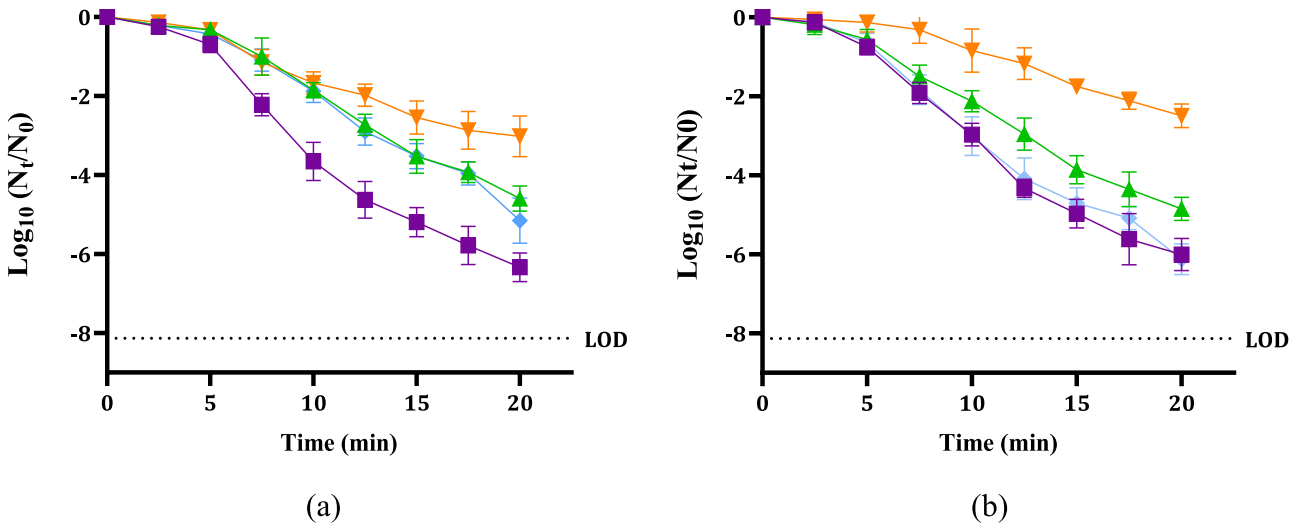

Fig. 3. Survival curves (Log_{10} N_t/N_0) of parental strain of *Listeria monocytogenes* EGD-e Lm_{Wt} (■) and the ciprofloxacin-resistant variants Lm_{Cip2} (▲), Lm_{Cip3} (▼), Lm_{Cip4} (◆), after heat treatment at 58 °C for 20 min in McIlvaine buffer at pH 7.0 **(A)** and in skimmed milk at pH 6.8 **(B)**. Error bars represent the standard deviation. LOD: limit of detection.

Table 3
Mutation identified in the oxytetracycline-resistant variant Lm_{Oxy}, in comparison with the parental strain of *Listeria monocytogenes* EGD-e (Lm_{Wt}). Mutation type is indicated as follows: Del, deletion.

Strain	Genome position	Mutation type	Change*	Predicted functional impact	Locus tag	CDS**	Description
Lm_{Oxy}	1304,806	Non-Frameshift Del	359_361delGTG → Gly120del	Moderate	*lmo1280*	*codY*	Global transcriptional regulator CodY

* Position with respect to the start of the coding region.
** CDS: the region of a gene that is translated into a protein, including the start codon and excluding the stop codon.

3.4.2. *Mutations consistent with ciprofloxacin resistance*

A more complex mutational landscape was observed in ciprofloxacin-RVs. Table 4 provides an overview of the mutations identified in the ciprofloxacin-RVs. A Venn diagram (Fig. 4) was generated to visualize the distribution of mutations across these RVs.

All ciprofloxacin RVs carried mutations in the *fepR* gene, which encodes a transcriptional repressor of *fepA*, the gene coding for an efflux pump associated with fluoroquinolone resistance (Douarre et al., 2022; Guérin et al., 2014). Although *fepR* was consistently affected, the nature of the mutations varied. In Lm_{Cip1}, the insertion (27_29dupTGC) affects the HTH TetR-type domain (position 1–60) without inducing frameshifts but introducing a new amino acid (Ala10). In contrast, Lm_{Cip2}, Lm_{Cip3}, Lm_{Cip4}, and Lm_{Cip5} harboured frameshift-inducing mutations, including insertions and deletions; and notably, Lm_{Cip4} and Lm_{Cip5} shared the

Table 4
Mutations identified in ciprofloxacin-resistant variants Lm_{Cip1} to Lm_{Cip5}, in comparison with the parental strain (Lm_{Wt}). Mutation types are indicated as follows: SNV, single nucleotide variant; Ins, insertion; Del, deletion; dup: insertion caused by direct duplication of the preceding bases; fs, frameshift.

Strain	Genome position	Mutation type	Change*	Predicted functional impact	Locus tag	CDS**	Description
Lm_{Cip1}	2019,429	Frameshift Ins	11dupG → Cys4fs	High	Lmo1944	*lmo1944*	Ferredoxin
	2167,914	Non-frameshift Ins	27_29dupTGC → Ala10dup	Moderate	Lmo2088	*fepR*	FepR transcriptional regulator
	2527,965	Frameshift Del	1217delA → His406fs	High	Lmo2456	*pgm*	2,3-bisphosphoglycerate-independent phosphoglycerate mutase
Lm_{Cip2}	906,251	Frameshift Ins	292_293dupCA → Val99fs	High	Lmo0866	*cshA*	ATP-dependent RNA helicase
	1117,083	Frameshift Del	545_548delATAA → Val182fs	High	Lmo1082	*rmlC*	dTDP-4-dehydrorhamnose 3,5-epimerase
	1311,883	Non-frameshift Del	1570_1572delTAT → Tyr524del	Moderate	Lmo1287	*parC*	DNA topoisomerase IV subunit A
	2167,593	Frameshift Ins	349_350dupGC →Asp118fs	High	Lmo2088	*fepR*	FepR transcriptional regulator
Lm_{Cip3}	1117,747	Frameshift Del	656delT → Val219fs	High	Lmo1083	*rmlB*	dTDP-glucose 4,6-dehydratase
	1310,670	SNV	356C>*A* → Ala119Glu	Moderate	Lmo1287	*parC*	DNA topoisomerase IV subunit A
	2167,425	Frameshift Ins	511_518dupGACCCGGA → Arg174fs	High	Lmo2088	*fepR*	FepR transcriptional regulator
	2609,218	Frameshift Ins	810_811dupTC → Arg271fs	High	Lmo2531	*atpA2*	ATP synthase subunit alpha 2
	2880,663	Frameshift Ins+ stop_gained	404_408dupTAGAC → Met137fs+ Codon stop	High	Lmo2794	*lmo2794*	Nucleoid occlusion protein
Lm_{Cip4}	1115,615	Frameshift Del	1861delA → Ile621fs	High	Lmo1080	*rmlT*	Glycosyltransferase
	1310,649	Non-frameshift Ins	336_341dupCGACGG → Gly114_Asp115insAspGly	Moderate	Lmo1287	*parC*	DNA topoisomerase IV subunit A
	2167,917	Frameshift Ins	25_26dupGC → Ala10fs	High	Lmo2088	*fepR*	FepR transcriptional regulator
Lm_{Cip5}	1310,852	SNV	538A>*G* → Thr180Ala	Moderate	Lmo1287	*parC*	DNA topoisomerase IV subunit A
	2167,917	Frameshift Ins	25_26dupGC → Ala10fs	High	Lmo2088	*fepR*	FepR transcriptional regulator

* Position with respect to the start of the coding region.
** CDS: region of the gene that is translated into a protein, including the start codon and excluding the stop codon.

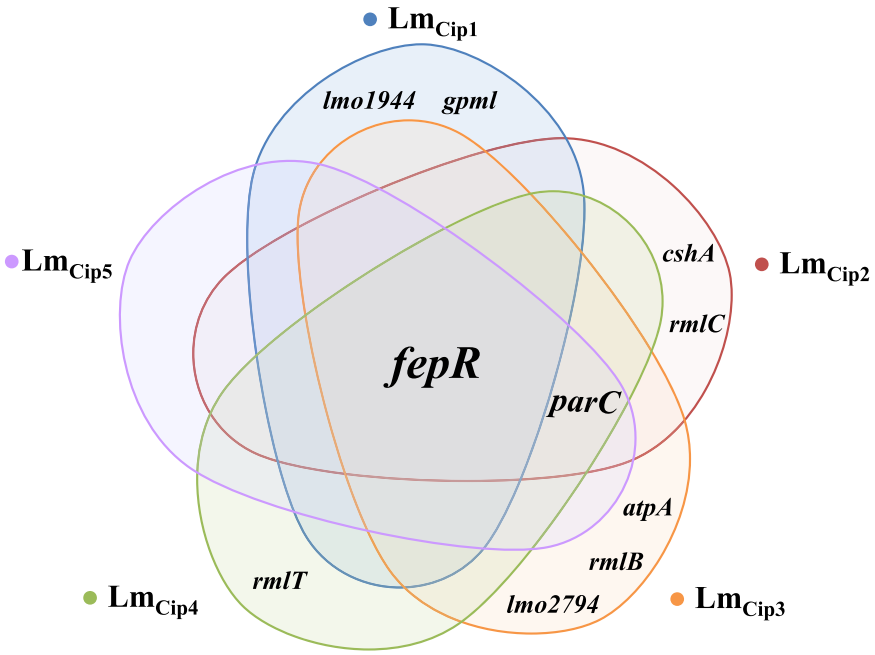

Fig. 4. Venn diagram showing the distribution of mutated genes among ciprofloxacin-resistant variants (RVs) of *Listeria monocytogenes* EGD-e. Overlapping regions indicate genes mutated in more than one RV.

same insertion (*25_26dupGC*) which also affected the HTH TetR-type domain (position 1–60). These mutations are likely to reduce or abolish *fepR* repression, thereby upregulating *fepA* expression and enhancing efflux-mediated resistance to ciprofloxacin in *L. monocytogenes* (Bland et al., 2021; Bolten et al., 2022; Douarre et al., 2022). This is further supported by Jiang et al. (2018), who provide evidence of increased efflux activity in ciprofloxacin-resistant bacteria obtained after ALE assays.

On the other hand, all ciprofloxacin RVs except Lm_{Cip1} also carried mutations in the *parC* gene, which encodes the A subunit of DNA topoisomerase IV, a well-known target of quinolones (Munita & Arias, 2016). Each RV carried a distinct mutation causing amino acid substitutions, insertions, or deletions. Although the mutations identified did not result in frameshifts, they may still alter the local structure of the enzyme and potentially affect antibiotic binding, thereby contributing to resistance. In fact, the role of *parC* mutations in quinolone resistance is well documented (Foster, 2017; Munita & Arias, 2016; Shariati et al., 2022).

In addition to these canonical resistance-associated changes, ciprofloxacin RVs also carried unique frameshift-inducing mutations in other loci (Table 4). Although their direct contribution to ciprofloxacin resistance remains unclear, such mutations may reflect compensatory or pleiotropic adaptations during antimicrobial stress, including metabolic rewiring, which has been proposed as a common response to antibiotic

exposure (Ahmad et al., 2025; Knudsen et al., 2016; Lopatkin et al., 2021). These additional adaptations may help explain the heterogeneous phenotypes observed across RVs under non-antibiotic stress conditions.

3.4.3. Candidate mutations potentially associated with increased heat resistance

To explore genetic features potentially linked to the heat cross-protection phenotype, we focused on mutations shared by RVs that exhibited increased survival to heat treatments. All RVs exhibiting cross-protection to heat (Lm_{Cip2}, Lm_{Cip3}, and Lm_{Cip4}) carried mutations in *rml* genes (*rmlC, rmlB*, and *rmlT*, respectively), which are involved in the biosynthesis of rhamnose-containing wall teichoic acids (WTAs) (Carvalho et al., 2015; Meireles et al., 2020). These cell wall components are known to influence antimicrobial susceptibility in Gram-positive bacteria (Carvalho et al., 2015; Meireles et al., 2020), though their exact role in AMR remains to be elucidated. Since bacterial envelopes are primary targets of heat inactivation (Russell, 2003), alterations in WTAs synthesis could contribute to the observed cross-protection.

Although mutations in *rml* genes appear to be responsible for increased resistance to heat treatment, differences in the specific genes and mutations, as well as the presence of additional alterations in other metabolic or regulatory genes, may also underlie the observed variation in thermoresistance among RVs and between different media (buffer at pH 7.0 or skimmed milk). Notably, Lm_{Cip4}, the only RV that did not show heat resistance in milk, carried a unique mutation in *rmlT*, whereas Lm_{Cip2} and Lm_{Cip3} harboured additional mutations in metabolic or regulatory genes.

In Lm_{Cip2}, *cshA* was mutated (292_293dupCA), affecting the helicase C-terminal domain (positions 214–374). This gene encodes a DEAD-box RNA helicase involved in ribosomal maturation (Netterling et al., 2015), which has been associated with repression of *L. monocytogenes* EGD-e growth at elevated temperatures (Markkula et al., 2012). Consequently, the observed mutation could reduce or abolish CshA function, potentially contributing to the increased heat resistance observed in this RV.

In Lm_{Cip3}, a mutation in *atpA2* was observed, a gene which encodes a subunit of ATP synthase required for energy homeostasis (UniprotKB, 2025). Additionally, *lmo2794*, which is associated with cell division processes (Camejo et al., 2009), showed a mutation (810_811dupTC) that resulted in a frameshift and a premature stop codon. The combined effect of these mutations may enhance energy-dependent repair mechanisms and help maintain cell integrity under heat stress, potentially explaining the notably high thermoresistance observed in this variant.

The bidirectional relationship between metabolism and antibiotic response suggests that metabolic mutations accumulated during antibiotic adaptation may have pleiotropic effects on stress resistance, potentially contributing to either cross-protection or cross-sensitization (Ahmad et al., 2025; Lopatkin et al., 2021).

Altogether, the genotypic profiles of ciprofloxacin-RVs reflect a complex interplay between canonical resistance mechanisms (e.g., efflux, target alteration), metabolic adaptation, and structural remodelling. However, the differences observed in MIC values and thermoresistance among RVs may not only result from mutations in different genes but also from epistatic interactions between mutations. Such interactions can modulate the phenotypic effects of individual mutations, potentially leading to distinct adaptive outcomes among closely related variants. Importantly, the increased heat survival observed in some ciprofloxacin-RVs could also be explained by alternative, non-mutually exclusive mechanisms, such as activation of general stress response pathways (e.g., σ^B/stressosome-regulated responses) (Guerreiro et al., 2020; Lobel & Herskovits, 2016; Zhou et al., 2012), cell envelope and membrane remodelling (Carvalho et al., 2015; Meireles et al., 2020), and other pleiotropic adaptations associated with efflux regulation and metabolic rewiring (Ahmad et al., 2025; Douarre et al., 2022; Guérin et al., 2014; Knudsen et al., 2016; Lopatkin et al., 2021). Therefore, the links between specific mutations and thermoresistance are presented as associations and mechanistic hypotheses, pending functional validation. These findings highlight the potential of antibiotic exposure for selecting antibiotic RVs with altered thermoresistance, which may be relevant when designing and validating mild heating steps or narrow-margin thermal hurdles within multi-hurdle preservation strategies in the food industry.

4. Conclusions

This study demonstrates that prolonged exposure of *L. monocytogenes* EGD-e to increasing antibiotics' concentrations can lead to the selection of RVs, particularly to oxytetracycline and ciprofloxacin.

Genomic analysis suggested that resistance to oxytetracycline was linked to a mutation in the global regulator *codY*, while resistance to ciprofloxacin was consistently associated with mutations in *fepR*, and, in most cases, in *parC,* supporting classical mechanisms such as efflux deregulation and target modification. Moreover, some ciprofloxacin-RVs exhibited enhanced thermoresistance under neutral conditions, both in buffer and skimmed milk, which may pose a concern to the food industry. Additional mutations in genes related to cell wall biosynthesis (*rml*), metabolism, and RNA or energy processing (e.g., *cshA, atpA2, lmo2794*) may enhance the ability of RVs to withstand or recover from heat-induced damage, leading to an increase in thermoresistance. Importantly, adaptive responses were heterogeneous: while some RVs exhibited cross-protection to heat, others showed cross-sensitization or no significant change.

Resistance outcomes varied among different RVs, depending on their genetic background but also with environmental context (pH and food matrix), underscoring the importance of assessing phenotypes under conditions that mimic food-processing environments.

Altogether, these results suggest that antibiotic-driven adaptation (particularly under ciprofloxacin selection) may be accompanied by altered heat resistance in a subset of *L. monocytogenes* variants, in a strain- and environment-dependent manner. Because our experiments were conducted in buffer and skimmed milk as model systems, validation across a broader range of food matrices and process-relevant heating profiles is required before extrapolating to industrial thermal processing. Nevertheless, the observed strain-dependent shifts in heat survival may be relevant when designing and validating mild heating steps or narrow-margin thermal hurdles within multi-hurdle preservation strategies, especially considering variability across strains and matrices.

Ethical statement

This study did not involve any experiments with human participants or animals. Therefore, ethical approval and informed consent were not required.

Funding sources

This work was supported by Project PID2021–123404NB-I00 funded by MICIU/AEI/10.13039/501100011033 and by "ERDF A way of making Europe", and by the Government of Aragón [Grant Grupo AESA A06_23R]. M.M. and I.G.-P. were supported by a predoctoral contract financed by the Government of Aragón (Spain). J.A. was supported by a predoctoral contract financed by the Vice-Rectorate for Scientific Policy of the University of Zaragoza, within the framework of the European University UNITA, under a cotutelle agreement. A.F. was supported by a predoctoral contract PRE2022–102372 funded by MICIU/AEI/10.13039/501100011033 and by "ESF Investing in your future". N.M. was supported by the postdoctoral contract Odón de Buen funded by the Government of Aragón (Spain).

CRediT authorship contribution statement

María Mei Martínez de Zuazo: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Jorge Andaluz-Arbe:** Investigation. **Alberto Fau:** Investigation, Formal analysis, Data curation. **Ivo García-Penas:** Formal analysis, Data curation. **Rafael Pagán:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Diego García-Gonzalo:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Natalia Merino:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation, 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.

Acknowledgments

No acknowledgments.

Data availability

Data will be made available on request.

References

Ahmad, M., Aduru, S. V., Smith, R. P., Zhao, Z., & Lopatkin, A. J. (2025). The role of bacterial metabolism in antimicrobial resistance. *Nature Reviews Microbiology, 23*(7), 439–454. https://doi.org/10.1038/s41579-025-01155-0

Augusto, P. E. D., Soares, B. M. C., & Castanha, N. (2018). Conventional technologies of food preservation. In F. J. Barba, A. S. Sant'Ana, V. Orlien, & M. Koubaa (Eds.), *Innovative technologies for food preservation: Inactivation of Spoilage and Pathogenic Microorganisms* (pp. 3–23). Academic Press. https://doi.org/10.1016/B978-0-12-811031-7.00001-7.

Berdejo, D., Chueca, B., Pagán, E., Renzoni, A., Kelley, W. L., Pagán, R., & García-Gonzalo, D. (2019). Sub-inhibitory doses of individual constituents of essential oils can select for *Staphylococcus aureus* resistant mutants. *Molecules (Basel, Switzerland), 24*(1), 170. https://doi.org/10.3390/molecules24010170. Article.

Berdejo, D., Gayán, E., Pagán, E., Merino, N., Campillo, R., Pagán, R., & García-Gonzalo, D. (2022). Carvacrol selective pressure allows the occurrence of genetic resistant variants of *Listeria monocytogenes* EGD-e. *Foods (Basel, Switzerland), 11*(20), 3282. https://doi.org/10.3390/foods11203282. Article.

Bintsis, T. (2017). Foodborne pathogens. *AIMS Microbiology, 3*(3), 529–563. https://doi.org/10.3934/microbiol.2017.3.529

Biswas, R., Sonenshein, A. L., & Belitsky, B. R. (2020). Genome-wide identification of *Listeria monocytogenes* CodY-binding sites. *Molecular Microbiology, 113*(4), 841–858. https://doi.org/10.1111/mmi.14449

Bland, R., Waite-Cusic, J., Weisberg, A. J., Riutta, E. R., Chang, J. H., & Kovacevic, J. (2021). Adaptation to a commercial quaternary ammonium compound sanitizer leads to cross-resistance to select antibiotics in *Listeria monocytogenes* isolated from fresh produce environments. *Frontiers in Microbiology, 12*, Article 782920. https://doi.org/10.3389/fmicb.2021.782920. Article.

Bolten, S., Harrand, A. S., Skeens, J., & Wiedmann, M. (2022). Nonsynonymous mutations in *fepR* are associated with adaptation of *Listeria monocytogenes* and other *Listeria* spp. To low concentrations of benzalkonium chloride but do not increase survival of *L. monocytogenes* and other *Listeria* spp. After exposure to benzalkonium chloride concentrations recommended for use in food processing environments. *Applied and Environmental Microbiology, 88*(11), Article e0048622. https://doi.org/10.1128/aem.00486-22. Article.

Byun, K.-H., Ha Han, S., Woo Choi, M., Hong Park, S., & Ha, S.-D. (2022). Effect of sublethal concentrations of bactericidal antibiotics on mutation frequency and stress response of *Listeria monocytogenes*. *Food Research International, 151*, Article 110903. https://doi.org/10.1016/j.foodres.2021.110903. Article.

Camejo, A., Buchrieser, C., Couvé, E., Carvalho, F., Reis, O., Ferreira, P., … Cabanes, D. (2009). In vivo transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection. *PLOS Pathogens, 5*(5), Article e1000449. https://doi.org/10.1371/journal.ppat.1000449

Campillo, R., García-Penas, I., López, N., Sánchez, A., Fau, A., Gómez, D., Berdejo, D., García-Gonzalo, D., & Pagán, R. (2025). Ciprofloxacin-resistant *Salmonella typhimurium* demonstrates cross-resistance to heat treatments in liquid food matrices. *Food Research International, 210*, Article 116330. https://doi.org/10.1016/j.foodres.2025.116330. Article.

Carvalho, F., Atilano, M. L., Pombinho, R., Covas, G., Gallo, R. L., Filipe, S. R., … Cabanes, D. (2015). L-rhamnosylation of *Listeria monocytogenes* Wall Teichoic Acids promotes resistance to antimicrobial peptides by delaying interaction with the membrane. *PLOS Pathogens, 11*(5), Article e1004919. https://doi.org/10.1371/journal.ppat.1004919

Clinical and Laboratory Standards Institute (CLSI). (2017). In P. A. Wayne (Ed.), *CLSI supplement M100: Performance Standards for Antimicrobial Susceptibility Testing Institute (27th ed)*.

Douarre, P.-E., Sévellec, Y., Le Grandois, P., Soumet, C., Bridier, A., & Roussel, S. (2022). FepR as a central genetic target in the adaptation to quaternary ammonium compounds and cross-resistance to ciprofloxacin in *Listeria monocytogenes*. *Frontiers in Microbiology, 13*. https://doi.org/10.3389/fmicb.2022.864576

Escolar, C., Gómez, D., Del Carmen Rota García, M., Conchello, P., & Herrera, A. (2017). Antimicrobial resistance profiles of *Listeria monocytogenes* and *Listeria innocua* isolated from ready-to-eat products of animal origin in Spain. *Foodborne Pathogens Disease, 14*(6), 357–363. https://doi.org/10.1089/fpd.2016.2248

European Medicines Agency (EMA). (2025a). Categorisation of antibiotics in the European Union: Answer to the request from the European Commission for updating the scientific advice on the impact on public health and animal health of the use of antibiotics in animals (EMA/CVMP/CHMP/682198/2017-Rev.1). Retrieved from https://www.ema.europa.eu/en/documents/report/categorisation-antibiotics-european-union-answer-request-european-commission-updating-scientific-advice-impact-public-health-animal-health-use-antibiotics-animals_en.pdf. Accessed March 5, 2026.

European Medicines Agency (EMA). (2025b). European sales and use of antimicrobials for veterinary medicine: Annual surveillance report 2024. ISBN 978-92-9155-147-7. Retrieved from: https://www.ema.europa.eu/en/documents/report/european-sales-use-antimicrobials-veterinary-medicine-annual-surveillance-report-2024_en.pdf. Accessed March 5, 2026.

European Food Safety Authority (EFSA) & European Centre for Disease Prevention and Control (ECDC). (2025). The European Union one health 2024 zoonoses report. *EFSA Journal, 23*(12), e9759. https://doi.org/10.2903/j.efsa.2025.9759

Foster, T. J. (2017). Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. *FEMS Microbiology Reviews, 41*(3), 430–449. https://doi.org/10.1093/femsre/fux007

Guérin, A., Bridier, A., Le Grandois, P., Sévellec, Y., Palma, F., Félix, B., … Soumet, C. (2021). Exposure to quaternary ammonium compounds selects resistance to ciprofloxacin in *Listeria monocytogenes*. *Pathogens (Basel, Switzerland), 10*(2). https://doi.org/10.3390/pathogens10020220

Guérin, F., Galimand, M., Tuambilangana, F., Courvalin, P., & Cattoir, V. (2014). Overexpression of the novel MATE fluoroquinolone efflux pump FepA in *Listeria monocytogenes* is driven by inactivation of its local repressor FepR. *PLOS One, 9*(9), Article e106340. https://doi.org/10.1371/journal.pone.0106340

Guerreiro, D. N., Arcari, T., & O'Byrne, C. P (2020). The σb-mediated general stress response of *Listeria monocytogenes*: Life and death decision making in a pathogen. *Frontiers in Microbiology, 11*, Article 1505. https://doi.org/10.3389/fmicb.2020.01505

Jiang, X., Yu, T., Xu, P., Xu, X., Ji, S., Gao, W., & Shi, L. (2018). Role of efflux pumps in the in vitro development of ciprofloxacin resistance in *Listeria monocytogenes*. *Frontiers in Microbiology, 9*, Article 2350. https://doi.org/10.3389/fmicb.2018.02350

Knudsen, G. M., Fromberg, A., Ng, Y., & Gram, L. (2016). Sublethal concentrations of antibiotics cause shift to anaerobic metabolism in *Listeria monocytogenes* and induce phenotypes linked to antibiotic tolerance. *Frontiers in Microbiology, 7*, Article 1091. https://doi.org/10.3389/fmicb.2016.01091

Liao, X., Ma, Y., Daliri, E. B.-M., Koseki, S., Wei, S., Liu, D., Ye, X., Chen, S., & Ding, T. (2020). Interplay of antibiotic resistance and food-associated stress tolerance in foodborne pathogens. *Trends in Food Science & Technology, 95*, 97–106. https://doi.org/10.1016/j.tifs.2019.11.006

Lima, V., Pinto, C. A., & Saraiva, J. A. (2023). The dependence of microbial inactivation by emergent nonthermal processing technologies on pH and water activity. *Innovative Food Science & Emerging Technologies, 89*, Article 103460. https://doi.org/10.1016/j.ifset.2023.103460. Article.

Lobel, L., & Herskovits, A. A. (2016). Systems level analyses reveal multiple regulatory activities of CodY controlling metabolism, motility and virulence in *Listeria monocytogenes*. *PLOS Genetics, 12*(2), Article e1005870. https://doi.org/10.1371/journal.pgen.1005870

Lopatkin, A. J., Bening, S. C., Manson, A. L., Stokes, J. M., Kohanski, M. A., Badran, A. H., Earl, A. M., Cheney, N. J., Yang, J. H., & Collins, J. J. (2021). Clinically relevant mutations in core metabolic genes confer antibiotic resistance. *Science (New York, N. Y.), 371*(6531), eaba0862. https://doi.org/10.1126/science.aba0862

Markkula, A., Lindström, M., Johansson, P., Björkroth, J., & Korkeala, H. (2012). Roles of four putative DEAD-box RNA helicase genes in growth of *Listeria monocytogenes* EGD-e under heat, pH, osmotic, ethanol, and oxidative stress conditions. *Applied and Environmental Microbiology, 78*(19), 6875–6882. https://doi.org/10.1128/aem.01526-12

Martini, A. M., Alexander, S. A., & Khare, A. (2025). Mutations in the *Staphylococcus aureus* Global Regulator CodY confer tolerance to an interspecies redox-active antimicrobial. *PLOS Genetics, 21*(3), Article e1011610. https://doi.org/10.1371/journal.pgen.1011610

Meireles, D., Pombinho, R., Carvalho, F., Sousa, S., & Cabanes, D. (2020). *Listeria monocytogenes* wall teichoic acid glycosylation promotes surface anchoring of virulence factors, resistance to antimicrobial peptides, and decreased susceptibility to antibiotics. *Pathogens (Basel, Switzerland), 9*(4), 290. https://doi.org/10.3390/pathogens9040290. Article.

Merino, N., Berdejo, D., Pagán, E., Girard, C., Kerros, S., Spinozzi, E., Pagán, R., & García-Gonzalo, D. (2023). Phenotypic and genotypic comparison of antimicrobial-resistant variants of *Escherichia coli* and *Salmonella typhimurium* isolated from evolution assays

with antibiotics or commercial products based on essential oils. *Pharmaceuticals (Basel, Switzerland), 16*(10), 1443. https://doi.org/10.3390/ph16101443. Article.
Mira, P., Lozano-Huntelman, N., Johnson, A., Savage, V. M., & Yeh, P. (2022). Evolution of antibiotic resistance impacts optimal temperature and growth rate in *Escherichia coli* and *Staphylococcus epidermidis*. *Journal of Applied Microbiology, 133*(4), 2655–2667. https://doi.org/10.1111/jam.15736
Munita, J. M., & Arias, C. A. (2016). Mechanisms of antibiotic resistance. *Microbiology Spectrum, 4*(2), Article VMBF-0016-2015. https://doi.org/10.1128/microbiolspec.VMBF-0016-2015
Netterling, S., Bäreclev, C., Vaitkevicius, K., & Johansson, J. (2015). RNA helicase important for *Listeria monocytogenes* hemolytic activity and virulence factor expression. *Infection and Immunity, 84*(1), 67–76. https://doi.org/10.1128/iai.00849-15
Olaimat, A. N., Al-Holy, M. A., Shahbaz, H. M., Al-Nabulsi, A. A., Abu Ghoush, M. H., Osaili, T. M., … Holley, R. A. (2018). Emergence of antibiotic resistance in *Listeria monocytogenes* isolated from food products: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety, 17*(5), 1277–1292. https://doi.org/10.1111/1541-4337.12387
Rippa, A., Bilei, S., Peruzy, M. F., Marrocco, M. G., Leggeri, P., Bossù, T., & Murru, N. (2024). Antimicrobial resistance of *Listeria monocytogenes* strains isolated in food and food-processing environments in Italy. *Antibiotics, 13*(6), 525. https://doi.org/10.3390/antibiotics13060525. Article.
Russell, A. D. (2003). Lethal effects of heat on bacterial physiology and structure. *Science Progress, 86*(1), 115–137. https://doi.org/10.3184/003685003783238699
Samtiya, M., Matthews, K. R., Dhewa, T., & Puniya, A. K. (2022). Antimicrobial resistance in the food chain: Trends, mechanisms, pathways, and possible regulation strategies. *Foods,, 11*(19), 2966. https://doi.org/10.3390/foods11192966. Article.
Shariati, A., Arshadi, M., Khosrojerdi, M. A., Abedinzadeh, M., Ganjalishahi, M., Maleki, A., Heidary, M., & Khoshnood, S. (2022). The resistance mechanisms of bacteria against ciprofloxacin and new approaches for enhancing the efficacy of this antibiotic. *Frontiers in Public Health, 10*, Article 1025633. https://doi.org/10.3389/fpubh.2022.1025633. Article.
Silva, A., Silva, V., Gomes, J. P., Coelho, A., Batista, R., Saraiva, C., … Poeta, P. (2024). *Listeria monocytogenes* from food products and food associated environments: Antimicrobial resistance, genetic clustering and biofilm insights. *Antibiotics (Basel, Switzerland), 13*(5). https://doi.org/10.3390/antibiotics13050447
Tavares da Silva, R., García-Penas, I., Espina, L., García-Gonzalo, D., Magnani, M., & Pagán, R. (2025). Meropenem-induced cross-protection in Salmonella enterica resistant variants: insights from adaptive laboratory evolution and whole genome sequencing. *Food Microbiology, 132*, 104823. https://doi.org/10.1016/j.fm.2025.104823
UniProtKB. (2025). Lmo2531. Retrieved from https://www.uniprot.org. Accessed November 24, 2024.
Walsh, D., Sheridan, J. J., Duffy, G., Blair, I. S., McDowell, D. A., & Harrington, D. (2001). Thermal resistance of wild-type and antibiotic-resistant *Listeria monocytogenes* in meat and potato substrates. *Journal of Applied Microbiology, 90*(4), 555–560. https://doi.org/10.1046/j.1365-2672.2001.01284.x
World Health Organization (WHO). (2023). Antimicrobial resistance. Retrieved from https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance. Accessed November 28, 2024.
World Health Organization (WHO). (2025). Foodborne diseases. Retrieved from https://www.who.int/health-topics/foodborne-diseases. Accessed January 29, 2025.
World Health Organization (WHO). (2024). WHO list of medically important antimicrobials: A risk management tool for mitigating antimicrobial resistance due to non-human use. ISBN 978-92-4-008461-2. Retrieved from https://cdn.who.int/media/docs/default-source/gcp/who-mia-list-2024-lv.pdf. Accessed November 15, 2024.
Zhou, Q., Wang, L., Yin, X., Feng, X., Shang, J., & Luo, Q. (2012). SigB-dependent tolerance to protein synthesis-inhibiting antibiotics in *Listeria monocytogenes* EGD-e. *Current Microbiology, 64*(3), 234–241. https://doi.org/10.1007/s00284-011-0058-3