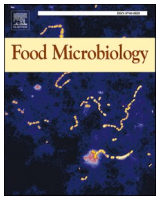
Food Microbiology 136 (2026) 105014

Contents lists available at ScienceDirect

Food Microbiology

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



Insights on the microbial resistance mechanisms of *Listeria monocytogenes* to Pulsed Electric Fields (PEF) treatments

Fotios Lytras [a], Muhammad Ahmed Ihsan [a], Georgios Psakis [a,c,*], Ruben Gatt [b], Guillermo Cebrián [d], Javier Raso [d], Vasilis P. Valdramidis [a,e,**]

[a] University of Malta, Faculty of Health Sciences, Department of Food Sciences & Nutrition, MSD 2080, Malta
[b] University of Malta, Faculty of Science, Metamaterials Unit, MSD 2080, Malta
[c] Malta College of Arts and Sciences, Institute of Applied Sciences, Main Campus, Paola, PLA 9032, Malta
[d] Food Technology, Facultad de Veterinaria, Instituto Agroalimentario de Aragón-IA2, Universidad de Zaragoza-CITA, Zaragoza, Spain
[e] National and Kapodistrian University of Athens, Department of Chemistry, Laboratory of Food Chemistry, Panepistimiopolis Zografou, Athens, 157 84, Greece

ARTICLE INFO

Keywords:
Pulsed electric fields
Listeria monocytogenes EGD-e
Mechanism of action
Transcriptomics
Assessment of mutants

ABSTRACT

This study aimed to identify the principal mechanisms of action by which *Listeria monocytogenes* EGD-e responds to pulsed electric field (PEF) treatments at pH 7.0, given its recognition as a robust target microorganism and strain. Microbiologically challenged buffer samples (pH 7.0) were subjected to pulses with an electric field strength of 20 kV/cm and their transcriptional response was assessed using RNA sequencing. Our analysis revealed 119 differentially expressed genes, 51 of which were upregulated and 68 downregulated. From the 51 upregulated genes, 4 were transcription regulators (*lmo1974, glnR, lmo806* and *lmo0371*) with the potential to influence the resistance of *L. monocytogenes* EGD-e. Additionally, assessment of 11 isogenic mutants at a PEF treatment (20 kV/cm, 184 kJ/kg) relative to the wild type identified the Δ*yneA* and Δ*clpB* deletion mutants as more resistant and more sensitive ($p < 0.05$). Finally, the isogenic mutant Δ*clpB* was assessed against the wild type at 25 kV/cm at different total specific energies (54, 113, 135 and 160 kJ/kg) resulting in statistical difference($p < 0.05$) only under the highest parameter. In conclusion, transcriptomic analysis revealed that the primary mechanistic pathways of *L. monocytogenes* in response to PEF involve the preservation of homeostasis, energy availability, and quorum sensing. Additionally, the increased sensitivity of the Δ*clpB* mutant highlights a supplementary mechanism related to protein disaggregation and refolding under high-energy. These findings suggest that *L. monocytogenes* mounts a complex and multifaceted response to PEF treatments. These results can provide insights and support PEF treatment decontamination alone or as pretreatment in combination with other hurdles.

1. Introduction

L. monocytogenes is a Gram-positive, rod shaped, facultative anaerobic, non-spore forming foodborne bacterium that can be found in a variety of environments, including soil, water, animal feeds, faecal matter, and the tissues of infected animals (Paudyal and Karatzas, 2016). *L. monocytogenes* can also be found in a variety of food products including milk, dairy products, fruit juices, vegetable juices, whole eggs, egg yolks, liquid eggs and mushrooms (Brackett and Beuchat, 1991; Mosqueda-Melgar et al., 2008; Kirchner et al., 2025). Phenotypic and molecular subtyping studies classify *Listeria monocytogenes* isolates into four lineages (I-IV), with most isolates falling under lineages I and II; lineage I includes serotypes such as 1/2b, 3b, 3c, and 4b, while lineage II comprises serotypes 1/2a, 1/2c, and (Wiedmann, 2002). From these *L. monocytogenes* serotypes, 1/2a, 1/2b, and 4b are mostly identified in clinical samples (Gorski, Flaherty and Mandrell, 2006). After consumption of *L. monocytogenes* contaminated foods/beverages, infected people may have a less severe, non-invasive listeriosis with mild symptoms including fever, muscle aches, nausea, vomiting and diarrhoea or a severe form of listeriosis, invasive and threatening with symptoms including headache, stiff neck, confusion, loss of balance and convulsions (U.S. Food and Drug Administration, 2025). Severe

* Corresponding author. University of Malta, Faculty of Health Sciences, Department of Food Sciences & Nutrition, MSD 2080, Malta
** Corresponding author. University of Malta, Faculty of Health Sciences, Department of Food Sciences & Nutrition, MSD 2080, Malta.
E-mail addresses: vasilis.valdramidis@um.edu.mt, Vasilis.Valdramidis@chem.uoa.gr (V.P. Valdramidis).

https://doi.org/10.1016/j.fm.2025.105014
Received 13 July 2025; Received in revised form 30 October 2025; Accepted 10 December 2025
Available online 11 December 2025

listeriosis is a public health concern worldwide due to its high morbidity and mortality with groups such as infants, pregnant women, elderly and immunocompromised individuals to be considered at high risk (U.S. Food and Drug Administration, 2025)). In 2023, 27 members of European Union (EU) reported 2952 cases of invasive human listeriosis corresponding to a EU notification rate of 0.66 cases per 100000 population which is the highest since 2007 (EFSA, 2023).

L. monocytogenes has the ability to survive and grow under extreme conditions including low temperatures, high acidity, high osmolarity (Mosqueda-Melgar et al., 2008; Bae, Crowley and Wang, 2011). This ability of *L. monocytogenes* to survive and proliferate under different environmental conditions is due to the complex gene regulatory network (Dou et al., 2024). In *L. monocytogenes*, the alternative sigma factor Sigma B (σB), and the virulence regulator (positive regulatory factor A) PrfA form overlapping transcriptional networks that enable transit from stress to virulence (Sibanda and Buys, 2022). The SigB is central to the robustness as a general stress response regulator which control the transcriptional response of approximately 300 genes (Guerreiro, Arcari and O'Byrne, 2020). *L. monocytogenes* mutants with deleted the *sigB* gene has shown sensitivity compared to the wild type for osmotic stress (Fraser et al., 2003; Sue et al., 2004), acid stress (Ferreira, O'Byrne and Boor, 2001; Sue et al., 2004; Wemekamp-Kamphuis et al., 2004), heat resistance (Somolinos et al., 2010) and high hydrostatic pressure (Wemekamp-Kamphuis et al., 2004). Additionally, depending on the type of stress, different genes can influence the adaptability of *L. monocytogenes*, such as the heat shock genes (*dnaK, dnaJ, groES*, and *groEL*, *clpP*, *clpC*, *clpE*, and *clpB*) under heat stress (Sibanda and Buys, 2022), transport systems (such as *betL gbuA*, *gbuB*, *gbuC, opuCA*, *opuCB*, *opuCC*, and *opuCD*) under hyperosmotic stress (Fraser et al., 2003), the glutamate decarboxylase (GAD) acid resistance system under acidic stress (Wemekamp-Kamphuis et al., 2004), and genes including the anti-oxidative *kat* gene, *sod* gene (superoxide dismutase), *fri* which encode ferritin detoxifies oxidative agents, and *perR* peroxide operon regulator under oxidative stress (Bucur et al., 2018).

Understanding the mechanisms that enable *Listeria monocytogenes* to adapt to various food processing environments, such as pulsed electric field (PEF) treatments, is crucial for evaluating the effectiveness of these interventions and their impact on the microbial ecology of food contaminants (Lytras et al., 2024b). Pulsed electric field (PEF) technology is described as a ''non-thermal'' method with potential as an alternative to thermal treatments for microbial inactivation due to its minimal impact on the nutritional value of products and its effective microbial inactivation capability (Peng et al., 2020). In general, PEF utilizes high-voltage electric pulses of a short duration (from micro-to milli--seconds) to increase the permeability of cell membranes, with an electric field strength from 15 to 40 kV/cm for microbial inactivation (Raso et al., 2016). The selection of PEF treatment parameters can lead to either reversible or irreversible electroporation, with the treatment intensity determining whether the electroporation results in temporary or permanent damage to microbial cells (Jaeger et al., 2009; Martínez et al., 2016). At an industrial scale, PEF technology is used at an electric field strength from 10 to 20 kV/cm for microbial inactivation (Toepfl, 2012). More specifically, PEF is based on the phenomenon of electroporation (or electropermeabilization) as a primary mechanism, whereby applying an electric field to microbial cells increases membrane permeability (Heinz et al., 2001). Depending on the PEF treatment conditions, the electroporation can be reversible or irreversible, with the intensity of the treatment determining whether the damage to microbial cells is temporary or permanent (Weaver and Chizmadzhev, 1996; Jaeger et al., 2009). The increase of the total specific energy leads to an increase in the medium's temperature which is known as Joule or Ohmic heating effect, and potentially due to thermal effects can enhance microbial inactivation while also impacting the product's quality characteristics (Schottroff et al., 2018). Thus, an increase of PEF treatment temperature can play a role in the inactivation efficiency by enhancing the permeability of cell membranes and/or microbial inactivation (Raso et al., 2016). Furthermore, the effectiveness of the technology for microbial inactivation is influenced by multiple factors including: microbial characteristics (such as species, strain, size and shape) (Heinz et al., 2001; Saldaña et al., 2009), growth phase of the microorganism (Álvarez et al., 2002), environmental conditions (such as pH, conductivity and water activity (a_W) (García et al., 2005a) and treatment parameters (including electric field strength, treatment time and total specific energy etc.) (Saldaña et al., 2009, 2010).

Industrially, the use of PEF has been regulated for commercial pasteurisation of fruit juices with the requirement of a 5 $\log_{10}$ reductions for the most resistant food-borne pathogens (U.S. Food and Drug Administration, 2000). Research has shown that under PEF treatments and in acidic conditions (pH 4.0) *L. monocytogenes* exhibit higher sensitivity and higher microbial inactivation compared to *E. coli*. This suggests that acidic environment may act as an additional hurdle for *L. monocytogenes*, making the microorganism less of a primary microbial target (Saldaña et al., 2009). However, studies have highlighted the resilience of *L. monocytogenes* under PEF at neutral pH (7.0) in comparison to other microorganisms, including *Escherichia coli*, *Lactiplantibacillus* plantarum, *Saccharomyces cerevisiae*, *Staphylococcus aureus*, *Salmonella* Typhimurium (Saldaña et al., 2009; Lytras et al., 2024a). This neutral pH is commonly associated with food products close to this pH value, such as liquid whole eggs, egg whites, and milk (Brackett and Beuchat, 1991; Mosqueda-Melgar et al., 2008). Additionally, this resistance of *L. monocytogenes* has also been observed in low-acid juices like those of watermelon (pH:5.46 ± 0.11) and melon (pH:5.82 ± 0.04), where *L. monocytogenes* showed lower inactivation rates in comparison to *E. coli* and *Salmonella* Enteritidis (Mosqueda-Melgar, Raybaudi-Massilia and Martín-Belloso, 2007). This phenomenon has been attributed to the size and shape of the microorganism which may provide a survival advantage during PEF treatments (Heinz et al., 2001).

The aim of the current study is to unravel the adaptation and stress-induced responses of *L. monocytogenes* by PEF treatment at pH 7.0. Additionally, the role of specific genes on the resistance and sensitivity of *L. monocytogenes* is evaluated with an overall aim to improve the understanding of the main cellular networks of *L. monocytogenes* likely to account for its resistance under PEF treatments.

2. Materials & methods

2.1. Bacterial strains and growth conditions

L. monocytogenes EGD-e strain and its 11 isogenic mutants (Δ*gadD1,* Δ*gadD2*, Δ*gadD3*, Δ*lmo0913,* Δ*sigB*, Δ*recA*, Δ*yneA*, Δ*hrcA,* Δ*clpB*, Δ*mogR*, Δ*dnaK*) were used in this study (Table 1). The microbial strains were stored at −70 °C by mixing volumes of log phase culture with glycerol (Merck, Germany) to achieve a freezing mix (70:30). Stock cultures were re-activated by inoculation onto solid media of Tryptic Soya Agar (Scharlab Spain) plates. The primary inoculum was prepared by selecting a single colony from the re-activated plate and incubating it in Tryptic Soya Broth without dextrose TSB-D (Scharlab, Spain) at 37 °C for 24 ± 2 h with shaking at 120 rpm. A subculture was also prepared in TSB-D (1 % v/v) at the same temperature at 37 °C and for 17 ± 1 h with shaking at 120 rpm allowing the bacteria to reach the stationary phase (10^8–10^9 log CFU/mL). After incubation, the culture was centrifuged (3000×*g*) for 20 min and washed with Phosphate Buffer Saline solution (PBS, Oxoid United Kingdom). Hereafter, the pellet was resuspended: a) in citrate-phosphate McIlvaine buffer (combination of citric acid and disodium hydroxide phosphate), of pH 7.0 ± 0.1 (Dawson et al., 1974) with a set conductivity of 2 mS/cm (measured at 20–25 °C; Alhborn conductivity probe (Alhborn, Almemo, Germany).

2.2. PEF treatment conditions

In this study, the same PEF system and set-up as previously described by Lytras et al. (2024) was used for both the transcriptomics and the

Table 1
Information on deleted genes of *L. monocytogenes* EGD-e isogenic mutants studied.

Gene	Protein	Function	Reference
gadD1, lmo0447	Glutamate decarboxylase	Amino acid transport and metabolism	(Feehily and Karatzas, 2013; Feehily, 2014; Boura, Brensone and Karatzas, 2020; Feehily and Karatzas, 2013; Feehily, 2014; Boura, Brensone and Karatzas, 2020)
gadD2, lmo2363	Glutamate decarboxylase	Amino acid transport and metabolism	(Feehily and Karatzas, 2013; Feehily, 2014; Boura, Brensone and Karatzas, 2020)[1]
gadD3, lmo2434	Glutamate decarboxylase	Amino acid transport and metabolism	(Feehily and Karatzas, 2013; Feehily, 2014; Boura, Brensone and Karatzas, 2020)
lmo0913	Succinate-semialdehyde dehydrogenase [NAD], Succinate-semialdehyde dehydrogenase [NADP+]	Energy production and conversion	(Feehily, O'Byrne and Karatzas, 2013; Boura, Brensone and Karatzas, 2020)
sigB, lmo0895	RNA polymerase sigma factor SigB	Transcription	Wemekamp-kamphuis et al. (2004)
recA, lmo1398	Recombinase A	Replication, recombination and repair	Veen et al. (2010)
yneA	Hypothetical protein	SOS response	Veen et al. (2010)
hrcA, lmo1475	Heat-inducible transcription repressor	Transcription	Veen and Abee (2010b)
clpB, lmo2206	ClpB protein	Posttranslational modification, protein turnover, chaperones	van der Veen et al. (2007)
mogR, lmo0674	Motility gene repressor MogR	Transcription	Veen et al. (2009)
dnaK, lmo1473	Molecular chaperone DnaK	Posttranslational modification, protein turnover, chaperones	Veen and Abee (2010b)

assessment of the resistance of *Listeria monocytogenes* mutants. Specifically, treatments were applied in continuous flow (5 L/h) using a treatment chamber equipped with parallel electrodes (0.4 cm gap, 3 cm length, 0.5 cm width). An electric field strength of 20 kV/cm was applied, with total treatment durations of 64.5 and 137.5 μs. These durations were calculated based on the calculation of residence time x frequency x pulse width, with residence time equal to 0.43 s, pulse frequencies of 30 and 64 Hz, and pulse width of 5 μs. The corresponding specific energy inputs were 88 kJ/kg and 184 kJ/kg, resulting in outlet temperatures of 41 ± 0.3 °C and 64 ± 0.7 °C, respectively. The total specific energy was estimated by calculating the temperature increase during pulses under presumed adiabatic conditions (Heinz et al., 2001) according to the following equation:

$$W = (T_{outlet} - T_{inlet}) \times C_p \quad (1)$$

where T_{outlet} is the temperature of the sample after the PEF treatment, T_{inlet} is the temperature of the sample just before entering the treatment chamber, and C_p is the specific heating capacity (C_p water: 4.186 kJ/kg in 20 °C). Furthermore, a higher electric field strength of 25 kV/cm was selected that can achieve greater microbial reductions for cross-validate our results and to assess the Log_{10} microbial reduction of Δ*clpB* against the wild type *L. monocytogenes* EGD-e in four different total specific energies. Specifically, an electric field strength of 25 kV/cm, with treatment times of 25.9, 53.3, 63.6, and 75.7 μs, outlet temperatures of 33 ± 0.3, 47 ± 0.5, 52.2 ± 0.5, 58.3 ± 0.7 °C and total specific energies of 54, 113, 135 and 160 kJ/kg were applied. Following PEF treatment, all samples were cooled within 5 s or less to maintain temperature control after processing, ensuring the temperature remained below 20 °C at the point of collection.

2.3. Transcriptomic analysis

2.3.1. Transcriptomic analysis: preliminary assessment of microbial viability

Three biological samples were analysed for the PEF treatment (20 kV/cm, 88 kJ/kg). Under this condition the log_{10} reduction was <1 (Log_{10} CFU/mL) for all the biological samples in accordance with previously presented results (Lytras et al., 2024). For enumeration purposes, the untreated and treated cell suspensions were diluted in (PBS) and 0.1 mL of the diluted sample was used for plating. The media used for the enumeration of the viable cells were the TSA agar and a selective medium (PALCAM). The samples were incubated for 48 ± 2 h at 37 °C. The use of selective medium was for the enumeration of the sublethal population. After incubation, 30–300 colonies were counted. Colony counts corresponded to the viable microorganisms were expressed as colony-forming unit per millilitre (CFU/mL) or its decimal logarithm (Log_{10} CFU/mL). The survival fraction was determined by dividing the number of microorganisms that persist after the treatment (N_t) with the initial count of viable cells (N_0).

$$\log_{10}\ reduction = \log_{10}\left(\frac{N_t}{N_0}\right) \quad (2)$$

2.3.2. Transcriptomic analysis: RNA extraction

For the RNA-extraction, after treatment, 15 mL of the treated and untreated samples were collected to falcon tubes and centrifuged for (3000×*g*) for 8 min at 4 °C. The resulting pellets were collected and resuspended to 1.5 mL for achieving a higher concentration (10^9-10^{10} log CFU/mL) and transferred to Eppendorf tubes. Subsequently, the suspensions were centrifuged to a microcentrifuge (10000×*g*) for 4 min at 4 °C. A protocol similar to the one described from Nadal et al. (2024) was used. The collected pellets were resuspended to 1.5 mL trizol (TRI Reagent, Sigma Aldrich) and subjected to a beat beating with Mini bead beater (Biospec) for 4 cycles each consisting of 1 min of beating followed by intermediate cooling on ice for 1 min after each cycle. After a 5-min incubation at ambient temperature, the sample's supernatant was transferred to a new tube, and 0.3 mL of chloroform (chloroform 99.5 %, Sigma Aldrich) was added. The sample was shaken for 15 s, incubated for 3 min at ambient temperature, and then centrifuged in a microcentrifuge (12000×*g*) for 15 min at 4 °C. Hereafter, the supernatant was removed (200 μL), lysis buffer was added (700 μL), and RNA extraction and purification were carried out using the standard Qiagen RNeasy Mini kit protocol. The RNA yield and purity were spectrophotometrically measured using the Spectrophotometer DS-11 FX (DeNovix, USA) and were then frozen at −80 °C.

2.3.3. Transcriptomic analysis: RNA sequencing, library construction and gene ontology analysis

Library construction and RNA Sequencing were conducted by Macrogen Inc. (Seoul, Republic of Korea). The RNA quantification was carried out using the fluorometric method with the 2100 Bioanalyzer Instrument (Agilent Technologies, United Kingdom). RNA samples with RNA integrity number (RIN) ≥ 6.8 were used for library preparation.

The RNA samples underwent rRNA depletion using the NEB Next rRNA depletion kit initially. Following this, the library preparation was conducted utilizing the Illumina TruSeq Stranded Total RNA (NEB Microbe) kit. Paired-end sequence reads were then generated on the Illumina NovaSeq 6000 system, with 2 × 100-bp reads. The generated FASTQ read sequences underwent processing, with expression profiles quantified as read counts. Normalization factors were computed, considering both transcript length and coverage depth. Raw reads underwent trimming, and the counts corresponding to mapped reads were normalized using the Fragments Per Kilobase of Transcript per Million Mapped Reads (FPKM) metric. Poor quality reads were removed, ensuring a paired score of >30 for all runs. Additionally, samples with more than one read count value of 0 were excluded from the analysis. *Listeria monocytogenes* EGD-e whole transcriptome sequencing was conducted to examine the different gene expression profiles, and to perform gene annotation on set of useful genes based on gene ontology pathway information. To map cDNA fragments obtained from RNA sequencing, ASM19603v1 was used as a reference genome (NCBI, US). Differential gene expression (DEG) analysis was performed on a comparison pair between treated and untreated samples and it included the application of statistical hypothesis testing techniques, including the fold change (FC), and *p-value* (Love, Huber and Anders, 2014). Identification of gene clusters ensued through the implementation of the hierarchical clustering method with Pearson correlation coefficient as a distance metric on iDEP (v2.01) online tool. To address the challenge of testing multiple genes concurrently, *p-values* underwent further adjustment via the Benjamini-Hochberg false discovery rate (FDR) method (Yoav Benjamini and Yosef Hochberg, 1995). These results were organized based on their expression levels. To understand the mechanisms and pathways involved, the identified genes were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis classifying gene functions in three main ontologies: molecular function, cellular component, and biological process.

2.3.4. Transcriptomics analysis: statistical analysis and software

Differential expression analysis of genes (DEGs) was performed using the edgeR and DESeq2 methods on iDEP.(v2.01) online tool (Ge et al., 2018). The heatmap were also conducted using iDEP (v2.01). The outcomes from these analyses included log2 fold changes (log2FC), and Benjamini-Hochberg false discovery rates (FDRs). In this study, the false discovery rate (FDR) criterium was set as 0.1 and log2FC > 1 for the screening of DEGs. To ensure data integrity, an initial quality assessment was conducted by Macrogen Inc. (Seoul, Republic of Korea) using FastQC version v0.11.7,(Bionfiromatics, Babahram, UK) and subsequent sequence trimming was performed using the Trimmomatic program (v0.38). DEGs list was further analysed with DAVID Bioinformatics Resources tool (http://david.abcc.ncifcrf.gov/) for gene functional annotations, set enrichment analyses per biological process (BP), cellular component (CC) and molecular function (MF), and KEGG analysis (Huang, Sherman and Lempicki, 2009; Sherman et al., 2022).

2.4. Assessment of mutants

2.4.1. Enumeration of viable cells

Three biological and two technical replicates were performed for *L. monocytogenes* wild type and its isogenic mutants. The untreated and treated cell suspensions were diluted in (PBS) and 0.1 mL of the diluted sample was used for plating. The media used for the enumeration of the viable cells were (TSA) and the samples were incubated for 48 ± 2 h at 37 °C. After incubation, counting of viable cells was performed as previously described in 2.3.1.

2.4.2. Statistical analysis

Three biological samples were analysed for each condition. The data values were expressed as the mean ± standard deviation. For the assessment of mutants, one-way analysis of variance (ANOVA) and Dunnett tests were performed to compare the mean of $\log_{10}$ cycles reduction of each isogenic mutant with the wild type. For the comparison between the isogenic mutant Δ*clpB* and *L. monocytogenes* EGD-e wild type, an unpaired *t*-test was performed. The analyses were performed using GraphPad Prism 10.0.2 (GraphPad Software, San Diego, California, United States). *Multiplicity adjusted P value* was calculated for each comparison as described by S.Paul Wright, 1992; Peter H. Westfall, Randall D. Tobias, (2000). Differences between $\log_{10}$ cycles reduction values lower than $p < 0.05$ were considered as significant.

3. Results

3.1. Transcriptomic analysis

In this study, the focus was on deciphering the main mechanisms of *L. monocytogenes* EGD-e under PEF. The selection of *L. monocytogenes* and the strain of EGD-e was due to the high resistance of the specific strain at PEF conditions at pH 7.0 when compared with *E. coli*, *L. plantarum* and *S. cerevisiae* as described in a previous investigation (Lytras et al., 2024a). As described above (section 2.2) the parameters selected were 20 kV/cm, 88 kJ/kg, 41° ± 0.3 °C for the transcriptomics analysis, which resulted in 0.48 ± 0.08 $\log_{10}$ reduction when plated to TSA agar and 0.94 ± 0.12 when plated to a selective medium PALCAM agar after the application of this PEF treatment, indicating sublethal damage.

3.1.1. Read trimming and analysis

The total number of reads obtained after trimming for each biological replicate of both untreated and treated samples is presented in Table 2. This analysis utilized 2,674 genes for statistical evaluation from an initial pool of 3,048 genes. The reads were aligned to a reference transcriptome ASM19603v1 to map existing gene annotations.

3.1.2. Differentially expressed genes (DEGs) and functional annotations to PEF treatment

Under these criteria the transcriptomic analysis after the application of PEF to *L. monocytogenes* EGD-e led to a total of 119 genes differentially expressed. Most of the genes were downregulated (68 genes, 57.1 % of the pool) and the minority were upregulated (51 genes, 42.9 % of the pool) (Fig. 1). From the pool of 51 upregulated genes, 4 of them were transcription regulators (with a potential to influence the resistance of *L. monocytogenes* EGD-e against PEF. Validation of the results was performed with qPCR (for randomly chosen genes: *tktB*, *cspD*, *glnR*, *ulaA*, *rpmA*) of three independent biological samples (Supplementary material).

3.1.3. Functional annotations

In our analysis, we identified significant functional annotations associated with DEGs from our RNA-seq dataset (Table 3). The analysis from the pool of the 51 upregulated genes, showed that genes are categorised to two biological processes: at a 21.6 % to transport and at a 3.9 % to amino-acid transport. Regarding the Protein Sequence Features genes were identified under the 3 terms of domain: ABC transmembrane type-1 (7.8 %), domain: carbohydrate kinase PfkB (3.9 %) and binding:

Table 2
Trimming data.

Sample	Total read bases	Total reads
T_1	2,678,563,188	26,669,020
T_2	2,778,272,208	27,699,718
T_3	2,689,095,867	26,752,354
U_1	3,562,052,193	35,426,436
U_2	1,993,996,026	19,889,136
U_3	3,633,513,640	36,119,022

Total read bases: Total number of read bases after trimming.
Total reads: Total number of reads after trimming.

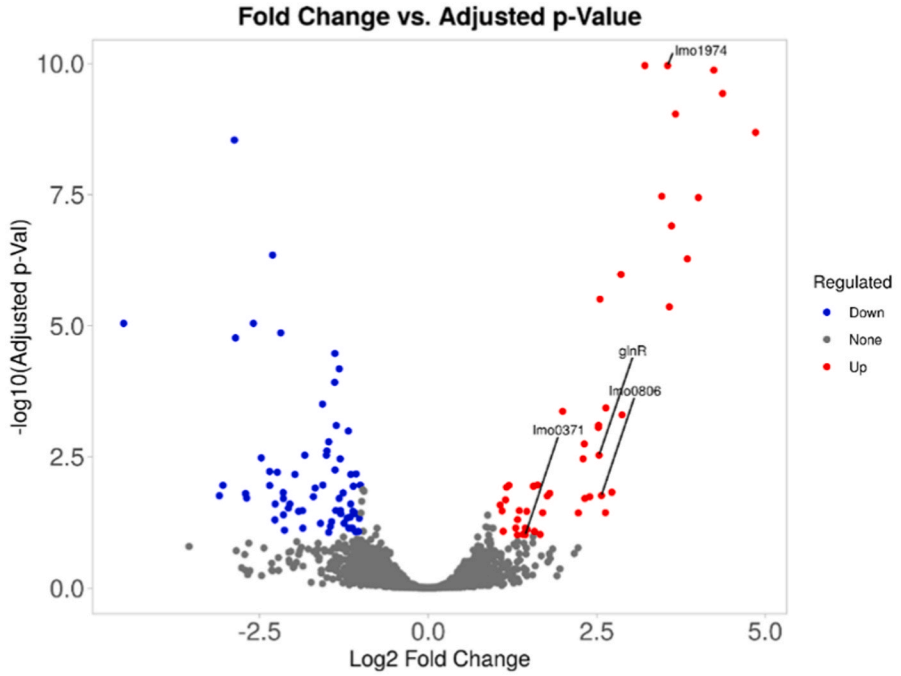


Fig. 1. Volcano plot showing differential gene expression with x-axis representing Log2 Fold change and y-axis $-\log_{10}$ adjusted p-value. Number of DEGs with FDR of 0.1 and |FC| >2. In red are the upregulated genes and in blue are the downregulated genes. The four upregulated transcriptional regulators *lmo1974*, *glnR*, *lmo806* and *lmo0371* are presented. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

via carmate group (3.9 %). From the 68 downregulated genes, a 22.1 % identified under the cellular component category at membrane level, 10.3 % identified at the molecular function category as ribosomal proteins and at a 10.3 % at the molecular function category as ribonucleoproteins.

3.1.4. Gene ontology analysis of DEGs

To emphasize the categories of differentially expressed genes (DEGs) under the PEF condition, we annotated all DEGs with relevant Gene Ontology (GO) terms. This comprehensive annotation allowed us to categorize the up-regulated and downregulated genes based on their biological processes, molecular functions, and cellular components (Table 4). The upregulated genes influenced transcriptomes responsible for biological processes, and more specifically processes related to the tRNA threonylcarbamoyladenosine modification and phosphate ion transport. The downregulated genes influenced transcriptomes related to biological processes, cellular components, and molecular functions related more specifically to translation, ribonucleoprotein complex, cytosolic large ribosomal subunit, ribosome, membrane and structural constituent of ribosome.

3.1.5. KEGG enrichment analysis

The pathway enrichment analysis of KEGG was also carried out to identify the pathways activated and de-activated after PEF treatment (Table 5). For the up-regulated genes, the KEGG enrichment analysis showed pahways relating to ABC transporters and biosynthesis of various secondary metabolites, whereas for the downregulated genes the most enriched pathway identified was relating to ribosomal subunit biosynthesis (11.5 %).

3.2. Assessment of the PEF resistance of selected L. monocytogenes isogenic knockout mutants

A pool of 11 isogenic mutants of *L. monocytogenes* EGD-e was selected based on their known importance in other stress responses. The aim was to identify additional genes and mechanisms involved under pulsed electric field (PEF) treatment at a higher intensity. The inactivation data of the *L. monocytogenes* EGD-e wild type and the poll of its 11 isogenic mutants' strains following the application of PEF treatment at 20 kV/cm, 184 kJ/kg were collected. This condition was selected due to the

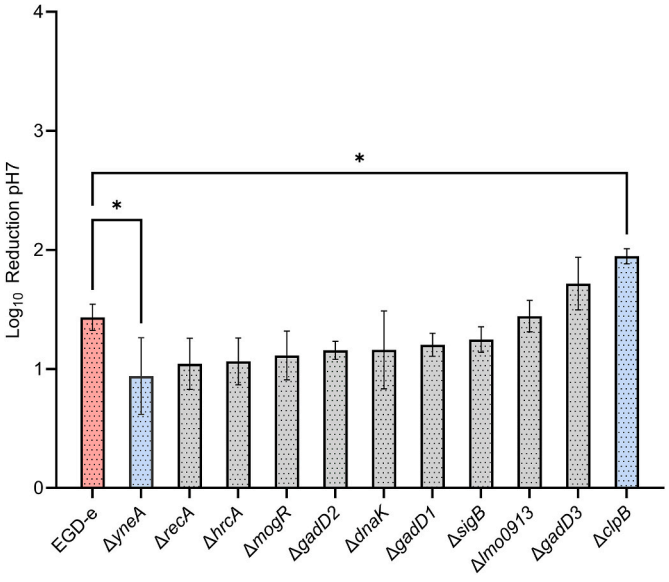


Fig. 2. Log_{10} reductions of *L. monocytogenes* and its isogenic mutants: (A) under 20 kV/cm, 184 kJ/kg at pH 7.0. Log_{10} reduction values of isogenic mutants with a statistical difference lower than $p < 0.05$ from the wild type are represented with (*; $p < 0.05$). The wild type is represented in dark grey. The isogenic mutants that do not exhibit a statistical difference ($p > 0.05$) from the wild type are shown in grey and the ones with a statistical difference are presented in blue. Experiments were performed in 3 biological replicates. Bars represent the standard deviations of these measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Functional annotations Overview of DEGs.

Upregulated genes					
Category	Term	Number of Genes	Percentage (%)	P-value	Fold Enrichment
Biological Process	Transport	11 (*lmo2419, lmo2418, ulaA, lmo0023, lmo2430, lmo0798, lmo2498, lmo2499, lmo0807, lmo0766, lmo0897*)	21.6	3.4E-2	1.8
Biological Process	Amino-acid transport	2 (*lmo2419, lmo0798*)	3.9	1.0E-1	18.3
Protein Sequence Features	Domain:ABC transmembrane type-1	4 (*lmo1652, lmo2418, lmo2498, lmo0766*)	7.8	5.7E-2	4.4
Protein Sequence Features	Domain:Carbohydrate kinase PfkB	2 (*ilvD, lmo1970*)	3.9	7.2E-2	26.5
Protein Sequence Features	Binding:via carbamate group	2 (*fruB, lmo2341*)	3.9	7.2E-2	26.5
Downregulated genes					
Category	**Term**	**Number of Genes**	**Percentage (%)**	**P-value**	**Fold Enrichment**
Cellular Component	Membrane	15 (*fxsA, lmo2219, lmo1230, lmo1526, lmo1529, lmo0778 ,lmo0954, lmo0994, lmo1665, lmo2169, lmo2269, lmo2574, lmo2706, lmo0477, lmo0478*)	22.1	6.2E-2	1.3
Molecular Function	Ribosomal protein	7 (*rpsR, rplS, rpmA, rpmC, rpmF, rpmG, rpmJ*)	10.3	1.7E-5	10.1
Molecular Function	Ribonucleoprotein	7 (*rpsR, rplS, rpmA, rpmC, rpmF, rpmG, rpmJ*)	10.3	1.7E-5	10.1

Table 4
Enrichment analysis of up-regulated and down-regulated *Listeria monocytogenes* EGD-e genes after PEF treatment.

Upregulated genes					
Category	Term	Number of Genes	Percentage (%)	P-value	Fold Enrichment
Biological Process	tRNA threonylcarbamoyladenosine modification	2 (*lmo2077, lmo2078*)	3.9	6.7E-2	28.4
Biological Process	phosphate ion transport	2 (*lmo2498, lmo2499*)	3.9	8.8E-2	21.3
Downregulated genes					
Category	**Term**	**Number of Genes**	**Percentage (%)**	**P-value**	**Fold Enrichment**
Biological process	translation	7 (*rpsR, rplS, rpmA, rpmC, rpmF, rpmG, rpmJ*)	10.3	7.3E-5	8.3
Cellular Component	ribonucleoprotein complex	3 (*rplS, rpmG, rpmJ*)	4.4	5.6E-2	7.5
Cellular Component	cytosolic large ribosomal subunit	3 (*rplS, rpmA, rpmC*)	4.4	7.1E-2	6.5
Cellular Component	ribosome	3 (*rplS, rpmG, rpmJ*)	4.4	7.6E-2	6.3
Cellular Component	membrane	12(*FxsA,lmo1230,lmo0778, lmo0954,lmo0994, lmo1665, lmo2169, lmo2269,lmo2574, lmo2706,lmo0477, lmo0478*)	17.6	9.9E-2	1.6
Molecular Function	Structural constituent of ribosome	7(*rpsR, rplS, rpmA, rpmC, rpmF, rpmG, rpmJ*)	10.3	2.6E-5	10.3

different apparatus between transcriptomics analysis and the assessment of mutants as at 88 kJ/kg the Log_{10} reduction for the wild type identified <1. After PEF treatment the Δ*yneA* was identified to be more resistant and the Δ*clpB* was more sensitive than the wild type *($p < 0.05$)* following PEF treatment at 20 kV/cm, and 184 kJ/kg (Fig. 2). At this PEF treatment all strains apart from Δ*yneA* identified to have more than 1 Log_{10} inactivation. The isogenic *L. monocytogenes* mutant of Δ*yneA* was the most resistant strain with a Log_{10} of 0.94 ± 0.26, when the most sensitive strain was the isogenic mutant Δ*clpB* with a Log_{10} inactivation of 1.95 ± 0.05 Log_{10} inactivation. The wild type showed a Log_{10} inactivation of 1.43 ± 0.09. A statistical difference *($p < 0.05$)* was identified for Δ*yneA* and Δ*clpB* against the wild type.

Fig. 3 illustrates that under PEF treatment at 135 kJ/kg the Log_{10} reduction was 1.91 ± 0.21 for the Δ*clpB* and 1.42 ± 0.14 for the wild type, with no significant difference between the two strains. Interestingly, a statistical difference was identified under the highest PEF condition of 25 kV/cm and 160 kJ/kg between Δ*clpB* and the wild type. Under this PEF conditions the Log_{10} reduction of Δ*clpB* was 2.87 ± 0.14 when this of the wild type's was 1.86 ± 0.11.

4. Discussion

4.1. Overview of PEF treatments and transcriptomic response

This research has focused on investigating the mechanistic responses of *Listeria monocytogenes* EGD-e when subjected to PEF treatments. The set-up of the PEF treatment was continuous, aligning with current industrial approaches. *Listeria monocytogenes* after exposed to stress, can undergo stress adaptation through activation of transcription of stress response genes (Foster, 2007), and response regulators important for tolerance (Kallipolitis and Ingmer, 2001). In general, under specific electric field strength and/or energy the electroporation is irreversible leading to loss of cell homeostasis, ultimately resulting in cell death (Mahnič-Kalamiza and Miklavčič, 2022).

Table 5
KEGG analysis.

Upregulated				
Term	Number of Genes	Percentage (%)	P-value	Fold Enrichment
ABC transporters	6 (*lmo1652, lmo2419, lmo2418, lmo2498, lmo2499, lmo0807*)	11.8	4.1E-2	2.9
Biosynthesis of various secondary metabolites	2 (lmo0372 *metK*)	3.9	9.6E-2	19.1
Downregulated				
Term	**Number of Genes**	**Percentage (%)**	**P-value**	**Fold Enrichment**
Ribosome	7(*rpsR, rplS, rpmA, rpmC, rpmF, rpmG, rpmJ*)	11.5	10.3	8.5

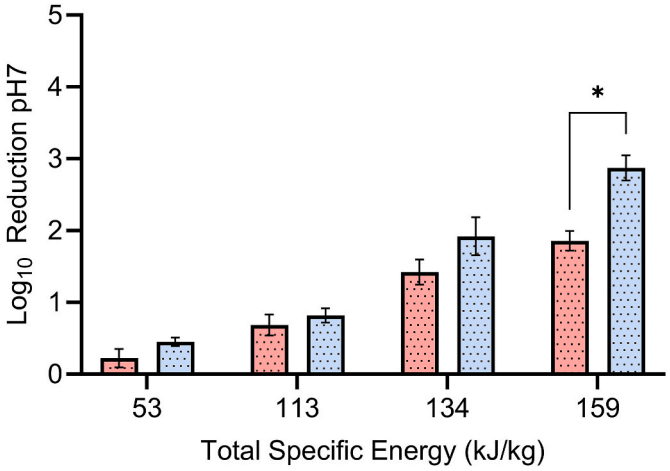


Fig. 3. Log_{10} reductions of *L. monocytogenes* and its isogenic mutants Δ*clpB* under 25 kV/cm at four different total specific energies (54, 113, 135 and 160 kJ/kg) at pH 7.0. Statistically significant differences ($p < 0.05$) in Log_{10} reduction values are represented with an asterisk (*; $p < 0.05$). The wild type is represented in pink. The isogenic mutant Δ*clpB* is presented in blue. Experiments were performed in 3 biological replicates. Bars represent the standard deviations of these measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.2. Transcriptional regulators involved in stress response

In this study, regarding the transcriptomic analysis, a mild-intensity PEF treatment (20 kV/cm, 5 μs, and 88 kJ/kg) was applied under conditions reflecting a technologically realistic processing environment to capture relevant cellular responses. Samples were collected immediately after the PEF treatment to ensure the transcriptomic profile reflected the immediate effects of the treatment. This led to the identification of 51 upregulated and 68 downregulated genes associated with *L. monocytogenes* EGD-e resistance to PEF and damage recovery. One of the transcriptional regulators identified was the gene *lmo0806* which is related to the MerR family of regulators. The majority of MerR family regulators have been associated with response to environmental stimuli including oxidative stress, heavy metals or antibiotics (Brown et al., 2003). Furthermore, *glnR* (a transcriptional regulator that controls the expression of critical genes of nitrogen metabolism, and is involved in ammonium uptake and biosynthesis of glutamine and glutamate) was also upregulated (Biswas, Sonenshein and Belitsky, 2020). In *Bacillus subtilis*, a system between GlnR and TnrA (a transcriptional factor related to nitrogen metabolism) and the global transcriptional regulator CodY is responsible for the regulation of many genes related to nitrogen metabolism (Biswas, Sonenshein and Belitsky, 2020). This example may suggest that a similar mechanism exist in the case of *L. monocytogenes*. Furthermore, GlnR has a similar role in *Saccharopolyspora erythraea* by regulating the expression of carbohydrate ATP-binding cassette (ABC) transporters, enabling efficient carbon uptake under conditions of nitrogen starvation (Liao et al., 2015). Additionally, *Streptomyces coelicolor* GlnR has been shown to play an important role in maintaining cellular osmotic balance and homeostasis (Shao et al., 2015).

Our results also indicate that another two transcriptional regulators (*lmo1974* and *lmo0371)* were also upregulated. These genes encode proteins that function as transcriptional regulators within the GntR family. This is recognized as a significant group of transcriptional regulators that influence metabolic processes across various bacterial species (Hillerich and Westpheling, 2006; Ogasawara et al., 2007). Regarding the *lmo0371* gene, a study by Hingston, Piercey and Hansen, (2015), has identified that a mutant harbouring a transposon in the intergenic region between the genes *lmo0371* and *lmo0372* was more sensitive in comparison with the wild type after desiccation on stainless steel coupons, exhibiting a greater inactivation (>4 log_{10} CFU/cm^2) after 7 days in comparison to a the wild type of *L. monocytogenes* 568 strain, serotype 1/2a, which exhibited a 1.72 log_{10} CFU/cm^2. Furthermore, the gene *lmo0372* which encodes a beta-glucosidase plays an important role in carbohydrate metabolism and potentially supports the energy production or maintenance of cellular homeostasis.

4.3. Phosphotransferase (PTS) and ABC transporter systems

In this study, alongside with the upregulation of *lmo1974*, genes of the phospotransferase system (PTS), including *lmo1971* (PTS sugar transporter subunits II) and *lmo1973* (PTS sugar transporter subunit IIA) were found upregulated. In *Listeria monocytogenes*, PTS transporters have been reported to play a role against oxidative stress (Chen et al., 2023). Supporting this, a study by Arvaniti et al. (2025) has also shown the upregulation of PTS system under the effect of peracetic acid stress to *L. monocytogenes* 104030s. Additionally, PTS system genes were also identified to be upregulated in different conditions of high hydrostatic pressure (200 and 400 MPa) for damage recovery (Duru et al., 2021), indicating the broader role of the PTS system in responding to multiple stress.

Additionally, to the PTS system, other transport mechanisms play significant roles in cellular adaptation after PEF stress. ATP-binding cassette (ABC) transporters, a superfamily of integral membrane proteins, facilitate the ATP-powered translocation of different substrates across cellular membranes (Rees, Johnson and Lewinson, 2009). The highly conserved ABC domains provide the nucleotide-dependent machinery that drives this transport process of various substrates (Rees, Johnson and Lewinson, 2009). In this study sugar, phosphate, methionine, spermidine/putrescine and ferrichrome ABC transporters were identified to be upregulated and playing a role as potential modulators of damage repair responses responses in *L. monocytogenes* after PEF treatment. Supporting the importance of energy dependent repair mechanisms, by García et al. (2006) showed that after PEF treatment, the population of sublethally membrane-injured *E. coli* (NCTC 5934) is heterogeneous: less than 5 % of the cells were able to repair their membranes immediately, while the remaining 95 % relied heavily on energy production and lipid synthesis to carry out membrane repair. This underlines that if the biosynthetic requirements of the cytoplasmic membrane are met, sub lethally injured cells can be repaired (García et al., 2006). Additionally, transcriptomic analysis by Chueca et al.

(2015) confirmed that following PEF treatment (20 kV/cm) to *E. coli* MG1655 cells at pH 4.0, genes involved in the tricarboxylic acid (TCA) cycle pathway were upregulated, reflecting the cellular demand for energy and reducing power during recovery from PEF-induced stress.

4.4. tRNA modification, methionine metabolism, and quorum sensing in L. monocytogenes stress adaptation

Furthermore, *lmo2077* and *tsaE* which are related with the tRNA threonylcarbamoyl adenosine modification biological processes by forming a TsaBDE complex, where TsaD catalyze the transfer of threonylcarbamoyl moiety onto tRNA, while TsaB and TsaE assist in completing this reaction, were also found upregulated. Under stress, tRNA modifications are proposed to help optimize cellular responses by affecting translational fidelity and selective protein expression (Fleming et al., 2022). Finally, genes with a potential role in the main mechanisms involved in restoring membrane integrity/functionality of *L. monocytogenes* EGD-e cells such as *metE* and *metI* which is a part of the methionine synthesis, were upregulated. Another upregulated gene was *metK* which is responsible for the conversion of methionine to S-adenosylmethionine synthetase (SAM). Potentially, after PEF treatment, *L. monocytogenes* may by using this integrated regulatory network including SAM signalling molecules and riboswitches (SreA and SreB) for PrfA regulation and adaptation to environmental stress (Meireles, Pombinho and Cabanes, 2024). Additionally, PrfA has been described as the main transcriptional regulator under virulence (Sibanda and Buys, 2022). In *Listeria monocytogenes* SAM is critical for quorum sensing (QS) systems and virulence, and particularly in the Lux system (Meireles, Pombinho and Cabanes, 2024). QS systems have been associated with bacterial communication about their density population and accordingly for the regulation of genes involved in, stress response, and resistance of the microorganism (Meireles, Pombinho and Cabanes, 2024).

In accordance to the previous upregulated genes, it was observed the downregulation of genes that were mostly related to the ribosome and ribosomal activity. Under stress, downregulation of the ribosome biogenesis and protein synthesis is expected, as a strategy to manage the high energy demands of both processes (Njenga et al., 2023). In addition to this, the reduced protein synthesis reduces the load for protein transport systems, which are required for maintaining the periplasmic, inner, and outer membrane sub proteomes (Njenga et al., 2023). Furthermore, in comparison with heat treatments that lead to denaturation of proteins, during PEF treatments proteins would not change their conformational state (Chueca, Pagán and García-Gonzalo, 2015). In accordance, García et al. (2006) have shown that after PEF treatment to *E. coli* cells, protein synthesis was not required to repair sublethal cells in the cytoplasmic membrane. To conclude, these findings suggest that cells prioritize energy conservation and rely on existing proteins and repair mechanisms rather than de novo protein synthesis to recover from PEF-induced stress.

4.5. Assessment of L. monocytogenes EGD-e mutants against the wild-type

The PEF resistance of 11 isogenic mutants was also assessed and compared with the wild type of *L. monocytogenes* EGD-e strain. As described above these mutants were chosen because the deleted genes have been associated to general stress, heat stress, oxidative stress, acid stress responses and repair mechanisms while they are also linked with the up-regulated and down-regulated *Listeria monocytogenes* that were found in the current study. The assessment of mutants was performed under the aforementioned PEF treatment leading to an inactivation of 1.44 ± 0.09 Log_{10} for the wild type of *L. monocytogenes* EGD-e. From the pool of 11 mutants, Δ*yneA* and Δ*clpB* were identified as more resistant and more sensitive *($p < 0.05$)* against the wild type, respectively. The *yneA* is a gene related to the SOS response which is a conserved bacterial pathway related to repair mechanism and it is regulated by RecA activator and LexA repressor (controlling genes related to DNA repair and cell division inhibition) (Veen and Abee, 2010a). In this study, although Δ*yneA* showed statistical difference against the wild type, the same was not observed for Δ*recA*. Furthermore, the Δ*clpB* mutant of *L. monocytogenes* EGD-e was examined against the wild type under four different total specific energy levels of PEF treatment. A statistically significant difference *($p < 0.05$)* between the mutant and wild type was observed only at the highest PEF intensity which indicates that the role of *clpB* is potentially intensity dependent. However, it is important to note that the observed statistically significant difference between the Δ*clpB* mutant and the wild at high intensities may partly reflect the reduced relative impact of experimental error at greater log reductions; conversely, under low inactivation conditions, the proportional experimental error is higher, making it more difficult to detect significant differences even if they exist. The *clpB* gene is a part of the CtsR regulon (class III heat shock genes), and is associated with enhanced thermotolerance due to its function in the proper folding of newly synthesized proteins and the refolding of aggregated proteins, thereby providing resistance to lethal temperatures (Nair et al., 2000; Chastanet et al., 2004). Additionally, *clpB* has shown to play a role also to resistance against high hydrostatic pressure (Bucur et al., 2018), which indicates the importance of the gene to other stress response. Thus, this indicates that the clpB influences the resistance to heat, high pressure and PEF stresses.

Other genes, such as *sigB,* appeared dispensable to PEF treatment, as both the wild type of *L. monocytogenes* EGD-e and its isogenic deletion mutant Δ*sigB* did not exhibit statistical differences in their PEF-inactivation profiles. Interestingly, other studies have shown the importance of alternative sigma factor B (SigB) as a regulator of over than 150 genes (Van Schaik and Abee, 2005), and its significance in promoting the resistance of *L. monocytogenes* to various stresses including oxidative stress, acid, heat, salt, and bile acids (Ferreira, O'Byrne and Boor, 2001; Somolinos et al., 2010). This result agrees with the study of Somolinos et al. (2010) that showed no differences for *L. monocytogenes* EGD-e and its isogenic deletion mutant Δ*sigB* under PEF treatment (30 kV/cm) at pH 4.0 and pH 7.0, which suggests that *SigB* does not play an important role in PEF-acquired resistance for *L. monocytogenes.* Additionally, a study by Ferreira, O'Byrne and Boor, (2001) has shown similar culture viabilities of *L. monocytogenes* 103040s wild type and the isogenic mutant Δ*sigB* under heat stress at 50 °C. Another two mutants that also showed no statistical differences against the wild type were Δ*hrcA* and Δ*dnaK*. HrcA is a regulator related to the class I proteins and the repressor of *dnaK* operon which is involved to the heat stress response (Hu et al., 2007). Thus, the related observation may not be directly related to the outlet temperature after PEF treatment but just to the intensity of the treatment.

In general, electroporation (or electropermeabilization) is the primary mechanism by which applying an electric field to microbial cells increases membrane permeability (Heinz et al., 2001). Depending on the PEF treatment conditions, the electroporation can be reversible or irreversible, with the intensity of the treatment determining whether the damage to microbial cells is temporary or permanent (Weaver and Chizmadzhev, 1996; Jaeger et al., 2009). The occurrence of sublethal population at pH 7.0 for *L. monocytogenes* (ATCC 15313) and PEF treatments of 19 and 25 kV/cm for 400 μs in citrate phosphate buffer of pH 7.0 has been previously described (García et al., 2005b). It is known that the post-treatment stage after electroporation can potential lead to the leakage of intracellular compounds to the medium and influx of extracellular substances, where the outcome can be pore resealing and membrane repairing or cell death due to loss of cell homeostasis (Saulis, 2010).

4.6. Implications and future research perspectives

Overall, the upregulation of genes for safekeeping of homeostasis, energy availability, quorum sensing and virulence were identified under

electroporation stress for *L. monocytogenes* EGD-e at pH 7.0. In parallel, it was identified through mutant assessment that under the highest total specific energy of 184 kJ/kg (outlet temperature of 64 ± 0.7 °C), ATP-dependent proteases (class III heat shock proteins) may also play an important role in the defence of the microorganism against PEF, which may be related to the ohmic heating effect. While providing valuable insights regarding the main mechanisms of *L. monocytogenes* EGD-e under PEF at a pH 7.0, this study could be further expanded focusing on proteomics (identification and characterization of specific stress response proteins) and metabolomics (metabolic profiling and identification of the resistance-related metabolites). While the studies need to be expanded and validated to actual food products, as a real food matrix may result in reduced microbial inactivation and altered stress response profiles. Finally, these results should be considered as a tool for understanding the mechanistic responses of *L. monocytogenes* for strategies related to PEF decontamination processes alone or in combination with other hurdles.

5. Conclusion

L. monocytogenes EGD-e is known for its resilience under PEF treatments at pH 7.0. The transcriptome analysis under PEF (88 kJ/kg) identified the upregulation of genes related to preservation of homeostasis, energy availability, quorum sensing and virulence regulation. Furthermore, a screening of The PEF resistance of 11 selected isogenic mutants showed that ATP-dependent proteases (class III heat shock proteins) may influence the resistance of the microorganism under higher total specific energy PEF treatments which indicates additional mechanisms of action when high specific energy treatments of this technology are applied (probably due to temperature effect). In summary, the resistance of *L. monocytogenes* identified as a multifaceted phenomenon involving complex gene regulation and adaptive mechanisms that ensure survival under different PEF treatments. Further, by strategically manipulating these molecular and cellular mechanisms, PEF applications can be optimized either as a stand-alone decontamination technology or as an effective pre-treatment in combination with other hurdles to overcome the robust defence capabilities of *L. monocytogenes*. This approach sensitizes the microorganism to subsequent control hurdles, paving the way for optimized combined hurdle technologies that enhance microbial safety and extend food shelf life through tailored PEF treatment conditions.

CRediT authorship contribution statement

Fotios Lytras: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Muhammad Ahmed Ihsan:** Writing – review & editing, Investigation. **Georgios Psakis:** Writing – review & editing, Supervision. **Ruben Gatt:** Writing – review & editing, Supervision, Funding acquisition. **Guillermo Cebrián:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Javier Raso:** Writing – review & editing, Supervision, Resources, Conceptualization. **Vasilis P. Valdramidis:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 955431.

Declaration of competing interest

None.

Acknowledgements

• The authors would like to thank and acknowledge Prof. Dr. Kimon-Andreas Karatzas from the University of Reading (United Kingdom) and Prof. Dr. Heidy den Besten from Wageningen University & Research (The Netherlands) for providing the microbial strains and mutants from their collections.

• The authors would like to thank and acknowledge the members of the University of Malta Bioinformatics Team, Dimosthenis Tzimotoudis, and Panagiotis Alexiou, for assistance provided under the ERA Chair of Bioinformatics for Genomics project (BioGeMT - HORIZON-WIDERA-2022 Grant ID: 101086768) at the University of Malta, for their invaluable assistance in the use of bioinformatics tools.

• The authors would like to thank and acknowledge Dr. Laura Nadal from the University of Zaragoza for her support during the optimization of the RNA extraction protocol.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2025.105014.

Data availability

Data will be made available on request.

References

Álvarez, I., et al., 2002. Environmental factors influencing the inactivation of Listeria monocytogenes by pulsed electric fields. Lett. Appl. Microbiol. 35 (6), 489–493. https://doi.org/10.1046/j.1472-765X.2002.01221.x.

Arvaniti, M., et al., 2025. Deciphering the molecular mechanism of peracetic acid response in Listeria monocytogenes. Journal of Food Protection. The Authors 88 (1), 100401. https://doi.org/10.1016/j.jfp.2024.100401.

Bae, D., Crowley, M.R., Wang, C., 2011. Transcriptome analysis of Listeria monocytogenes grown on a ready-to-eat meat matrix. J. Food Protect. 74 (7), 1104–1111. https://doi.org/10.4315/0362-028X.JFP-10-508.

Benjamini, Yoav, Yosef, Hochberg, 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Roy. Stat. Soc. B 57 (1), 289–300.

Biswas, R., Sonenshein, A.L., Belitsky, B.R., 2020. Role of GlnR in controlling expression of nitrogen metabolism genes in Listeria monocytogenes. J. Bacteriol. 202 (19). https://doi.org/10.1128/JB.00209-20.

Boura, M., Brensone, D., Karatzas, K.A.G., 2020. A novel role for the glutamate decarboxylase system in Listeria monocytogenes; protection against oxidative stress. Food Microbiol. 85 (July 2019), 103284. https://doi.org/10.1016/j.fm.2019.103284. Elsevier Ltd.

Brackett, R.E., Beuchat, L.R., 1991. Survival of Listeria monocytogenes in whole egg and egg yolk powders and in liquid whole eggs. Food Microbiol. 8 (4), 331–337. https://doi.org/10.1016/S0740-0020(05)80007-3.

Brown, N.L., et al., 2003. The MerR family of transcriptional regulators. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 27 (2–3), 145–163. https://doi.org/10.1016/S0168-6445(03)00051-2.

Bucur, F.I., et al., 2018. Resistance of Listeria monocytogenes to stress conditions Encountered in food and food processing environments. Front. Microbiol. 9 (NOV), 1–18. https://doi.org/10.3389/fmicb.2018.02700.

Chastanet, A., et al., 2004. clpB, a novel member of the Listeria monocytogenes CtsR regulon, is involved in virulence but not in general stress tolerance. J. Bacteriol. 186 (4), 1165–1174. https://doi.org/10.1128/JB.186.4.1165-1174.2004.

Chen, M., et al., 2023. Stress tolerance via regulation of the phosphoenolpyruvate-. American Society for Microbiology 11 (5).

Chueca, B., Pagán, R., García-Gonzalo, D., 2015. Transcriptomic analysis of Escherichia coli MG1655 cells exposed to pulsed electric fields. Innov. Food Sci. Emerg. Technol. 29, 78–86. https://doi.org/10.1016/j.ifset.2014.09.003. Elsevier Ltd.

Dawson, R.M.C., Elliot, D.C., Elliot, W.H., Jones, K.M., 1974. 'No title', pH and buffers. In: Data for Biochemical Research. C. Press, Oxford, pp. 475–508.

Dou, X., et al., 2024. Whole-Genome Analysis of Stress Resistance-Related Genes in Listeria monocytogenes 2, pp. 1–24. https://doi.org/10.1101/2024.01.30.577952.

Duru, I.C., et al., 2021. High-pressure processing-induced transcriptome response during recovery of Listeria monocytogenes. BMC Genomics. BMC Genomics 22 (1), 1–20. https://doi.org/10.1186/s12864-021-07407-6.

EFSA, 2023. Listeria monocytogenes Story Map. Available at: https://storymaps.arcgis.com/stories/629e6627e6c64111bfd5b9257473c74a. (Accessed 26 March 2025).

Feehily, C., 2014. Elucidation of the Role of the Glutamate Decarboxylase System and the Γ -aminobutyric Acid Shunt Pathway in the Stress Response of Listeria monocytogenes. PhD thesis. Available at: https://aran.library.nuigalway.ie/handle/10379/4628.

Feehily, C., Karatzas, K.A.G., 2013. Role of glutamate metabolism in bacterial responses towards acid and other stresses. J. Appl. Microbiol. 114 (1), 11–24. https://doi.org/10.1111/j.1365-2672.2012.05434.x.

Feehily, C., O'Byrne, C.P., Karatzas, K.A.G., 2013. Functional γ-aminobutyrate shunt in listeria monocytogenes: role in acid tolerance and succinate biosynthesis. Appl. Environ. Microbiol. 79 (1), 74–80. https://doi.org/10.1128/AEM.02184-12.

Ferreira, A., O'Byrne, C.P., Boor, K.J., 2001. Role of σB in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in Listeria monocytogenes. Appl. Environ. Microbiol. 67 (10), 4454–4457. https://doi.org/10.1128/AEM.67.10.4454-4457.2001.

Fleming, B.A., et al., 2022. A tRNA modifying enzyme as a tunable regulatory nexus for bacterial stress responses and virulence. Oxford University Press 50 (13), 7570–7590.

Foster, P.L., 2007. Stress-induced mutagenesis in bacteria. Crit. Rev. Biochem. Mol. Biol. 42 (5), 373–397. https://doi.org/10.1080/10409230701648494.

Fraser, K.R., et al., 2003. Role of σB in regulating the compatible solute uptake systems of Listeria monocytogenes: osmotic induction of opuC is σB dependent. Appl. Environ. Microbiol. 69 (4), 2015–2022. https://doi.org/10.1128/AEM.69.4.2015-2022.2003.

García, D., Gómez, N., Raso, J., et al., 2005a. Bacterial resistance after pulsed electric fields depending on the treatment medium pH. Innov. Food Sci. Emerg. Technol. 6 (4), 388–395. https://doi.org/10.1016/j.ifset.2005.04.003.

García, D., Gómez, N., Mañas, P., et al., 2005b. Occurrence of sublethal injury after pulsed electric fields depending on the micro-organism, the treatment medium ph and the intensity of the treatment investigated. J. Appl. Microbiol. 99 (1), 94–104. https://doi.org/10.1111/j.1365-2672.2005.02611.x.

García, D., et al., 2006. Biosynthetic requirements for the repair of sublethal membrane damage in Escherichia coli cells after pulsed electric fields. J. Appl. Microbiol. 100 (3), 428–435. https://doi.org/10.1111/j.1365-2672.2005.02795.x.

Ge, S.X., Son, E.W., Yao, R., 2018. iDEP: an integrated web application for differential expression and pathway analysis of RNA-Seq data. BMC Bioinformatics. BMC Bioinformatics 19 (1), 1–24. https://doi.org/10.1186/s12859-018-2486-6.

Gorski, L., Flaherty, D., Mandrell, R.E., 2006. Competitive fitness of Listeria monocytogenes serotype 1/2a and 4b strains in mixed cultures with and without food in the U . S. Food and Drug Administration Enrichment Protocol 72 (1), 776–783. https://doi.org/10.1128/AEM.72.1.776.

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. Front. Microbiol. 11 (July), 1–11. https://doi.org/10.3389/fmicb.2020.01505.

Heinz, V., et al., 2001. Preservation of liquid foods by high intensity pulsed electric fields - basic concepts for process design. Trends Food Sci. Technol. 12 (3–4), 103–111. https://doi.org/10.1016/S0924-2244(01)00064-4.

Hillerich, B., Westpheling, J., 2006. A new GntR family transcriptional regulator in Streptomyces coelicolor is required for morphogenesis and antibiotic production and controls transcription of an ABC transporter in response to carbon source. J. Bacteriol. 188 (21), 7477–7487. https://doi.org/10.1128/JB.00898-06.

Hingston, P.A., Piercey, M.J., Hansen, L.T., 2015. Genes associated with desiccation and osmotic stress in Listeria monocytogenes as revealed by insertional mutagenesis. Appl. Environ. Microbiol. 81 (16), 5350–5362. https://doi.org/10.1128/AEM.01134-15.

Hu, Y., et al., 2007. Transcriptomic and phenotypic analyses suggest a network between the transcriptional regulators HrcA and □ B in, 73 (24), 7981–7991. https://doi.org/10.1128/AEM.01281-07.

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4 (1), 44–57. https://doi.org/10.1038/nprot.2008.211.

Jaeger, H., et al., 2009. Protective effect of milk constituents and sublethal injuries limiting process effectiveness during PEF inactivation of Lb. rhamnosus. Int. J. Food Microbiol. 134 (1–2), 154–161. https://doi.org/10.1016/j.ijfoodmicro.2009.06.007. Elsevier B.V.

Kallipolitis, B.H., Ingmer, H., 2001. Listeria monocytogenes Response Regulators Important for Stress Tolerance and Pathogenesis, vol 204.

Kirchner, M., et al., 2025. A binational sample-initiated retrospective outbreak investigation of Listeria monocytogenes infections in the United States and Canada linked to Enoki Mushrooms imported from China 2022–2023. J. Food Protect. 88 (1). https://doi.org/10.1016/j.jfp.2024.100413.

Liao, C., et al., 2015. Nitrogen regulator GlnR controls uptake and utilization of non-phosphotransferase-system carbon sources in actinomycetes, 112 (51), 1–6. https://doi.org/10.1073/pnas.1508465112.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15 (12), 1–21. https://doi.org/10.1186/s13059-014-0550-8.

Lytras, F., Psakis, G., Gatt, R., Hummerjohann, J., et al., 2024a. Evaluation of strain variability of food microorganisms in response to decontamination by pulsed electric fields and thermal treatments. Innov. Food Sci. Emerg. Technol. 95 (June), 103731. https://doi.org/10.1016/j.ifset.2024.103731. Elsevier Ltd.

Lytras, F., Psakis, G., Gatt, R., Cebrián, G., et al., 2024b. Exploring the efficacy of pulsed electric fields (PEF) in microbial inactivation during food processing: a deep dive into the microbial cellular and molecular mechanisms. Innov. Food Sci. Emerg. Technol. 95 (June). https://doi.org/10.1016/j.ifset.2024.103732.

Mahnič-Kalamiza, S., Miklavčič, D., 2022. The phenomenon of electroporation. Food Engineering Series. https://doi.org/10.1007/978-3-030-70586-2_3.

Martínez, J.M., et al., 2016. Release of mannoproteins during saccharomyces cerevisiae autolysis induced by pulsed electric field. Front. Microbiol. 7 (SEP), 1–8. https://doi.org/10.3389/fmicb.2016.01435.

Meireles, D., Pombinho, R., Cabanes, D., 2024. Signals behind Listeria monocytogenes virulence mechanisms. Gut Microbes 16 (1), 1–27. https://doi.org/10.1080/19490976.2024.2369564. Taylor & Francis.

Mosqueda-Melgar, J., Raybaudi-Massilia, R.M., Martín-Belloso, O., 2007. Influence of treatment time and pulse frequency on Salmonella Enteritidis, Escherichia coli and Listeria monocytogenes populations inoculated in melon and watermelon juices treated by pulsed electric fields. Int. J. Food Microbiol. 117 (2), 192–200. https://doi.org/10.1016/j.ijfoodmicro.2007.04.009.

Mosqueda-Melgar, J., et al., 2008. Effects of pulsed electric fields on pathogenic microorganisms of major concern in fluid foods: a review. Crit. Rev. Food Sci. Nutr. 48 (8), 747–759. https://doi.org/10.1080/10408390701691000.

Nadal, L., Mañas, P., Cebrián, G., 2024. The sigma factor σB is required for the development of the growth phase- and temperature-dependent increases in thermoresistance and membrane rigidity in Staphylococcus aureus. Lwt 210 (August). https://doi.org/10.1016/j.lwt.2024.116814.

Nair, S., et al., 2000. CtsR controls class III heat shock gene expression in the human pathogen Listeria monocytogenes. Mol. Microbiol. 35 (4), 800–811. https://doi.org/10.1046/j.1365-2958.2000.01752.x.

Njenga, R., et al., 2023. Coping with stress : how bacteria fi ne-tune protein synthesis and protein transport. Journal of Biological Chemistry. The Authors 299 (9), 105163. https://doi.org/10.1016/j.jbc.2023.105163.

Ogasawara, H., et al., 2007. PdhR (pyruvate dehydrogenase complex regulator) controls the respiratory electron transport system in Escherichia coli. J. Bacteriol. 189 (15), 5534–5541. https://doi.org/10.1128/JB.00229-07.

Paudyal, R., Karatzas, K.A., 2016. Stress Adaptation of Listeria monocytogenes in Acidic ready-to-eat Products, Food Hygiene and Toxicology in Ready-to-Eat Foods. Elsevier Inc. https://doi.org/10.1016/B978-0-12-801916-0.00010-8

Paul Wright, S., 1992. Adjusted P-Values for simultaneous inference. Science 48, 1005–1013. https://doi.org/10.1126/science.87.2249.103-a. Statistics Department, University of Tennessee, Knoxville, Tennessee 37996, U.S.A.: BIOMETRICS.

Peng, K., et al., 2020. Effect of pulsed electric fields on the growth and acidification kinetics of Lactobacillus delbrueckii Subsp. bulgaricus. Foods 9 (9), 1–11. https://doi.org/10.3390/foods9091146.

Peter, H. Westfall, Randall, D., Tobias, D.R., 2000. Multiple Comparisons and Multiple Tests (Text and Workbook Set). Sas Inst.

Raso, J., et al., 2016. Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes. Innov. Food Sci. Emerg. Technol. 37, 312–321. https://doi.org/10.1016/j.ifset.2016.08.003. Elsevier Ltd.

Rees, D.C., Johnson, E., Lewinson, O., 2009. ABC transporters: the power to change. Nat. Rev. Mol. Cell Biol. 10 (3), 218–227. https://doi.org/10.1038/nrm2646.

Saldaña, G., et al., 2009. Comparing the PEF resistance and occurrence of sublethal injury on different strains of Escherichia coli , Salmonella Typhimurium , Listeria monocytogenes and Staphylococcus aureus in media of pH 4 and 7. Innov. Food Sci. Emerg. Technol. 10 (2), 160–165. https://doi.org/10.1016/j.ifset.2008.11.003. Elsevier Ltd.

Saldaña, G., et al., 2010. Inactivation kinetics of pulsed electric field-resistant strains of Listeria monocytogenes and Staphylococcus aureus in media of different pH. Food Microbiol. 27 (4), 550–558. https://doi.org/10.1016/j.fm.2010.01.002.

Saulis, G., 2010. Electroporation of cell membranes: the fundamental effects of pulsed electric fields in food processing. Food Eng. Rev. 2 (2), 52–73. https://doi.org/10.1007/s12393-010-9023-3.

Schottroff, F., et al., 2018. Sublethal injury and viable but non-culturable (VBNC) state in microorganisms during preservation of food and biological materials by non-thermal processes. Front. Microbiol. 9 (NOV), 1–19. https://doi.org/10.3389/fmicb.2018.02773.

Shao, Z.H., et al., 2015. GlnR-mediated regulation of ectABCD transcription expands the role of the GlnR regulon to osmotic stress management. J. Bacteriol. 197 (19), 3041–3047. https://doi.org/10.1128/JB.00185-15.

Sherman, B.T., et al., 2022. DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). Nucleic Acids Research. Oxford University Press 50 (W1), W216–W221. https://doi.org/10.1093/nar/gkac194.

Sibanda, T., Buys, E.M., 2022. Listeria monocytogenes pathogenesis: the role of stress adaptation. Microorganisms 10 (8). https://doi.org/10.3390/microorganisms10081522.

Somolinos, M., et al., 2010. SigB absence decreased Listeria monocytogenes EGD-e heat resistance but not its Pulsed Electric Fields resistance. Int. J. Food Microbiol. 141 (1–2), 32–38. https://doi.org/10.1016/j.ijfoodmicro.2010.04.023. Elsevier B.V.

Sue, D., et al., 2004. σB-dependent gene induction and expression in Listeria monocytogenes during osmotic and acid stress conditions simulating the intestinal environment. Microbiology 150 (11), 3843–3855. https://doi.org/10.1099/mic.0.27257-0.

Toepfl, S., 2012. Stewart Postharvest Review Commercial Applications. https://doi.org/10.2212/spr.2012.2.4 (September).

U.S. Food and Drug Administation, 2000. No Title, CFR - Code of Federal Regulations Title 21. Available at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=179.26.

U.S. Food and Drug Administration, 2025. Listeria (Listeriosis). Available at: https://www.fda.gov/food/foodborne-pathogens/listeria-listeriosis. (Accessed 26 March 2025).

Veen, S. Van Der, Abee, T., 2010a. Dependence of continuous-flow biofilm Formation by Listeria monocytogenes EGD-e on SOS response factor YneA. Appl. Environ. Microbiol. 76 (6), 1992–1995. https://doi.org/10.1128/AEM.02680-09.

Veen, S. Van Der, Abee, T., 2010b. HrcA and DnaK are Important for Static and continuous-flow Biofilm Formation and Disinfectant Resistance in Listeria monocytogenes, pp. 3782–3790. https://doi.org/10.1099/mic.0.043000-0.

van der Veen, S., et al., 2007. The heat-shock response of Listeria monocytogenes comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. Microbiology 153 (10), 3593–3607. https://doi.org/10.1099/mic.0.2007/006361-0.

Veen, S. Van Der, et al., 2009. Genome-Wide Screen for Listeria monocytogenes Genes Important for Growth at High Temperatures. https://doi.org/10.1111/j.1574-6968.2009.01586.x (1990).

Veen, S. Van Der, et al., 2010. The SOS Response of Listeria monocytogenes is Involved in Stress Resistance and Mutagenesis, pp. 374–384. https://doi.org/10.1099/mic.0.035196-0.

Weaver, J.C., Chizmadzhev, Y.A., 1996. Theory of electroporation: a review. Bioelectrochem. Bioenerg. 41 (2), 135–160. https://doi.org/10.1016/S0302-4598(96)05062-3.

Wemekamp-kamphuis, H.H., et al., 2004. Identification of Sigma Factor □ B -Controlled Genes and Their Impact on Acid Stress , High Hydrostatic Pressure , and Freeze Survival in Listeria monocytogenes EGD-e, vol. 70, pp. 3457–3466. https://doi.org/10.1128/AEM.70.6.3457, 6.

Wemekamp-Kamphuis, H.H., et al., 2004. Identification of sigma factor σB-controlled genes and their impact on acid stress, high hydrostatic pressure, and freeze survival in Listeria monocytogenes EGD-e. Appl. Environ. Microbiol. 70 (6), 3457–3466. https://doi.org/10.1128/AEM.70.6.3457-3466.2004.

Wiedmann, M., 2002. Methods in nutrition science subtyping of bacterial foodborne pathogens. Nutr. Rev. 60 (7), 201–208.