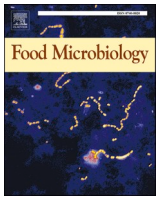
Food Microbiology 137 (2026) 105042

Contents lists available at ScienceDirect

Food Microbiology

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



Modelling gene-dependent PEF resistance of *E. coli* K-12

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ARTICLE INFO

Keywords:
Pulsed electric fields
Escherichia coli K12
Mutants
Mathematical modelling
Polynomial model

ABSTRACT

The current study investigated the antimicrobial mechanisms of Pulsed Electric Fields (PEF) by evaluating the resistance of 22 *Escherichia coli* K12 mutants. Initial screening at PEF treatment (23 kV/cm, 53.3 μs, 95.4 kJ/kg), pH 7.0, revealed increased sensitivity ($p < 0.05$) of Δ*clpB*, Δ*rpoS*, and Δ*dnaK* expressed in Log_{10} reductions. Further inactivation kinetic analysis of 8 selected strains at pH 7.0 and 4.0 revealed a non-linear, polyphasic behaviour. This was described by a global modelling approach combining a log-linear primary model with a second-order polynomial model incorporating treatment time, total specific energy, and survival data as variables. The calculated model parameters (C_1, C_2, and C_3) significantly differed ($p < 0.05$) among strains at pH 7.0, but not at pH 4.0. Furthermore, the calculated inactivation rates, k_{max}, varied in relation to the total specific energy. At pH 7.0, k_{max} was higher at low (0–40 kJ/kg) and high (140–180 kJ/kg) total specific energies, while at pH 4.0, it raised at high total specific energies (120–160 kJ/kg). In conclusion, *E. coli* response to PEF was dependant on the stress regulator *rpoS*. This response also involved genes which encode molecular chaperones such as *dnaK*, *clpB* and *recA*, related proteins for DNA repair. In conclusion, resistance to PEF was found to be influenced by pH and total specific energy, indicating that *E. coli* mounts a multifaceted response to PEF treatments, informing advanced microbial inactivation strategies for food safety.

1. Introduction

Escherichia coli is a Gram-negative, non-spore-forming, rod-shaped bacterium (Khan et al., 2021). As a member of the bacterial family *Enterobacteriaceae*, it is one of the most common commensal inhabitants of the gastrointestinal tract of humans and warm-blooded animals, living in mutually beneficial association with its hosts and rarely causing disease (Allocati et al., 2013). Wildlife, livestock, and humans can be occasional carriers of pathogenic *E. coli* (Shiga toxin-producing *E. coli* (STEC), such as *E. coli* O157:H7), which may contaminate meats and food crops (U.S. Food and Drug Administration, 2020). For example, in Europe, European Food Safety Authority (EFSA) reported an outbreak related Shiga-toxin producing *E. coli* (STEC), serotype O104:H4 related to fresh salad vegetables (EFSA, 2011). While, in the U.S, different outbreaks of *E. coli* (STEC), including *E. coli* O157:H7 have been reported for various food products: leafy greens, sprouts, raw milk cheeses, raw beef and poultry (U.S. Food and Drug Administration, 2019).

In general, *E. coli* can adapt to a wide range of environmental stresses and survive within the host during infection (Abdelwahed et al., 2022). Under stress conditions, the bacterial response network can detect, respond to, and adapt to various chemical and physical challenges, including changes in pH, temperature, nutrient deprivation, and oxidative stress. (Abdelwahed et al., 2022). In *E. coli*, the primary general stress regulator is Rpos, which is activated under a variety of stress conditions, including oxidative stress, hyperosmolarity, UV radiation, heat, acidity and ethanol (Hengge-Aronis, 2002; Battesti et al., 2011). Rpos, also referred to as σ^S, is a subunit of RNA polymerase that, under several stress conditions, can largely substitute the σ^{70} (RPOD), the main sigma factor responsible for the transcription of genes related to essential cellular functions (Hengge-Aronis, 2002). Additional regulators may play also a role under different stresses, for example under oxidative stress and specifically under hydrogen peroxide H_2O_2, OxyR is activated and leads to the expression of 40 genes for protecting the cell from hydrogen peroxide toxicity (Chiang and Schellhorn, 2012). Additionally, another system with an important role in *E. coli*, is the soxRS

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https://doi.org/10.1016/j.fm.2026.105042
Received 8 September 2025; Received in revised form 13 January 2026; Accepted 23 January 2026
Available online 24 January 2026

regulon that is expressed by the SoxR and SoxS regulators in a two stage regulatory network system with importance to the resistance against organic compounds, antibiotics, nitric oxide radicals and heavy metals (Semchyshyn et al., 2005). Furthermore, dependent on the stress other mechanisms may exist, such as heat shock proteins (i.e., molecular chaperones and proteases) which help the cells to adapt to environmental and metabolic changes, including the DnaK and GroE chaperone systems formed by DnaK, DnaJ and GrpE, and GroEL and GroES responding to stress (Arsène et al., 2000).

Currently, the focus of industry and research is at the replacement of thermal treatment with innovative technologies including pulsed electric fields (PEF), ultrasound (US), pulsed light (PL), cold atmospheric plasma (CAP), ultraviolet light, and ozonation as arises the desire to preserve food safety while improving and maintaining food quality (White et al., 2025). PEF technology is an emerging method for microbial inactivation that uses high-voltage electric pulses of very short duration (ranging from microseconds to milliseconds), with electric field strengths between 15 and 40 kV/cm for achieving an effective decontamination (Raso et al., 2016). It is believed that the main mechanism of action of PEF is based primarily on the phenomenon of electroporation (or electropermeabilization), whereby the application of an electric field to microbial cells leads to the increase of membrane permeability (Coster and Zimmermann, 1975; Heinz et al., 2001; Mañas and Pagán, 2005). Additionally, the increase in specific energy applied to generate the electric field raises the temperature of the treatment medium through Joule (ohmic) heating, and this temperature rise enhances the effectiveness of PEF treatments by promoting greater microbial inactivation (Saldaña et al., 2010). The outcome of electroporation induced by pulsed electric field (PEF) treatment depends largely on the specific treatment parameters, with the applied electric field intensity determining whether the disruption of microbial cell membranes is transient and reversible, allowing potential cell recovery, or causing irreversible damage that potentially leads to cell death (Weaver and Chizmadzhev, 1996; Jaeger et al., 2009). Furthermore, the effectiveness of PEF technology for microbial inactivation depends on multiple factors, including microbial characteristics (species, strain, as well as size, and shape), growth phase, environmental conditions (pH, conductivity, and water activity), and treatment parameters (electric field strength, treatment time, and total specific energy) (Raso et al., 2016; Lytras et al., 2024). The microbial cell death after PEF treatments is due to the leakage of intracellular contents caused by increased membrane permeability, structural alterations of the membrane, and osmotic or swelling effects (Min et al., 2007; Golberg et al., 2010). Industrially, PEF technology has been approved for commercial pasteurization of fruit juices, requiring at least 5-$\log_{10}$ reductions of the most resistant relevant foodborne pathogen (U.S. Food and Drug Administation, 2000).

Under environmental stresses such as temperature fluctuations and osmotic changes, alterations in the organization and structure of membrane lipids can occur, which in turn modulate various cellular activities (Los and Murata, 2004). Following PEF treatment, cell recovery is an active process that depends on complex cellular machinery to restore membrane integrity and reestablish normal function (Batista Napotnik et al., 2021). Gene expression alterations and differential protein synthesis before and after PEF treatments can reveal useful information for elucidating PEF-microbial inactivation and resistance mechanisms. Different studies have reported that multiple mechanisms that potentially contribute to microbial resistance against PEF, including alterations in the synthesis of proteins involved in membrane function and transport, enhanced membrane repair, adaptation to oxidative stress, and changes in cellular energy metabolism (Lytras et al., 2024). However, despite these advancements, the primary mechanisms driving microbial resistance to PEF remain not fully understood.

Accurate mathematical prediction models are essential for designing an effective PEF treatment (Álvarez et al., 2003). These mathematical prediction models correlate various PEF treatment parameters with survival fractions to estimate the level of microbial inactivation (Singh et al., 2017). Survival curves typically follow a first-order log-linear pattern for low $\log_{10}$ reductions, but alternative mathematical prediction models are required when the curves deviate from this behaviour (Rivas et al., 2006). Previous studies have employed various models to describe the microbial inactivation data of *E. coli* strains to PEF treatments, including the first-order kinetic model (Amiali et al., 2007), Arrhenius model (Alkhafaji and Farid, 2008), Bigelow (Rivas et al., 2006), Hülsheger (Rivas et al., 2006), Weibull (Rivas et al., 2006), and quadratic response model (Mosqueda-Melgar et al., 2007; Saldaña et al., 2011).

This study aims to implement a comprehensive analysis of microbial inactivation kinetics of *E. coli* K12 under PEF using a global modelling approach. The specific objectives are to characterize and compare the kinetic inactivation patterns and microbial reductions of *E. coli* K12 and selected isogenic mutants subjected to PEF treatments, with particular emphasis on strain-dependent differences in resistance to PEF. Inactivation kinetics were evaluated under different environmental conditions, including two pH levels, to assess the impact of the genetic background and the physicochemical factors on the microbial susceptibility to PEF. By integrating kinetic modelling with comparative strain analysis, this study advances into PEF resistance microbial mechanisms and supports the further optimisation of PEF processing either alone or in combination with other preservation methods.

2. Materials & methods

2.1. Strains, medium and culture conditions

The bacterial strains used were *E. coli* K12 BW25113 wild type (with genotype: Δ(*araD-araB*)*567* Δ*lacZ4787*(*::rrnB-3*) λ^- *rph-1* Δ(*rhaD-rhaB*) *568 hsdR514*, and with complete genome sequence deposited in GenBank with accession number CP009273.1) and its 22 isogenic mutants (Δ*clpB*, Δ*rpoS*, Δ*recA*, Δ*leuO*, Δ*rmpF*, Δ*dnaK*, Δ*ompT, ΔcadC, ΔoxyR, ΔbetI, ΔsoxS, ΔkatG, ΔsdhC, ΔtrxC, ΔnudE, ΔappY, ΔcbI, ΔcspD, ΔtktB, ΔpstB, ΔoppB, ΔcyoA*) (Table 1). All strains were obtained from the National Bio-Resource Project (NIG, Japan) (Baba et al., 2006). Microbial strains were stored at −70 °C by mixing volumes of late exponential-phase cultures with glycerol (Merck, Germany) (70:30 v/v). Stock cultures were re-activated by inoculation onto solid media of Tryptic Soya Agar (Scharlab, Spain) plates. A single colony from the re-activated plate was selected to prepare the primary inoculum, which

Table 1
Function of the selected *E. coli* K12 mutants and their deleted genes based on Baba et al. (2006).

Gene	Function
clpB	Protein disaggregation chaperone
rpoS	RNA polymerase sigma factor
recA	DNA recombination/repair
leuO	Global transcription factor
rmpF	Ribonucleoprotein
dnaK	Chaperone protein
ompT	Outer membrane porin C
cadC	*cadBA* operon transcriptional activator
oxyR	Oxidative and nitrosative stress transcriptional regulator
betI	Choline-inducible *betIBA-betT* divergent operon transcriptional repressor
soxS	Superoxide response regulon transcriptional activator; autoregulator
katG	Catalase-peroxidase HPI
sdhC	Succinate dehydrogenase
trxC	Thioredoxin 2 (Trx-2)
nudE	Adenosine nucleotide hydrolase: substrates include Ap3A
appY	Global transcriptional activator; DLP12 prophage
cbI	Colicin-B immunity protein
cspD	Inhibitor of DNA replication
tktB	Transketolase 2
pstB	Phosphate transporter subunit
oppB	Oligopeptide transporter subunit
cyoA	Cytochrome o ubiquinol oxidase subunit II

was then incubated in Tryptic Soya Broth without dextrose (TSB-D; Scharlab, Spain) at 37 °C for 24 ± 2 h. Subsequently, a subculture was generated by transferring 1 % (v/v) of this culture into fresh TSB-D and incubating it at the same temperature for 17–18 h, allowing the bacterial population to reach the stationary phase (10^8–10^9 Log_{10} 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 of each strain was resuspended: in citrate-phosphate McIlvaine buffer (combination of citric acid and disodium hydroxide phosphate), of pH 7.0 ± 0.1 or 4.0 ± 0.1 (Dawson et al., 1974) with a set conductivity of 2 mS/cm. The pH and electrical conductivity were measured at 20–25 °C with a benchtop multi-parameter-meter (Hanna Instruments, model H5522, Romania).

2.2. PEF treatments

The EPULSUS-BM1A-12 (3 kW, Energy Pulse System Lisbon, Portugal) pulse generator was used in this study with the capability of providing monopolar square wave pulses (1–200 μs), with a maximum output voltage of 12 kV, a current of 200 A and frequencies of up to 200 Hz. The system operated in a continuous mode with a flow rate of 5 L/h, pumping the sample through a treatment chamber with parallel titanium electrodes chamber of 0.4 cm gap,3 cm length, 0.5 cm width (Lytras et al., 2024) leading to a residence time of 0.43 s (Fig. 1).

Prior to PEF treatment, samples were tempered to an inlet temperature of 20 °C using a coil immersed in a water bath. After PEF treatment, the samples were cooled to ambient temperature using a cooling coil. The sample spent 1 s in the pipeline connecting the treatment chamber's exit to the cooling heat exchanger. The residence time within the cooling heat exchanger was 3 s while after the sample collection, it remained in a sterile falcon tube which was inserted in an ice bath until the temperature was dropped below 20 °C (Fig. 2). The actual voltage applied during processing was monitored through an integrated oscilloscope, from Pico Technologies. The actual voltage applied during processing was monitored through an integrated oscilloscope, from Pico Technologies. Inlet and outlet temperature measurements were constantly monitored with a thermocouple (OM-HL-EH-TC, OMEGA, Netherlands) (Fig. 2).

Square pulses of 4 μs width at an electric field strength of 23 kV/cm, repetition rates of (0–58 Hz) were applied, leading to outlet temperatures of (20–62.7 °C). The duration of the treatments derived from theoretical values (number of pulses x pulse width), leading to 0–99.8 μs. The total specific energy for the 9 tested conditions (from 0 to 178.7 kJ/kg) (Table 2) was estimated by calculating the temperature increase during pulses under presumed adiabatic conditions (Heinz et al., 2001) according to the following equation:

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

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). For the initial screening, all strains were treated under a mid-PEF treatment (23 kV/cm, 53.3 μs, 95.4 kJ/kg, and 42.8 °C) at pH 7.0 by assessing microbial survival (Section 2.3). Further analysis was performed on selected strains at both pH 7.0 and 4.0.

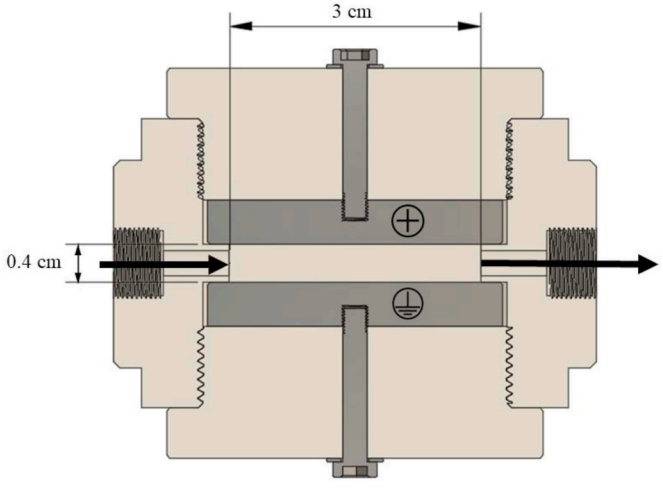

Fig. 1. PEF treatment chamber with parallel plate electrodes (3 cm) configuration and a gap of 0.4 cm between the two electrodes (as presented previously Lytras et al., 2024a).

2.3. Enumeration of viable cells

The untreated and treated cell suspensions were diluted in (PBS) and 0.1 mL (or 1 mL divided at three different plates) of the diluted sample was used for surface spread plating. Three biological replicates were performed for each strain and for each biological replicate two technical replicates were performed. The media used for the enumeration of the viable cells was TSA. Samples were incubated for 48 ± 2 h at 37 °C before performing microbial count enumeration. Colony counts corresponded to the viable microorganisms were expressed as colony forming units per millilitre (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) (Eq. (1)).

$$\mathrm{Log}_{10}\, reduction = \mathrm{Log}_{10}\left(\frac{N_t}{N_0}\right) \quad (1)$$

2.4. Kinetic modelling analysis

Following the microbial survival studies, selected mutants of the most sensitive and resistant strains were kinetically analysed at dynamic specific energies (W_T) for PEF treatments at pH 7.0 and 4.0. The data were fitted by applying a global modelling approach, which involves simultaneously fitting of the primary and secondary model into the experimental data, for estimating the microbial kinetic parameters. The Log-linear model was used as the primary model (Eq. (2)) (Geeraerd et al., 2005) while a second order polynomial type model (Eq. (3)) was used as the secondary model to determine the total specific energy dependence of k_{max}.

$$\frac{dN_t}{dt} = -k_{max} \times N_t \quad (2)$$

$N(t)$ (CFU/mL) is the microbial cell density at time t, and k_{max} (1/μs) is the specific inactivation rate.

$$k_{max} = C_1 + (C_2 \times WT) + (C_3 \times WT^2) \quad (3)$$

C_1: baseline inactivation constant, C_2: linear coefficient describing the sensitivity of the inactivation rate with respect to the total specific energy, C_3: quadratic coefficient capturing the nonlinear effects of the total specific energy on the microbial population, and WT: the total specific energy.

Regression analysis for the estimation of the primary and secondary model parameters was performed using Matlab ® Version 6.1 (The Mathworks, MA, USA). Furthermore, parameter optimisation was carried out using the optimisation function Isqnonlin from the Matlab Optimisation Toolbox. The model's performance was assessed using metrics such as Mean Square Error (*MSE*) and Regression coefficient (R^2) (Eq. (4)). The regression coefficient R^2 value, ranges from 0 (indicating a poor fit) to 1 (indicating a perfect fit) provides information about the proportion of the variance in the data explained by the model (Den Besten et al., 2006). The regression coefficient R^2 is defined as:

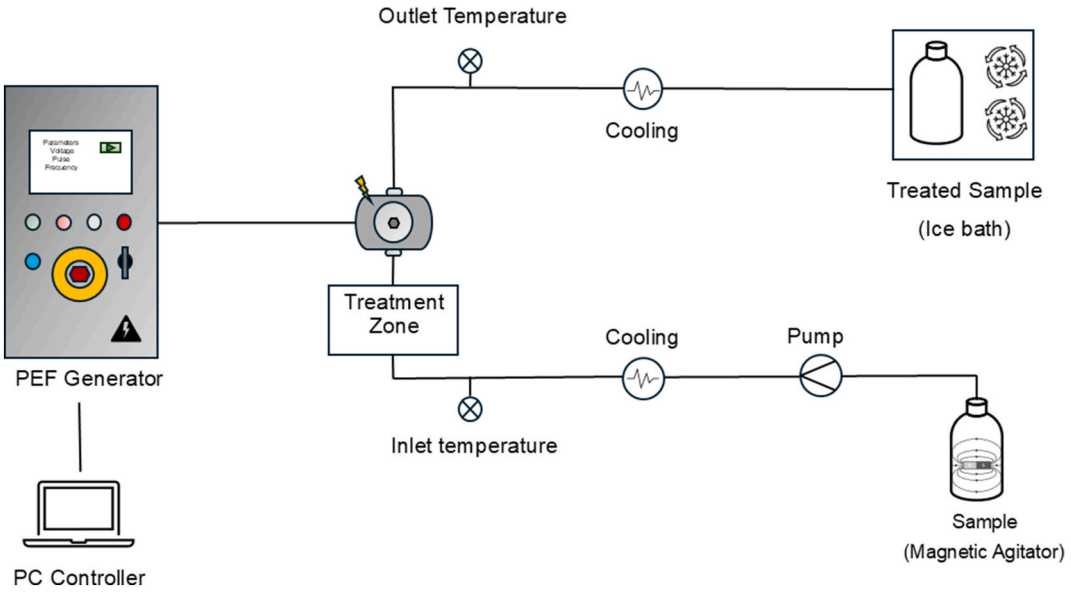

Fig. 2. Set-up of PEF equipment for continuous process for microbial inactivation.

Table 2
PEF process parameters for microbial inactivation.

Flow rate (L/h)	Electric Field Strength (kV/cm)	Total Specific Energy (kJ/kg)	Pulse width (μs)	Frequency (Hz)	Treatment time (μs)	Pulse number (n)	Outlet Temperature (°C)
5	0	0.0	0	0	0	0	20
5	23	14.7	4	6	10.3	2.58	23.5
5	23	33.9	4	11	18.9	4.73	28.1
5	23	52.3	4	17	29.2	7.31	32.5
5	23	74.1	4	24	41.3	10.32	37.7
5	23	95.4	4	31	53.3	13.33	42.8
5	23	113.9	4	37	63.6	15.91	47.2
5	23	135.6	4	44	75.7	18.92	52.4
5	23	157.0	4	51	87.7	21.93	57.5
5	23	178.7	4	58	99.8	24.94	62.7

$$R^2 = 1 - \frac{RSS}{TSS} \tag{4}$$

Where *RSS* is the residual sum of squares and *TSS* is the total sum of squares

Furthermore, the *MSE* provides information on the differences between the experimental data and the estimated values of the model (Buzrul and Alpas, 2004). Therefore, the larger the *MSE* value, the higher the discrepancy between the experimental data and the predicted output of the model. As a result, lower MSE predicts better fitting capacity and model performance (Eq. (5)). *MSE* is defined by the following equation:

$$MSE = \frac{1}{n}\sum_{i-1}^{n}\left(y_{exp}(t_i) - y(t_i, p_{ls})\right)^2 \tag{5}$$

where n is the total number of observations made, $y_{exp}(t_i)$ is the experimental data and $y(t_i, p_{ls})$ is the predicted value from the mathematical model.

The Akaike information criterion (*AIC*) is employed to evaluate how well the model fits the experimental data. The smaller the *AIC*, the better the corresponding model's ability to describe the data.(Johnson Esua et al., 2022).

$$AIC = 2k + n[ln(2\pi \cdot MSE) + 1] \tag{6}$$

Where n is the total number of observations made, k is the number of model parameters, *MSE* (Mean Squared Error) and *SSE* (Sum of Squared Errors).

2.5. Statistical analysis

Three biological samples were analysed for each strain and condition. For the mutants screening data, the data values were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) and Dunnett tests were performed to compare the mean of Log_{10} cycles reduction of each microorganism against the wild type K12 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 (Wright, 1992; Peter et al., 2000). Differences between Log_{10} cycles reduction values lower than $p < 0.05$ were considered as significant. For the parameter estimated data (C_1, C_2, C_3) one-way Analysis of Variance (ANOVA) and Tukey tests (post-hoc test) were performed using GraphPad Prism 10.0.2 (GraphPad Software, San Diego, California, United States) for the identification of significant differences between the C_1, C_2, C_3. Multiplicity adjusted *P value* was calculated for each comparison as described by (Wright, 1992; Peter et al., 2000). The differences between the parameter's estimated values lower than $p < 0.05$ were considered as significant.

3. Results

This study investigated the mechanistic responses of *E. coli* K12 when

subjected to pulsed electric field PEF treatments. A continuous processing system was intentionally used to mimic industrial applications. The research focused on comparing *E. coli* K12 isogenic mutants with the wild type to better understand their responses to PEF treatment and to identify genes that may be crucial for adapting to PEF-induced stress.

3.1. PEF inactivation screening of E. coli K12 isogenic mutants at pH 7.0

The inactivation data of the *E. coli* K12 and its 22 isogenic mutants (Δ*clpB*, Δ*rpoS*, Δ*recA*, Δ*leuO*, Δ*rmpF*, Δ*dnaK*, Δ*ompT*, Δ*cadC*, Δ*oxyR*, Δ*betI*, Δ*soxS*, Δ*katG*, Δ*sdhC*, Δ*trxC*, Δ*nudE*, Δ*appY*, Δ*cbI*, Δ*cspD*, Δ*tktB*, Δ*pstB*, Δ*oppB*, Δ*cyoA*) following the application of a specific PEF treatment (23 kV/cm, 53.3 μs, 95.4 kJ/kg, 42.8 °C) were collected. Differences in microbial resistance were apparent for three isogenic *E. coli* K12 mutants (Δ*clpB*, Δ*rpoS* and Δ*dnaK*) in comparison to the wild type. A statistical difference ($p \leq 0.01$) was noted between the Log_{10} reductions of these mutants and the wild type (Fig. 3). The most sensitive mutants were Δ*clpB*, Δ*rpoS* and Δ*dnaK* showing Log_{10} reductions of 2.97, 2.96 and 2.95, respectively. The three consecutively sensitive mutants were Δ*recA*, Δ*leuO*, Δ*rmpF* with Log_{10} reductions of 2.73, 2.68, and 2.62, respectively. Lastly, the Δ*ompT* with Log_{10} reduction of 1.56 was the most resistant mutant. These sensitive and resistant strains were further investigated for assessing the microbial inactivation kinetics under pulsed electric field (PEF) treatments at both pH 7.0 and 4.0.

3.2. Modelling of selected strains at pH 7.0 and 4.0

E. coli K-12 and its seven selected isogenic mutants (Δ*clpB*, Δ*rpoS*, Δ*dnaK*, Δ*recA*, Δ*leuO*, Δ*rmpF*, Δ*ompT*) were evaluated under pulsed electric field (PEF) treatments at an electric field strength of 23 kV/cm, with treatment durations ranging from 0 to 99.8 μs, total specific energies between 0 and 178.7 kJ/kg, and an outlet temperature between 20 and 62.7 °C, at two different pH levels (7.0 and 4.0) (Fig. 4). Microbial data were analysed using a global modelling approach to estimate the inactivation kinetic parameters, which involved simultaneously fitting both the primary and secondary models (Eqs. (2) and (3)) to the experimental data. In this study, the Log_{10} (CFU/mL) survival data of the tested strains exhibited a non-linear, multi-phase pattern in their inactivation kinetics in response to PEF treatments.

Differences were observed in the behaviour of the Log_{10} survival data depending on the type of strain used and the pH conditions (7.0 and 4.0). The results suggest that the pH influences the responses of *E. coli* K12 wild type and mutants differently. At pH 7.0, the microbial inactivation data followed a concave-upward shape, with a gradual, continuous decline in microbial counts. While, at pH 4.0 the microbial inactivation curve displays a shoulder concave-downward shape with low microbial inactivation at the initial points (up to 41.3 μs, 74.1 kJ/kg) and accelerated inactivation.

The estimated model parameters (C_1, C_2, C_3) alongside with the regression coefficient R^2, the Akaike information criterion (*AIC*) and the mean square error (*MSE*) were calculated and are presented in Table 3. The calculated regression coefficients (R^2) ranged from 0.978 to 0.998 across all datasets, indicating a good model fit, as values close to 1 are considered acceptable. The AIC was found to be between −13.17 and 12.18. Furthermore, the MSE values ranged between 0.005 and 0.088 for all the different strains.

The calculated model parameters C_1, C_2, and C_3 were found to be strain-dependent and exhibited statistically significant differences ($p < 0.05$) at pH 7.0 between strains as noted, Tables 3 and 4. More specifically, the Δ*clpB*, Δ*rpoS*, Δ*dnaK,* and Δ*recA* showed differences in the calculated parameters indicating sensitivity in comparison with the wild type at pH 7.0. The Δ*dnaK* pH 7.0 was identified as the most sensitive strain with the highest inactivation rate due to the statistical differences ($p < 0.05$) from the most resistant strains to all model parameters C_1, C_2 and C_3. Furthermore, Δ*clpB*, Δ*rpoS*, and Δ*recA* were different ($p < 0.05$) from K12 and Δ*rpmF* regarding the C_2 model parameter which indicates the highest inactivation rate of the strains. Regarding C_1, strains K12, Δ*leuO,* Δ*rpmF,* and Δ*ompT* were identified to have the lowest values in comparison with the other strains of Δ*clpB*, Δ*rpoS*, Δ*dnaK,* and Δ*recA*. At pH 4.0, there were no statistically significant differences ($p < 0.05$) among the strains concerning the calculated model parameters (C_1, C_2, C_3). However, the sensitivity of the isogenic mutants (Δ*clpB*, Δ*rpoS*, Δ*dnaK,* and Δ*recA*) compared to the wild type was observed at the highest levels of total specific energies (>113 kJ/kg), indicating that,

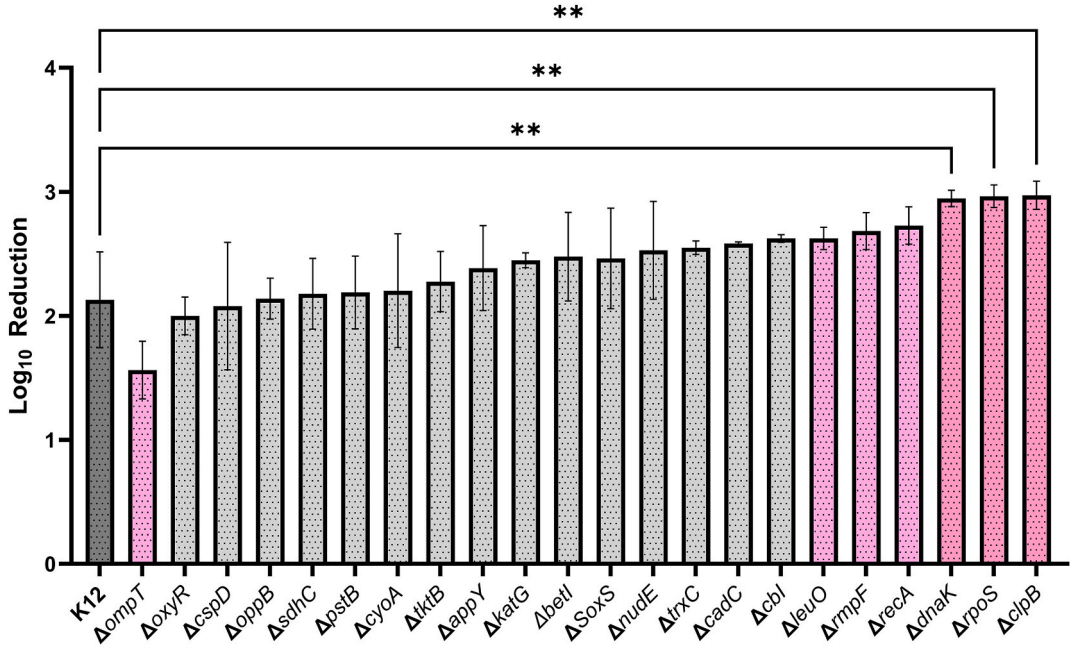

Fig. 3. Log_{10} reductions of *E. coli* K12 and its isogenic mutants: (A) under 23 kV/cm, 95.4 kJ/kg and 42.8 °C at pH 7.0. Log_{10} reduction values of isogenic mutants with a statistical difference lower than $p < 0.05$ from the wild type are represented with (*; $p < 0.05$, **; $p \leq 0.01$). The wild type is represented in dark grey. The isogenic mutants that are presented in pink were selected and further assessed. Bars represent the standard deviations of these measurements. Experiments were performed in 3 biological replicates.

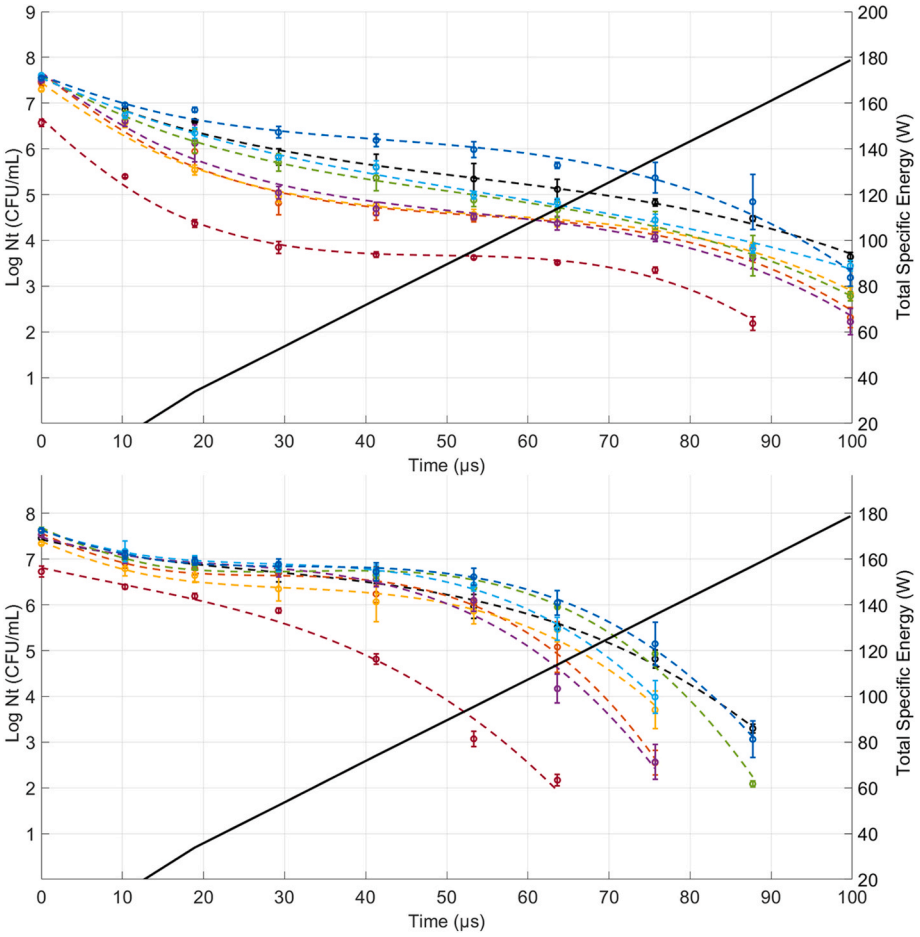

Fig. 4. Survival curves of *E. coli* K12 (■) and its isogenic mutants Δ*clpB* (■), Δ*rpoS* (■), Δ*recA* (■), Δ*leuO* (■), Δ*rmpF* (■), Δ*dnaK* (■), Δ*ompT* (■) based on experimental data describing the changes in Log_{10} CFU/mL with treatment time (μs) and total specific energy (kJ/kg) at pH 7.0 (A) and 4.0 (B). Experimental data and error bars represent the mean values of three biological replicates and standard deviations.

Table 3
Parameter estimation from the model for *E. coli* K12 and its isogenic mutants at pH 7.0 and 4.0. Values are expressed as mean ± standard error (SSE).

pH 7.0	C_1	C_2	C_3	Log_{10} N_0 (Log CFU/mL)	R^2	MSE	AIC	SSE
K12	$17.8 \pm 2.38 \times 10^{-2}$	$-26.8 \pm 6.48 \times 10^{-4}$	$14.0 \pm 3.60 \times 10^{-6}$	7.55 ± 0.12	0.992	0.017	−4.37	0.10
Δ*clpB*	$33.8 \pm 4.20 \times 10^{-2}$	$-64.5 \pm 11.0 \times 10^{-4}$	$28.6 \pm 6.26 \times 10^{-6}$	7.67 ± 0.20	0.986	0.051	6.52	0.30
Δ*rpoS*	$30.1 \pm 4.94 \times 10^{-2}$	$-55.6 \pm 13.0 \times 10^{-4}$	$33.5 \pm 7.40 \times 10^{-6}$	7.68 ± 0.24	0.978	0.071	9.94	0.43
Δ*recA*	$30.3 \pm 3.08 \times 10^{-2}$	$-54.4 \pm 8.35 \times 10^{-4}$	$28.1 \pm 4.62 \times 10^{-6}$	7.45 ± 0.15	0.989	0.027	0.34	0.16
Δ*leuO*	$22.0 \pm 2.12 \times 10^{-2}$	$-35.4 \pm 5.74 \times 10^{-4}$	$10.3 \pm 3.18 \times 10^{-6}$	7.55 ± 0.10	0.995	0.013	−6.93	0.08
Δ*rpmF*	$17.6 \pm 1.53 \times 10^{-2}$	$-21.2 \pm 4.17 \times 10^{-4}$	$19.4 \pm 2.32 \times 10^{-6}$	7.59 ± 0.08	0.998	0.007	−13.17	0.04
Δ*dnaK*	$39.3 \pm 3.05 \times 10^{-2}$	$-87.3 \pm 9.44 \times 10^{-4}$	$49.4 \pm 5.94 \times 10^{-6}$	6.67 ± 0.13	0.992	0.020	−1.52	0.10
Δ*ompT*	$16.2 \pm 3.19 \times 10^{-2}$	$-34.9 \pm 8.71 \times 10^{-4}$	$23.3 \pm 4.85 \times 10^{-6}$	7.61 ± 0.16	0.987	0.031	1.65	0.19
pH 4.0	C_1	C_2	C_3	Log_{10} N_0 (Log CFU/mL)	R^2	MSE	AIC	SSE
K12	$9.05 \pm 2.66 \times 10^{-2}$	$-21.4 \pm 8.25 \times 10^{-4}$	$22.5 \pm 5.21 \times 10^{-6}$	7.43 ± 0.12	0.994	0.016	−3.85	0.08
Δ*clpB*	$11.4 \pm 7.30 \times 10^{-2}$	$-43.3 \pm 26.0 \times 10^{-4}$	$54.3 \pm 19.3 \times 10^{-6}$	7.47 ± 0.28	0.983	0.088	11.22	0.35
Δ*rpoS*	$19.8 \pm 4.56 \times 10^{-2}$	$-76.1 \pm 17.0 \times 10^{-4}$	$76.4 \pm 12.2 \times 10^{-6}$	7.57 ± 0.17	0.992	0.034	3.55	0.13
Δ*recA*	$17.7 \pm 4.27 \times 10^{-2}$	$-57.1 \pm 16.0 \times 10^{-4}$	$51.9 \pm 11.4 \times 10^{-6}$	7.39 ± 0.16	0.986	0.030	2.54	0.12
Δ*leuO*	$14.2 \pm 1.83 \times 10^{-2}$	$-51.3 \pm 6.63 \times 10^{-4}$	$51.7 \pm 4.88 \times 10^{-6}$	7.63 ± 0.07	0.998	0.005	−11.04	0.02
Δ*rpmF*	$19.6 \pm 3.90 \times 10^{-2}$	$-69.4 \pm 12.0 \times 10^{-4}$	$59.7 \pm 7.63 \times 10^{-6}$	7.68 ± 0.17	0.992	0.034	3.16	0.17
Δ*dnaK*	$8.91 \pm 9.71 \times 10^{-2}$	$-10.0 \pm 43.0 \times 10^{-4}$	$33.0 \pm 38.0 \times 10^{-6}$	6.77 ± 0.31	0.0971	0.11	12.18	0.10
Δ*ompT*	$15.2 \pm 1.85 \times 10^{-2}$	$-51.5 \pm 5.73 \times 10^{-4}$	$45.0 \pm 3.61 \times 10^{-6}$	7.64 ± 0.08	0.998	0.007	−10.50	0.04

under these acidic conditions and high-intensity stress, the mutants exhibit high sensitivity.

Curves presenting the inactivation rates, k_{max}, of *E. coli* K12 and its isogenic mutants in relation to the total specific energy are presented in Fig. 5. Interestingly, the strains showed various inactivation rates with different k_{max} values under different PEF treatments (total specific energy). More specifically, at pH 7.0, the inactivation rate (k_{max}) peaked at both low (0–40 kJ/kg) and high (140–180 kJ/kg) total specific energies,

Table 4
Statistical differences of the model parameters C_1, C_2, C_3 of *E. coli* K12 and its isogenic mutants at pH 7.0 and 4.0. The letters a, b, and c denote groups of values that were compared using statistical analysis (ANOVA followed by a post-hoc Tukey's test).

pH 7.0	C_1	C_2	C_3
K12	c	a, b	b
Δ*clpB*	a,c	a, c	a, b
Δ*rpoS*	a, b	b, c	a, b
Δ*recA*	a, c	a, c	a, b
Δ*leuO*	c	4 a	b
Δ*rpmF*	c	4 a, b	b
Δ*dnaK*	a	c	a
Δ*ompT*	c	a, b	b
pH 4.0	***C₁***	***C₂***	***C₃***
K12	a	a	a
Δ*clpB*	a	a	a
Δ*rpoS*	a	a	a
Δ*recA*	a	a	a
Δ*leuO*	a	a	a
Δ*rpmF*	a	a	a
Δ*dnaK*	a	a	a
Δ*ompT*	a	a	a

whereas at pH 4.0, k_{max} increased predominantly at high total specific energies (120–160 kJ/kg), demonstrating that k_{max} varies depending on the total specific energy and pH conditions. Overall, differences were observed in the behaviour of the Log_{10} survival data depending on the isogenic mutant, PEF intensity and the pH conditions (7.0 and 4.0).

4. Discussion

In this study, the effects of pH and PEF intensity were highlighted as the main critical factors influencing the behaviour of *E. coli* strains and their subsequent microbial inactivation rates. In general, earlier studies under PEF treatment observed a linear relationship in survival curves (Heinz, 1999; Reina, 1998). However, this may have been related to the limited inactivation levels <4.0 Log_{10}, which occurred only when the PEF treatments were applied at low electric field strengths or for short durations. In this study, the survival profiles of the tested strains revealed a complex, non-linear, multi-phase inactivation pattern across various PEF treatments and pH levels with >4.0 Log_{10} reductions under specific strains and PEF conditions, supporting the use of a global modelling approach for fitting the survival data. Similarly with the current study, more recent research on PEF treatments on *E. coli* has shown a non-linear behaviour(Rodrigo et al., 2003; Timmermans et al.,

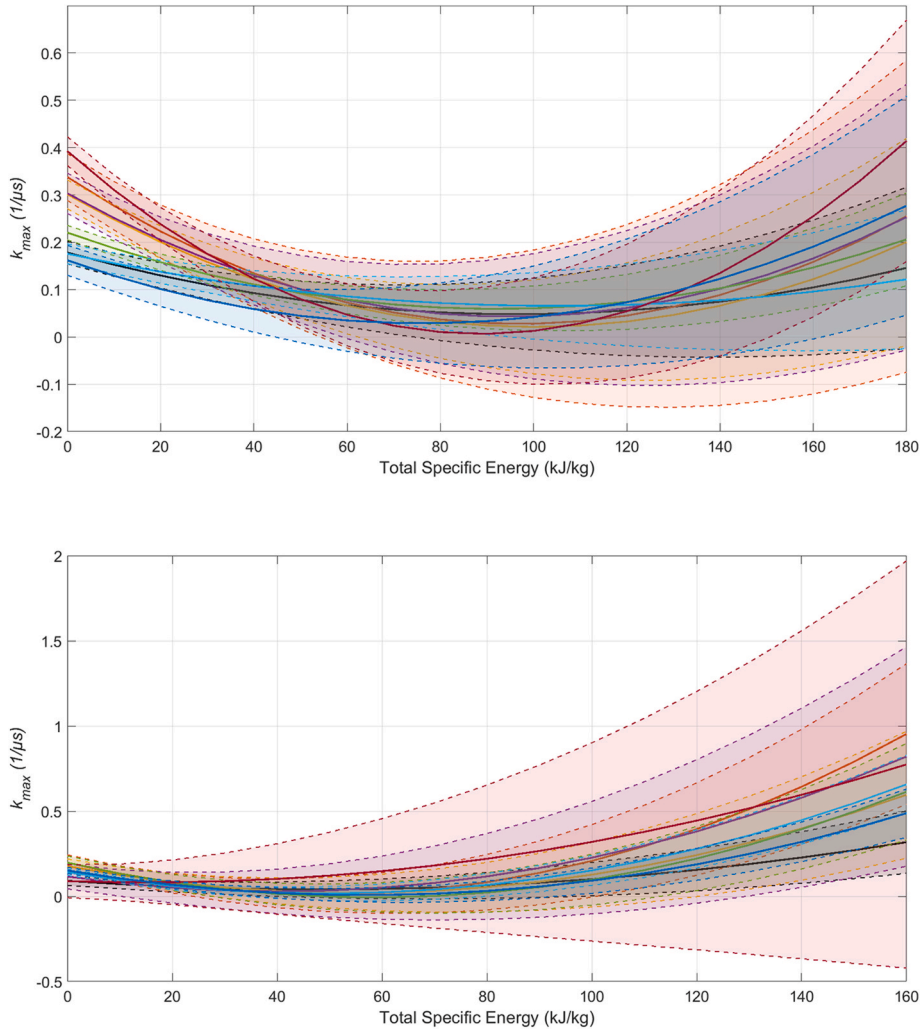

Fig. 5. Survival curves of *E. coli* K12 (■) and its isogenic mutants Δ*clpB* (■), Δ*rpoS* (■), Δ*recA* (■), Δ*leuO* (■), Δ*rmpF* (■), Δ*dnaK* (■), Δ*ompT* (■) based on based on model-derived k_{max} (1/μs) values as a function of total specific energy (kJ/kg) at pH 7.0 (A) and 4.0 (B). The k_{max} values were theoretically estimated using fitted second order polynomial type of model from experimental data. Dashed lines represent prediction bands obtained via error propagation of the calculated k_{max} estimates.

2014). In addittion, these findings build upon previous research studies by Saldaña et al. (2009),García et al. (2005) and Saldaña et al. (2010), which assessed the effect of pH levels on microbial inactivation in *E. coli*, including kinetic studies by Álvarez et al. (2003) and Saldaña et al. (2010). Specifically, Saldaña et al.(2009) and García et al. (2005) showed that *E. coli* exhibits higher PEF sensitivity at pH 7.0 than 4.0, demonstrating that pH significantly influences the microbial response and inactivation during PEF treatments. In accordance Saldaña et al. (2010) who investigated the effect of different pH levels on PEF inactivation kinetics under static conditions, showed different responses dependent on the pH value in *E. coli* and consistent with Álvarez et al. (2003) a convex shape of microbial inactivation However, these studies were conducted in a batch mode, applying pulses at a frequency of 1 Hz, so the experiments focused solely on the electric field effect without a significant temperature increase (<35 °C) (Álvarez et al., 2003; Saldaña et al., 2010). In the present study, a continuous mode was used; under these conditions, the treatment chamber functions as an adiabatic system because the short residence time inhibits energy dissipation, resulting in the conversion of applied energy into heat and consequently raising the sample temperature (Saldaña et al., 2014). This temperature rise improves the effectiveness of PEF treatments, as higher treatment temperatures have been shown to increase microbial inactivation efficiency (Saldaña et al., 2014). Thus, due to this synergistic effect and the higher total specific energy achievement, in our findings, we revealed inactivation kinetics with concave shapes, in comparison to convex shapes observed in batch mode studies. Consistent with these findings, a recent study by Thamsuaidee et al. (2024) observed concave-shaped inactivation kinetics during PEF treatments in continuous mode. More specifically, in this study, for the different pH levels, at pH 7.0 revealed a concave-upward shape with a steady and continuous decrease in microbial counts, while at pH 4.0, the curve exhibited a shouldered, concave-downward pattern marked by minimal initial inactivation (up to 41.3 μs, 74.1 kJ/kg) followed by a more rapid reduction in microbial numbers. Furthermore, at pH 7.0 the inactivation rate (k_{max}) was identified at its peak at both low (0–40 kJ/kg) and high (140–180 kJ/kg) total specific energies, whereas at pH 4.0, k_{max} increased predominantly at high total specific energies (120–160 kJ/kg), indicating the importance of the PEF intensity in the microbial inactivation.

It is generally accepted that PEF similar to most environmental stresses, do not act as "all or nothing" events (Wang et al., 2015), resulting in a sub-lethally injured population that can potentially recover if the PEF intensity is insufficient (Jaeger et al., 2009). Zhao et al. (2011) reported that under high PEF treatments >25 kV/cm, irreversible electroporation occurs, whereas at lower intensity 10–20 % of cells undergo reversible electroporation, highlighting the role of PEF intensity in the generation of sublethal populations. Furthermore, Saldaña et al. (2009), after exposing *E. coli* strains to PEF treatments of different intensity (between 20 and 30 kV/cm, 85–180 kJ/kg, <35 °C), reported higher levels of sub-lethally injured cells at pH 4.0 in comparison to pH 7.0, emphasizing the differences in PEF efficiency and the potential sublethal population dependent on the pH. Thus, the different behaviour and initial resistance observed in the microbial inactivation at pH 4.0 in comparison to pH 7.0 may be related to the presence of a sublethal population capable to recover under low-intensity PEF treatments, while at higher intensities, this effect is diminished due to the greater efficacy of the PEF treatment.

Exposure to stress prompts *E. coli* cells to adapt by activating the transcription of stress response genes and key response regulators that enhance their tolerance (Chung et al., 2006). Among the mutants examined in this research, the Δ*rpoS* strain demonstrated notable sensitivity to PEF. The *rpoS* gene is a critical sigma factor, functioning as the key regulator for the general stress response in many Gram-negative bacteria, including *E. coli* (Batista Napotnik et al., 2021; Bouillet et al., 2024); while it is known to be the primary controller for activating the expression of over 500 genes (Hengge, 2009). In our study, Δ*rpoS* was more sensitive in comparison with the wild type *E. coli* K12 at both pH levels (7.0 and 4.0). However, this was dependent on the total specific energy. For example, the Log_{10} reduction at a PEF treatment (23 kV/cm, 113.9 kJ/kg) at both pH 7.0 and 4.0, was 3.08 and 2.46 for Δ*rpoS*, and 2.35 and 1.94 for *E. coli* K12, respectively, indicating the higher sensitivity of the mutant. Yun et al. (2016) in a study focusing on the Gram-negative bacterium of *Salmonella* Typhimurium, demonstrated the potential role of the alternative sigma factors (*rpoS*, *rpoE* and *rpoH* genes) as regulators of the bacterial response to PEF. Similarly, Guillén et al. (2023), reported that *Salmonella enterica* utilizes RpoS to enhance its resistance to PEF and UV-C treatments. Furthermore, Somolinos et al. (2008) found that that the *rpoS* null mutant, *E. coli* BJ4L1, exhibited decreased PEF resistance as compared with its wild-type counterpart, BJ4. The same study by Somolinos et al. (2008) has observed minimal differences between the counts of the *rpoS* null mutant, *E. coli* BJ4L1 in selective and non-selective media, suggesting low repair capacity of the strain relative to the wild type. Thus, RpoS is suggested to play an important role in protein synthesis, among other functions, for the repairment of cell membrane damage caused by PEF which is the primary cause of cell death due to the technology (Somolinos et al., 2008).

Furthermore, our findings support that *dnaK* and *clpB* potentially play an important role in the resistance mechanisms of *E. coli* against PEF. DnaK is the primary bacterial Hsp70 chaperone, and one of the most abundant proteins in the *E. coli* cytosol, expressed both constitutively and in response to stress (Bukau and Walker, 1989). In general, under heat stress, dnaK has an important role; however, under moderate, non-stressful temperatures, it is not essential for the bacterium's survival (Bukau and Walker, 1989). The Δ*dnaK* was identified to have the highest statistical difference ($p < 0.05$) in comparison with the wild type *E. coli* K12 at the calculated model parameters C_1, C_2 and C_3 indicating the high sensitivity of the strain at pH 7.0. On the contrary, at pH 4.0, no statistical differences were observed between the calculated model parameters C_1, C_2 and C_3 between the Δ*dnaK* and the wild type. However, at PEF treatments of 23 kV/cm with 74.1 and 95.4 kJ/kg the Δ*dnaK* showed Log_{10} reductions of 3.65 and 4.55, while the *E. coli* K12 wild type showed 1.49 and 1.94, respectively indicating the sensitivity of the strain at high total specific energies. ClpB, is a well-known member of the Hsp100/Clp (caseinolytic protease) family found in various organisms. It is a heat shock protein that functions as a molecular chaperone involved in protein disaggregation (Eriksson and Clarke, 2000). In this study, a statistical difference ($p < 0.05$) was identified for the calculated model parameter C_2 of the Δ*clpB* when compared with the wild type at pH 7.0. At pH 4.0 and under a PEF of treatment of 23 kV/cm at 95.4 kJ/kg, Δ*clpB* showed a Log_{10} reduction of 3.36, indicating that, as with Δ*dnaK,* statistically significant differences in inactivation were observed at high intensities of PEF treatments. ClpB, in cooperation with its cochaperones DnaK, DnaJ, and GrpE (collectively known as the KJE system), utilizes energy from ATP hydrolysis to disassemble protein aggregates (Motohashi, 1999; Doyle et al., 2007). Meury and Kohiyama, (1991)reported that *E. coli* O157:H7 exposed to heat shock conditions (47.5 °C for 10 min) in dairy compost exhibited upregulation of heat shock genes such as *clpB*, *dnaK*, *groEL*, along with the alternative sigma factor *rpoH*. This suggests that the heat shock response acts as a protective mechanism during composting, contributing to bacterial survival (Singh and Jiang, 2015). These findings align with the current observation that *E. coli* Δ*clpB* and Δ*dnaK* mutants may cooperate in resistance to PEF treatment. Meanwhile, previous study by Lytras et al. (2026) compared *L. monocytogenes* EGD-e wild type against the homologous mutants of Δ*clpB* and Δ*dnaK* under PEF treatment (20 kV/cm, 184 kJ/kg). It revealed statistically significant difference in Log_{10} reductions only between the wild type and Δ*clpB* mutant indicating potential differences between *E. coli* and *L. monocytogenes* (Lytras et al., 2026). In addition to Δ*clpB*, Δ*rpoS*, and Δ*dnaK* deletions, this study identified Δ*recA* (Log_{10} reduction of 2.73) as one of the most sensitive mutants at PEF treatment of 23 kV/cm,53.3 μs, 95.4 kJ/kg, at 42.8 °C, while a statistically significant difference ($p < 0.05$) was identified for the calculated model parameter C_2 between the mutant and the wild type at

pH 7.0. The *recA* gene encodes the RecA protein, which is essential for multiple enzymatic activities pivotal to homologous recombination and DNA repair in *E. coli* (Clyman and Cunningham, 1987). RecA plays a central role in orchestrating the bacterial SOS response, a complex regulatory network triggered by DNA damage alongside the LexA repressor (Clyman and Cunningham, 1987). This is consistent with a proteomic study by Rivas et al. (2013) that has shown increased levels of proteins associated with the recovery of *E. coli*, with direct or indirect functions with RecA, after PEF treatment including hosphoheptose isomerase (GmhA; involved in the biosynthesis of cell wall lipopolysaccharide), cytosolic phosphorylase A (ClpA; involved in n the degradation of unfolded or abnormal proteins), ribosomal protein S6 in the 30S ribosomal subunit (RS6; involved in the translation stage of protein biosynthesis), enzyme deoxyuridine 5′-triphosphate nucleotidohydrolase (Dut; responsible of the production of dUMP and involved in nucleic acid metabolism) and ferritin A (FtnA; involved in storing iron). When PEF treatments are insufficiently applied, they can result in sub-lethally injured *E. coli* cells-damaged but capable of recovery (Jaeger et al., 2009). Therefore, the increased sensitivity observed in the Δ*recA* mutant compared to *E. coli* K-12 is likely due to its impaired ability to recover from PEF-induced damage.

The isogenic *ompT* gene deletion was identified as the most resistant strain under PEF treatments and especially at pH 7.0. For example, at the initial PEF treatment (23 kV/cm, 53.3 μs, 95.4 kJ/kg, 42.8 °C) screening at pH 7.0, the Log_{10} reduction of Δ*ompT* was 1.56 Log_{10} CFU/mL. Outer membrane porins are transmembrane proteins that form water-filled channels through their β-barrel architecture, facilitating the passive diffusion of hydrophilic molecules across the bacterial outer membrane (Choi and Lee, 2019). The *ompT* gene encodes OmpT, an outer membrane protein of *E. coli*, which plays an in proteolysis and immune invasion (Grodberg and Dunn, 1988), while the omptin genes of *ompT* and *ompP* are transcriptionally regulated by PhoPQ system. A study by Tan et al., (2017)demonstrated that deletion of *ompF*, which is another outer membrane related gene encoding OmpF (accession W9ADK8) that forms pores and contributes to the structural stability of *E. coli* cells, enhances membrane integrity, suggesting that the absence of this porin can strengthen the bacterial membrane barrier. Although, it is known that Δ*ompT* alter membrane dynamics, including outer membrane vesicle production (OMV) as described by Premjani et al. (2014), the deletion of *ompT* may result in a less permeable and more rigid membrane that is better able to withstand the effects of PEF. On the other hand, PhoPQ may regulate changes in the outer membrane structure, while altered resistance to antimicrobial peptides contributes to complex membrane-level adaptations that improve survival under PEF.

Our findings demonstrate that *E. coli* mounts a sophisticated and coordinated response to PEF treatment, involving the combined actions of heat shock proteins, global stress regulators, and DNA repair systems. Deletion of the chaperone genes *dnaK* or *clpB* significantly reduced cell viability under PEF stress, indicating that protein denaturation is a major factor in PEF-induced damage and that these heat shock proteins play a protective role similar to their function of heat shock response. This critical role of heat shock proteins, especially DnaK and ClpB, aligns with the expanded σˆ32 regulon characterized by (Nonaka et al., 2006) which revealed that the σ^{32} controlled heat shock response not only includes molecular chaperones like DnaK, GroEL/S, and ClpB but also incorporates regulatory feedback mechanisms essential for maintaining protein homeostasis under stress conditions. Alongside, the general stress sigma factor RpoS regulates numerous genes that contribute to oxidative stress defense, metabolic adjustments, and various repair mechanisms, all of which enhance *E. coli*'s ability to survive the stresses induced by PEF treatment. Crucially, RecA facilitates DNA repair by initiating homologous recombination and the SOS response to counteract PEF-induced DNA damage, ensuring genomic stability. Thus, we propose that these interconnected pathways related to protein quality control via σ^{32}, broad stress adaptation through RpoS, and genomic maintenance by RecA constitute a comprehensive defense strategy that enables *E. coli* to withstand and recover from the diverse cellular damages inflicted by PEF treatment and influences by the intensity of the treatment and the pH.

5. Conclusion

In conclusion, this study investigated the mechanistic responses of *E. coli* K12 and its isogenic mutants to PEF treatments at pH 7.0 and 4.0. Firstly, an initial screening at PEF treatment (23 kV/cm, 53.3 μs, 95.4 kJ/kg, 42.8 °C) of 22 mutants, 3 were identified (Δ*clpB*, Δ*rpoS*, Δ*dnaK*) showing a statistical difference ($p \leq 0.01$) in terms of Log_{10} reduction when compared with the wild type at pH 7.0. In addition to these mutants, three equally sensitive, i.e., Δ*recA*, Δ*leuO*, Δ*rmpF*, and one more resistant, i.e., Δ*ompT* were further assessed, under PEF treatments at pH 7.0 and 4.0. The experimental findings demonstrated that microbial inactivation kinetics under PEF exhibited non-linear behavior which was best described by a second-order polynomial model, with model parameters (C_1, C_2, C_3) significantly differing between strains at neutral pH 7.0 but not at acidic pH 4.0. Nonetheless, under high total specific energy (>113 kJ/kg) stress at acidic pH, the same mutants showed increased sensitivity in comparison with the wild type, emphasizing that pH and energy input, modulate bacterial resistance mechanisms. In summary, the behaviour and the inactivation rate of *E. coli* K12 and its isogenic mutants was dependent on the pH and the total specific energy. Further research into the key resistant mechanisms of *E. coli* will deepen our understanding of its response to PEF, potentially improving the process's efficacy through the development of PEF-based hybrid approaches that combine physical or chemical hurdles.

CRediT authorship contribution statement

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

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

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.

Acknowledgements

•The authors would like to thank and acknowledge Prof. Dr. Kimon-Andreas Karatzas from the University of Reading (United Kingdom) for providing the *Escherichia coli* K12 and its mutants from their collection.

Data availability

Data will be made available on request.

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