CELLULAR EVENTS INVOLVED IN *E. coli* CELLS INACTIVATION BY SEVERAL AGENTS FOR FOOD PRESERVATION: A COMPARATIVE STUDY.

María Marcén, Guillermo Cebrián, Virginia Ruiz-Artiga, Santiago Condón and Pilar Mañas*

Running title: Cellular events in treated *E. coli*

Tecnología de los Alimentos, Facultad de Veterinaria de Zaragoza, Instituto Agroalimentario de Aragón– IA2 - (Universidad de Zaragoza-CITA), Zaragoza, Spain

*Corresponding author: Pilar Mañas

Postal address: Tecnología de los Alimentos, Facultad de Veterinaria, C/ Miguel Servet, 177, 50013, Zaragoza, Spain.

Tel No.: +34876554136

E-mail: manas@unizar.es

ABSTRACT

Traditional and novel technologies for food preservation are being investigated to obtain safer products and fulfil consumer demands for less processed foods. These technologies inactivate microorganisms present in foods through their action on different cellular targets, but the final cause of cell loss of viability often remains not well characterized. The main objective of this work was to study and compare cellular events that could play a role on *E. coli* inactivation upon exposure to treatments with technologies of different nature. *E. coli* cells were exposed to heat, high hydrostatic pressure (HHP), pulsed electric fields (PEF) and acid treatments, and the occurrence of several alterations, including presence of sublethal injury, membrane permeabilization, increased levels of reactive oxygen species (ROS), DNA damage and protein damage were studied. Results reflected differences among the relevance of the several cellular events depending on the agent applied. Sublethally injured cells appeared after all the treatments. Cells consistently recovered in a higher percentage in non-selective medium, particularly in minimal medium, as compared to selective medium; however this effect was less relevant in PEF-treated cells. Increased levels of ROS were detected inside cells after all the treatments, although their order of appearance and relationship with membrane permeabilization varied depending on the technology. A high degree of membrane permeabilization was observed in PEF treated cells, DNA damage appeared as an important target in acid treatment, and protein damage, in HHP treated cells. Results obtained help to understand the mode of action of food preservation technologies on bacterial cells.

KEYWORDS: heat, acid, PEF, HHP, sublethal injury, cellular alterations.

1. INTRODUCTION

Food preservation is a continuous fight against pathogenic and spoilage microorganisms in order to obtain safe products. However, consumers not only expect safe products, but also demand products which keep their nutritional and sensorial properties. With the purpose of achieving consumer demands, the food industry investigates novel technologies for food preservation. Some of the most investigated technologies are non-thermal technologies such as high hydrostatic pressure (HHP) and pulsed electric fields (PEF). Besides, optimization of traditional techniques such as heat treatments, acidity, natural antimicrobials, packaging systems, etc., continues to be an important research topic. To obtain a better profit of all these technologies and to design appropriate combined processes, it is necessary to gain deeper insight into their mode of action on microorganism.

Within a bacterial cell, there are many potential cellular targets that may be affected by a given stressing agent; some of them are considered as critical targets, whereas others are not essential for bacterial survival (Miles, 2006). In addition, there are tight interrelationships between the various structures and cellular functions; therefore, the alteration of a particular structure or function may indirectly affect another one (Mackey and Mañas, 2008). Furthermore, the degree of damage exerted to the different cellular targets is also an important factor, since damages in critical components at low intensity may produce sublethal injury; similarly, damages in non-critical components may also render sublethally injured cells. These cells can repair their damages and resume growth only if the environmental conditions are appropriate (Mackey, 2000). In summary, identifying the particular structures and processes whose alteration leads to cell death is a difficult task.

Nowadays, it is generally accepted that heat treatment has a multi-target mode of

action on microorganisms, since it is able to cause alterations at different cellular levels. These include cytoplasmic membrane damage, outer membrane damage, DNA strand breaks, inactivation of enzymes, protein coagulation, etc (Lado and Yourself, 2002; Mackey, 2000). HHP is also considered a multi-target technology, affecting envelopes integrity, DNA and protein conformation, ribosomes configuration etc. (Aertsen et al., 2005; Mackey and Mañas, 2008; San Martín et al., 2002). Studies about PEF have described that electroporation in the cytoplasmic membrane is the main cellular alteration during exposure to this technology (Mañas and Pagán, 2005). On the other hand, although much less information is available, envelopes, protein and DNA damage are considered as important targets to inactivate microorganisms by acid pH (Richard and Foster, 2004; Van de Guchte et al., 2002). Despite the different mode of action of the various technologies, the cytoplasmic membrane appears as a common target in most cases.

It is important to note that, despite these general observations about the different technologies are generally assumed, the precise mode of action on bacterial cells, and the relative importance of the various cellular alterations on cell survival or inactivation remains largely understudied. Currently, a new aspect is gaining importance as a possible common mechanism of microbial inactivation of all of these technologies: the oxidative component (Mols and Abee, 2011). There are scattered reports of increased ROS levels in cells treated by physical and chemical agents. In this way, it has been proven that higher levels of ROS are detected in heat treated *E. coli* cells (Baatout et al., 2005; Marcén et al., 2017). Besides, some authors demonstrated that HHP treatment induces cytoplasmic oxidative stress in *E. coli* (Aertsen et al., 2005; Malone et al., 2006). Pakhomova et al. (2012) suggested that in PEF treatment (nanosecond pulses) the increase in non-selective transmembrane cation conductance may be in

part mediated by oxidative stress, and ROS formation could be a factor contributing to the cytotoxic effects in eukaryotic cells. Finally, Mols et al. (2010) supported the idea that acid stress also induced oxidative stress. These authors proposed that the formation of radicals such as $OH^{\bullet -}$ may be a common mechanism of cellular death when bacteria are exposed to different stress conditions. Increased ROS levels have been attributed to disturbances in the electron transport as a consequence of alterations in cytoplasmic membrane integrity and functionality. However, levels of ROS within bacterial cells may also increase depending on other factors such as the loss of activity of scavenging enzymes, the loss of antioxidant molecules, or the presence of free iron in the cytoplasm, among other factors (De Spiegeleer et al., 2004; Gusarov and Nudler, 2005; Imlay, 2013).

This research was aimed to obtain robust and systematic data in order to clarify the main cellular events involved in the inactivation of bacteria by food preservation technologies of different nature and the possible relationship between them. To reach this objective, the presence of ROS and the permeabilization of the membrane after heat, HHP, PEF and acid treatments was determined and comparisons among the different agents were made. Moreover, other cellular alterations that could be involved in cell inactivation were also studied in order to gain knowledge about the mode of action of these stressing agents on bacteria.

2. MATERIAL AND METHODS

2.1. Bacterial strains and growth conditions

Escherichia coli BW25113 and its isogenic mutants JW0013 (*ΔDnaK:kan*) and, JW2669 (*ΔRecA*:*kan*) were used in this study. The strains were stored at -80ºC. To prepare preculture, a flask containing 10 mL of sterile TSBYE (Tryptic Soy Broth with 0.6% Yeast Extract, Oxoid, Basingstoke, UK) was inoculated with one single

colony from a TSAYE plate (Tryptic Soy Agar with 0.6 % Yeast Extract, Oxoid) for parental strain, and a TSAYE plus kanamycin (0.05 mg/mL) plate for mutant strains. The preculture was incubated overnight at 37ºC, under agitation. Subsequently, a culture was obtained by inoculating a flask with 50 mL TSBYE with 100 µL of the preculture, and incubating under agitation at 37ºC for 18-24 hours until stationary phase of growth (1-2×10^9 CFU/ mL, approximately).

2.2. Heat treatment

Heat treatments were carried out in glass tubes, which contained 4.5 mL of sterile PBS (Phosphate Buffered Saline, Sigma, San Louis, Missouri, USA) as treatment medium, submerged and prewarmed at 58ºC in a thermostated water bath. Five hundred µL of the bacterial culture was inoculated to reach an initial concentration of 10^8 CFU/ mL, approximately, and after different heating times, samples were collected, immediately cooled and kept for further analysis.

2.3. High hydrostatic pressure treatments

Stansted Fluid Power S-FL-085-09-W (Harlow, London, England) equipment was used to carry out HHP treatments (Ramos et al., 2015). A mixture of propylene glycol and distilled water (50/50, v/v) was used as the pressure transmitting fluid. An automatic device was employed to set and/or record the pressure, time and temperature during the pressurization cycle. Cell suspensions were centrifuged and diluted to a cell concentration of 10^8 CFU/ mL in PBS, approximately. Samples were packed in plastic bags, which were sealed and introduced in the equipment treatment chamber. Treatments were applied at 300 MPa during different treatment times up to 30 min, and temperature never exceeded 40ºC.

2.4. Pulsed electric field treatment

PEF equipment used in this investigation was supplied by ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden). The equipment and treatment chamber used in this investigation were previously described by Saldaña et al. (2010). For PEF treatments, cells were dissolved in McIlvaine buffer, which allowed a simultaneous adjustment of pH (7.0) and conductivity (1 mS/cm), at a concentration of 10^8 CFU/ mL. Samples were introduced in the treatment chamber of the PEF equipment, which had a gap of 0.25 cm. A square pulse with a width of 3 µs, a frequency of 0.5 Hz (1 pulse per 2 seconds), electric field strength of 20 kV/cm and <40ºC of temperature were used during the treatment. Under these experimental conditions, the energy per pulse was 1.20 kJ/kg. Treatments of up to 100 pulses (300 µs) were applied.

2.5. Acidity treatment

TSBYE was acidified to pH 3.0 with lactic acid and then filter-sterilized. Cells were added to a concentration of 10^8 CFU/ mL, and temperature was kept constant at 25ºC. One hundred µL samples were withdrawn at intervals, up to 120 min, and transferred into 900 µL of TSBYE for neutralization (Cebrián et al., 2010).

The treatment parameters for heat, HHP, PEF and acidity (intensity and exposure times) were chosen from preliminary experiments (data not shown), in order to achieve a slow *E. coli* inactivation, to study damages in different cellular targets under equivalent lethality conditions for the four agents.

2.6. Recovery after treatments and survival curves

After each treatment, samples were serially diluted in MRD (Maximum Recovery Diluent, Oxoid) and pour-plated in TSAYE for survival counts. Plates were incubated at 37ºC in aerobic conditions and after 24-72 h CFU were counted. With the purpose of constructing survival curves, the fraction of survivors (Log N_t/N_0) was represented *vs* the treatment time (min for heat, HHP and acid treatments, and µs in PEF treatment). Under

most experimental conditions deviations from linearity were observed, and therefore the Geeraerd inactivation model-fitting tool (GInaFiT) (Geeraerd et al., 2005) was used to fit survival curves and to calculate resistance parameters. As survival curves obtained in this investigation generally showed shoulders, the log linear regression plus shoulder model was used in the majority of the experiments (Equation 1). However, in the particular case of PEF treatments, survival curves showed a linear portion plus a tail, so in this case the log linear regression plus tail was used (Equation 2) (Geeraerd et al., 2000).

$$N(t) = N(0) \cdot e^{-k_{max} \cdot t} \cdot \left[\frac{e^{k_{max} \cdot S_l}}{1 + \left(e^{k_{max} \cdot S_l} - 1\right) \cdot e^{-k_{max} \cdot t}} \right] \quad (1)$$

$$N(t) = \left(N(0) - N_{res}\right) \cdot e^{-k_{max} \cdot t} + N_{res} \quad (2)$$

In these equations *N(t)* represents the number of survivors, *N(0)* the initial count and *t* the time for treatments. Furthermore, to describe the survival curves, these two models use the following parameters: shoulder length (S_l), defined as the time before the exponential inactivation begins; inactivation rate (k_{max}), defined as the slope of the exponential portion of the survival curve; and N_{res} which describes the residual population density (tail).

The traditional decimal reduction time value (*D*) of each survival curve was calculated from k_{max} (Equation 3).

$$D = 2.303/k_{max} \quad (3)$$

2D values were also calculated. In this case *2D* is defined as the dose necessary to inactivate 2 Log_{10}-cycles of the initial population, and is calculated by Equation 4.

$$2D = S_l + 2 \times D \quad (4)$$

Where S_l is the shoulder length duration and *D* is the inactivation parameter calculated from Equation 3.

Where indicated in the text, cells were also recovered in minimal M9 glucose-salts agar

enriched with 3 mM L-cysteine HCl (MM-cys) (Sigma Aldrich, Milan, Italy) under anaerobic atmosphere (MACS VA500 Microaerophilic Workstation, DW Scientific, UK). M9 agar was prepared as described previously (Gerhardt et al., 1994), supplemented with $FeSO_4$ (10 mg/L) to improve bacterial growth (Stanier et al., 1992), and with cysteine to create a low redox potential medium (Gerhardt et al., 1994; Suh and Knabel, 2000). On the other hand, a selective medium which consisted of TSAYE with the maximum non inhibitory concentration of NaCl (4%) determined in previous experiments (data not shown) (Panreac S.A, Barcelona, Spain), was used to detect the number of sublethally damaged cells (Mackey, 2000). In order to quantify and compare the proportion of sublethally damaged cells appearing after exposing cells to the different agents, the area under the curve (time units × Log N_t/N_0) was calculated with the GraphPad PRISM 5 software (GraphPad Software, Inc., San Diego, CA, USA) as described by Lou and Yousef (1997), fixing a treatment time corresponding to 2 Log cycles of inactivation under standard recovery conditions (TSAYE). The population displaying sublethal damage corresponds to the area under the curve of cells recovered in selective medium, minus the area under the curve of cells recovered in non-selective one for a fixed exposure time. Since survival curves corresponding to the different technologies were not directly comparable because of their different treatment lengths; the ratio between both areas was calculated, instead of the difference, in order to establish meaningful comparisons.

2.7. ROS and membrane permeabilization determinations

The presence of ROS and membrane permeabilization in cells after exposure to the treatments was studied through staining with specific fluorochromes followed by epifluorescence microscopy. Treated cells were collected and stained separately with DHE (dihydroethidium) (Life Technologies), HPF (hydroxyphenyl fluorescein) (Sigma

Aldrich) and PI (propidium iodide) (Sigma Aldrich). In some experiments, where indicated in the text, double staining was performed, by the use of two dyes combined simultaneously. The combinations used were DHE plus HPF, and HPF plus PI. Treated cells were incubated with the fluorescent dye at a cell concentration of approximately 10^8 CFU/mL, then centrifuged and resuspended in PBS. A positive and a negative control were always included. Staining conditions were 50 µM/90 min for DHE and HPF and 3 µM/30 min for PI (Klotz et al. 2010; Marcén et al., 2017; Mols et al., 2009; Patsoukis et al., 2005). The results obtained were analyzed by phase contrast and fluorescence microscopy (Nikon Eclipse E400, Nikon Corporation, Japan), in order to obtain the percentage of stained cells. Images were obtained with a high resolution camera (AxioCam MRc, Zeiss, Germany) and processed with the software ZEN 2012 (Zeiss, Germany). Total and fluorescent cells were counted from photographs taken from each sample, and at least three different representative microscopic fields, containing 100-200 cells, approximately, were used for quantification.

2.8. Measurement of DNA damage by qualitative PCR assays.

Bacterial DNA damage was measured semi quantitatively following the fundamentals of the method reported by Park and Imlay (2003), based on the fact that damaged DNA renders a less effective amplification with a high fidelity polymerase. Total genomic DNA, either from untreated or from treated cells, was isolated from 1 mL of culture using a genomic DNA extraction kit (Realpure, Real Laboratory, Valencia, Spain). DNA was quantified spectrophotometrically and diluted in MilliQ water to 10 ng/µL (Simplinano, Biochrom, Cambridge, UK). A 1860 pb fragment upstream and downstream *rpoS* gene was used for amplification. Primer sequences were as follows: 5'-ACTGTCAGCAGTACATCAACCAGTA (forward primer) and 5'-GTTACCAGCCGCATTTATTATTTC (reverse primer). The 20 µL PCR mixture

contained 20 ng of genomic DNA as a template, a 40 µM concentration (each) of the two primers, 20 mM of the deoxynucleotide mix (Sigma), 5X Phusion HF Buffer and 1 µL of Phusion DNA Polymerase 2 U/µL (Thermo Scientific, Massachusetts, USA). Thermal cycling was performed with a MultiGene II Personal Thermal Cycler (Labnet Biotecnica, Madrid, Spain). The genomic DNA was initially denatured for 3 min at 98°C, and then the DNA was subjected to 30 cycles of PCR, with 1 cycle consisting of denaturation at 98°C for 10 s and annealing at 66ºC for 30 s and extension at 72°C for 90 s. A final extension step at 72°C was performed for 10 min at the completion of the profile. PCR products were separated by 1% agarose gel electrophoresis, stained with SYBR safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and photographs were obtained with Gel Doc XR (Bio-Rad Laboratories, Hercules, CA, USA).

2.9. Statistical analysis

All the experimental determinations were performed at least in triplicate with independent microbial cultures, and data in Figures correspond to the average and the mean standard deviation (error bars). Survival curve fitting was carried out individually for each replicate, resistance parameters obtained (S_l, k_{max}, N_{res}, D, $2D$) and included in Table 1 as the average and the mean standard deviation. Fitting performance was indicated by the parameters RMSE (root mean squared error) and R^2. Student´s t tests were carried out using the GraphPad PRISM 5 software (GraphPad Software, Inc., San Diego, CA, USA), and differences were considered significant for $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Survival of *E. coli* to heat, HHP, PEF and acid.

The resistance of *E. coli* cells to different agents was evaluated. In this way, cells were treated by heat (58ºC), HHP (300 MPa), PEF (20 kV/cm), and lactic acid (pH 3.0). Figure 1 shows the survival curves of *E. coli* to the four technologies, obtained in

TSAYE, TSAYE-NaCl and MM-Cys. The level of inactivation of *E. coli* cells to heat, HHP, PEF, and lactic acid obtained was similar to that reported by other authors, using a complex nutritional medium for the recovery of cells (Aertsen et al., 2005; Álvarez et al., 2003; Benito et al., 1999; Cebrián et al., 2007). Experimental data were fitted to the Geeraerd equation, and the parameters obtained are included in Table 1. All the survival curves were adequately described by the Geeraerd equations ($R^2 \geq 0.97$), except the curve corresponding to acid treatment and MM-cys as recovery medium, due to the insufficient inactivation attained after the longest exposure time tested. As it can be observed in the graphs, the shape of the survival curves depended on the agent applied. Convex or close to straight curves were obtained for heat, HHP and acid treatments, whereas for PEF treatment the profile of the survival curves was concave. For this reason, curves were fitted to the shoulder log-linear Geeraerd, except those corresponding to PEF treatments, which needed the log-linear Geeraerd equation provided with the mathematical adjustment to describe the tail portion. In this study, tails appeared at an inactivation level of 3 Log cycles, approximately. The presence of tails in PEF survival curves has been frequently reported and has been associated to different phenomena, such as the presence of cells in the population with different resistance to the treatment, adaptation phenomena along the treatment time or heterogeneity within the treatment chamber. In contrast, convex curves are often associated with the presence of a shoulder phenomenon, which has been attributed to the occurrence of sublethally injured cells within the treated population (Cebrián et al., 2007; Lou and Yourself, 1997; Mackey, 2000; Mañas and Pagán, 2005). To this regard, the occurrence of an injured population is frequently estimated by the difference between the number of viable cells in non selective medium (TSAYE) and in selective medium, and normally the loss of the ability to grow in a selective medium with

sodium chloride (TSAYE-NaCl) is interpreted as the loss of functionality of the cytoplasmic membrane (Mackey, 2000; Wuytack et al., 2003). In this research we also used minimum salts-glucose media with cysteine (MM-cys) under anaerobic conditions to improve cells recovery. As Figure 1 shows, the four technologies here studied lead to the appearance of sublethally damaged cells. For the four agents, statistically significant differences (p<0.05) were found among *2D* values obtained for the three different recovery conditions, and these differences were highly significant (***) for heat, HHP and acidity.

According to our results, the relevance of sublethal damage seemed to be lower in PEF-treated cells than in the other three technologies, since survival curves obtained under the three recovery conditions used were closer in the case of PEF treatment, which, in addition, showed absence of shoulder phenomena. The parameter area under the curve (AUC, Table 1) offers an overall indication of the relative inactivation provided by each treatment, therefore meaningful comparisons among recovery conditions can be made. The ratios between the areas associated to the survival curves obtained in TSAYE and in TSAYE- NaCl for heat, HHP, PEF and acid treatments were 2.8, 2.2, 1.5 and 4.5, respectively (calculated from values in Table 1). As a whole, these results would indicate that in PEF treated cells, and under the experimental conditions here used, damage to the cytoplasmic membrane would be more difficult to repair, and thus, probably more directly related to cell inactivation.

Conversely, when cells were recovered in minimal medium MM-cys and anaerobic atmosphere, the amount of survivors was higher for the four technologies studied (Fig 1, Table 1). Thus, these later conditions can be considered as more adequate for cell repair after all the treatments applied. It has to be noted that anaerobic atmosphere was not the only factor that favored cell recovery, since the number of survivors

obtained in TSAYE and anaerobiosis was very similar to that obtained under aerobic recovery (data not shown). Also in this case, the ratios between the areas associated to survival curves obtained in MM-cys and in TSAYE (3.0, 3.6, and 1.9 for heat, HHP and PEF respectively) confirmed that for PEF treatments, the amount of cells with sublethal injuries, able of recover and resume growth, was always lower. With regards to the higher cell recovery observed in minimal medium MM-cys and anaerobic atmosphere, it is reasonable to think that the protective effect observed in MM-cys medium could be ascribed to its low redox potential and to the absence of oxidative species generated during autoclaving, as previously demonstrated by other authors (Mackey, 2000). These observations suggested that an oxidative component was involved in cell inactivation and/or recovery after treatment, for the four technologies here studied. It is important to keep in mind that, according to recovery data, also cytoplasmic membrane alterations seemed to occur in cells treated by the four agents.

3.2. Exposure of *E. coli* cells to different technologies caused increase of ROS levels and membrane permeabilization.

Treated cells were stained with different dyes to evaluate ROS presence. DHE (dihydroethidium) was used for the detection of $O_2^{\bullet-}$ due to its relative specificity for this radical. Superoxide oxidizes the dye producing oxyethidium, which binds to DNA showing strong red fluorescence (Gomes et al., 2005), although other reactive species can also react with DHE (Zielonka and Kalyanaraman, 2010). We also used HPF 3′-(p-hydroxyphenyl-fluorescein) which is oxidized by hydroxyl radicals producing green fluorescence (Gomes et al., 2005). On the other hand, propidium iodide was used to detect membrane permeabilization after treatment. This dye is not permeable, thus it does not penetrate the cell unless the membrane is damaged. Once inside, it binds to DNA and RNA and produces red fluorescence.

Figure 2 shows the percentage of stained and inactivated cells along time for each treatment. It has to be noted that the inactivation is represented in a linear scale to allow comparisons with the amount of stained cells. Treatments were applied in order to obtain up to 90-99% of inactivation under standard recovery conditions (TSAYE, aerobiosis) to enable comparisons among different treatments. Results showed that there was an increase in ROS level and membrane permeabilization in *E. coli* cells upon exposure to the four agents. The kinetics and order of appearance of both phenomena differed among the technologies.

Inactivation of heat treated cells proceeded progressively as the treatment time increased, following a profile similar to the acquisition of DHE staining (Fig 2A). Staining with HPF occurred later in time. The last phenomenon to happen was permanent permeabilization to PI. For instance, after 2 min of exposure to 58ºC, only 20% of the cells were permeable to PI, whereas 90% of the cells were stained with DHE and also 90% were inactivated, approximately. Under these treatment conditions, 70% of the cells, approximately, presented staining with HPF. The response of *E. coli* to HHP treatments (Fig 2B) was similar, although in this case DHE staining was faster and slightly above cell inactivation, in the first min of treatment. Also in this case the percentage of cells permeabilized to PI remained always lower than the percentage of inactivated cells.

For PEF treated cells (Fig 2C) close percentages of cell inactivation, membrane permeabilization to PI and DHE staining were observed, whereas HPF staining took place in a lower percentage of cells. Finally, acid-treated cells (Fig 2D) presented a high percentage of staining with HPF and DHE, which was higher than that of inactivated cells. The percentage of permeabilization to PI remained always below 30%, even when 90% of cells appeared to be inactivated.

Thus, results obtained in this study revealed that the kinetics and order of appearance

of oxidative species and membrane permeabilization differed among the technologies. Membrane permeabilization to PI seemed to occur simultaneously to cell inactivation only in PEF treated cells, whereas in the other three technologies, a large percentage of inactivated cells maintained a non permeable membrane to PI. Thus, inactivation by heat, HHP and acidity seems to require additional cellular events other than loss of permeability to PI of the cytoplasmic membrane. Although we cannot discard that more subtle membrane alterations, that may contribute to cell inactivation, occurred in these cells before permanent permeabilization to PI takes place, it seems clear from our data that the degree of membrane alteration and its relevance in cell survival is different depending on the technology applied. The results here obtained confirm the pivotal role of the cytoplasmic membrane in cell inactivation by PEF, in comparison to other agents. It is noteworthy, according to our results, that the use of the so-called vital dyes to estimate bacterial viability requires a careful interpretation, since the response to PI of *E. coli* seems to depend on the agent applied.

On the other hand, also the order of appearance of the different ROS seemed to vary depending on the treatment applied. For heat, HHP and PEF treatments, the amount of cells stained with HPF was lower than the amount of cells stained with DHE, and only after longer treatments, able to attain more than 95% of inactivation, both staining percentages were similar. However, in the case of acid-exposed cells, the amount of HPF-stained cells was slightly superior to that of DHE-stained cells.

In order to confirm the order of appearance of these phenomena, DHE-HPF and HPF-PI double staining was carried out. Cells were stained simultaneously with the two dyes, and the amount of cells stained in red, in green, and with both colors was estimated. The results are shown in Figure 3. We would like to point out that these experiments were repeated at different treatment intensities for each agent, and the observations were similar (data not

shown). As shown in Fig 3A, when cells were exposed to heat, the appearance of HPF staining in a particular cell was always accompanied by DHE staining. On the contrary, there was a certain percentage of cells (55%) which presented DHE staining, that did not show HPF staining. On the other hand, every heat-treated cell with a PI-permeabilized membrane showed HPF staining, but a certain percentage of cells (67%) with HPF staining maintained a non permeabilized membrane. For HHP treated cells, results (Fig 3B) indicated an order of cellular events similar to that observed for heat-treated cells.

Results obtained for PEF treated cells were different (Fig 3C), since a great percentage of cells (73%) with a PI-permeabilized membrane did not show HPF staining. Finally, in acid-treated cells (Fig 3D), it is remarkable that the appearance of increased levels of DHE staining was always accompanied by HPF staining, indicating a different mechanism involved in ROS formation and/or elimination in these cells. For heat, HHP and PEF treated cells, hydroxyl radicals could be formed as a consequence of superoxide radicals presence. According to some authors, under physiological conditions, ROS production takes place in the order $O_2^{\bullet-} \rightarrow H_2O_2 \rightarrow OH^{\bullet}$ (superoxide→hydrogen peroxide→hydroxyl), due to the consecutive addition of one electron to the oxygen molecule (Imlay, 2003; Lushchak, 2011). For acid- treated cells hydroxyl radicals appeared more intensely than in cells treated by the other agents, suggesting a different origin of the oxidative radicals present.

As a whole view, and taking into account results from Fig 2 and 3, increased levels of ROS and membrane permeabilization were detected in cells treated by the four technologies, therefore the two phenomena could be involved in cell inactivation. However, the relative importance of the two cellular events on cell survival and inactivation seems different. We can hypothesize that increased ROS levels could be

the consequence of an extensively damaged cytoplasmic membrane in the case of PEF treatment, but not in the case of the other three technologies. Thus, no clear-cut relationship between these two phenomena can be established, although the occurrence of subtle membrane alterations leading to increased ROS levels, such as for instance damages in particular enzymes in the respiratory chain, cannot be ruled out. Besides, in the particular case of acid-treated cells, probably additional cellular mechanisms are taking place giving rise to an increased level of hydroxyl radicals. For instance, if particular enzymes or structures involved in iron homeostasis were more affected by acid exposure, increased intracellular Fenton reaction could rise to uncontrolled hydroxyl levels inside the cell.

Relationship between cell recovery and cellular staining

Although results obtained so far suggested that increased levels of ROS after treatments could be related to cell inactivation in some of the technologies studied, it has to be kept in mind that apparent cell inactivation may widely vary depending on the recovery conditions (Fig 1, Table 1). Figure 4 represents the percentage of inactivated cells, measured as cells unable to grow in TSAYE, in TSAYE-NaCl, and in MM-cys, for cells treated with the four technologies. Treatment conditions applied were chosen to obtain an inactivation close to 60% for the four technologies, measured in standard recovery conditions (TSAYE, aerobiosis). The percentage of cells with positive staining with DHE, HPF and PI has also been included in the graph for comparison purposes. Data in Fig 4 show that the percentage of cells with ROS (DHE staining for heat, HHP and PEF or HPF staining for acid) was coincident with loss of viability measured in TSAYE-NaCl, for the four technologies ($p>0.05$). These results were also observed for other experimental conditions (data not shown). This fact could indicate that cells with increased levels of ROS would present sublethal

injuries, able to be repaired by the cellular machinery. The relationship was lost when cells were recovered under milder environmental conditions, for instance TSAYE or, most notably, minimal medium plus anaerobic atmosphere (MM-cys). Thus, these latter conditions could help cells to effectively control ROS levels and to repair their damages. Therefore, the role of increased levels of ROS on cell inactivation and survival remains unclear, since a certain proportion of cells with increased levels of ROS were able to recover and survive under appropriate environmental conditions. Whether increased ROS levels arise from unbalances in the electron transport chain located in the membrane, as suggested by other authors (Mols and Abee, 2011), or from other cellular alterations such as the massive loss of activity of detoxifying enzymes, disassembly of intracellular iron-sulfur clusters, or loss of antioxidant intracytoplasmic molecules (for instance glutathione), is something that remains to be investigated. Also, the exact reason behind the protective effect of MM-cys-anaerobiosis is not known. As discussed above, the most plausible explanation is that it could be ascribed to the fact that it represents a lower oxidative-stress burden, as compared to other recovery conditions. In any case, these results underscore the need to optimize recovery media and conditions, in order to more accurately evaluate the number of survivors to food processes. From our results it can be concluded that complex media under aerobic recovery conditions underestimate the number of survivors to all the technologies studied.

A great percentage of heat-, HHP-, and lactic acid-treated cells was unable to grow in the presence of NaCl, while maintained impermeability to PI (Fig 4). Only in the particular case of PEF, both percentages were coincident, results that are in concordance with those previously reported (Cebrián et al., 2016). These results indicate that the nature, magnitude and relevance of membrane damage are different

depending on the technology used. We can hypothesize that heat, HHP and acid may be either affecting structures other than the membrane itself, but also involved in NaCl homeostasis, or, alternatively, may be causing subtle alterations in the cytoplasmic membrane, unable to increase permeability to PI, but still relevant to NaCl homeostasis.

3.3. Protein and DNA damage

In order to obtain a deeper insight into the mechanisms of inactivation of the four technologies, we studied the importance of two additional cellular events: protein and DNA damage. On the one hand, we checked the inactivation of two mutant strains defective either in the chaperone DnaK, involved in refolding of aberrant proteins within the cell, or in the RecA protein, responsible for the DNA-repair mechanism through homologous recombination (Doyle et al., 2015; Sharma et al., 2013). And on the other hand, we directly evaluated the degree of DNA damage, through agarose electrophoresis of a fragment amplified from DNA extracted from treated cells, respectively (Park and Imlay, 2003).

Figure 5 shows the difference in Log cycles of inactivation of the mutant strains as compared to the parental BW25113. Results showed that the *ΔDnaK* mutant strain was particularly sensitive to HHP treatments, suggesting a primary role of protein damage and recovery, mainly in HHP inactivation.

Conversely, the *ΔRecA* mutant strain was more sensitive to heat treatment and to acid treatment. In fact its sensitivity to acid treatment was especially notable. Neither of the two mutant strains showed more sensitivity than the parental to PEF treatments, suggesting that this technology would cause neither DNA nor protein damage in cells. From the results in Figure 5 it can be inferred that DNA is a primary cellular target upon acid exposure. Fig 6 shows the DNA electrophoresis of an 1860 bp fragment amplified

from DNA extracted from BW25113 cells treated by the four technologies applied at equivalent lethality. As it can be observed in the Figure, DNA from acid-exposed cells showed poor amplification, in comparison to DNA from native cells or from heat-, HHP- and PEF-treated cells. This poor amplification can be interpreted as a consequence of extensive damage, and could be possibly related to the increased presence of hydroxyl radical (Fig 2, Fig 4), which is recognized as highly toxic for the genetic material (Park and Imlay, 2003).

Conclusions

In summary, results show that increased ROS levels are found inside *E. coli* cells after all the treatments applied and their appearance is progressive along treatment time, despite the different nature of these agents. Only in the particular case of PEF-treated cells occurrence of increased ROS level was coincident with permanent permeabilization to PI, which can be considered as indicative of seriously compromised membranes. On the contrary, for the other three technologies, the presence of increased ROS levels occurred well before permeabilization to PI. In cells treated by heat, HHP and PEF, DHE staining preceded HPF staining, and only in the particular case of acid-treated cells, HPF staining preceded DHE staining, suggesting that in these cells, hydroxyl radicals were formed through a different mechanism, and possibly played a more relevant role, particularly in relation to DNA damage.

Results obtained in this investigation add new data to help to understand the mode of action of food preservation technologies on bacterial cells. This knowledge would help in the design of more effective processes.

Acknowledgements

The authors would like to thank the European Regional Development Fund, MINECO-CICYT (AGL2012-33522, AGL2015-69565-P), the Department of Innovation Research

and University of the Aragon Government and European Social Fund (FSE) for the support (predoctoral grant M. Marcén, C093/2014).

REFERENCES

Aertsen A, De Spiegeleer P, Vanoirbeek K, Lavilla M, Michiels CW. 2005. Induction of oxidative stress by high hydrostatic pressure in *Escherichia coli*. Appl. Environ. Microbiol. 71:2226-2231.

Álvarez, I, Virto R, Raso J, Condón S. 2003. Comparing predicting models for the *Escherichia coli* inactivation by pulsed electric fields. Innov. Food Sci. Emerg. Technol. 4:195-202.

Baatout S, De Boever P, Mergeay M. 2005. Temperature- induced changes in bacterial physiology as determined by flow cytometry. Ann. Microbiol. 55:73-80.

Benito A, Ventoura G, Casadei M, Robinson T, Mackey B. 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses. Appl. Environ. Microbiol. 65:1564-1569.

Cebrián G, Sagarzazu N, Pagan R, Condón S, Mañas P. 2007. Heat and pulsed electric field resistance of pigmented and non-pigmented enterotoxigenic strains of *Staphylococcus aureus* in exponential and stationary phase of growth. Int. J. Food Microbiol. 118:304-311.

Cebrián G., Sagarzazu N., Pagán R., Condón S., Mañas P. 2010. Development of stress resistance in *Staphylococcus aureus* after exposure to sublethal environmental conditions. Int. J. Food Microbiol. 140:26-33.

Cebrián G, Condón S, Mañas P. 2016. Influence of growth and treatment temperature on *Staphylococcus aureus* resistance to pulsed electric fields. Relationship with membrane fluidity. Innov. Food Sci. Emerg. Technol. 37:161-169.

De Spiegeleer P, Sermon J, Lietaert A, Aertsen A, Michiels C. 2004. Source of

tryptone in growth medium affects oxidative stress resistance in *Escherichia coli*. J. Appl. Microbiol. 97:124-133.

Doyle SM, Shastry S, Kravats AN, Shih YH, Miot M, Hoskins JR, Stan G, Wickner S. 2015. Interpay between *E. coli* DnaK, ClpB and GepE during protein disaggregation. J. Mol. Biol. 427:312-327.

Geeraerd A, Herremans C, Van Impe J. 2000. Structural model requirements to describe microbial inactivation during a mild heat treatment. Int. J. Food Microbiol. 59:185-209.

Geeraerd A, Valdramidis V, Van Impe J. 2005. GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. Int. J. Food Microbiol. 102:95-105.

Gerhardt P, Murray RGE, Wood WA, Krieg NR. 1994. Methods for General and Molecular Bacteriology, vol 1325. American Society for Microbiology, Washington, DC.

Gomes A, Fernandes E, Lima JL. 2005. Fluorescence probes used for detection of reactive oxygen species. J. Biochem. Biophys. Meth. 65:45-80.

Gusarov I, Nudler E. 2005. NO-mediated cytoprotection: instant adaptation to oxidative stress in bacteria. Proceedings of the National Academy of Science USA 102:13855-13860.

Imlay JA. 2003. Pathways of oxidative damage. Ann. Rev. Microbiol. 57:395-418.

Imlay JA. 2013. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat. Rev. Microbiol. 11:443.

Klotz B, Mañas P, Mackey BM. 2010. The relationship between membrane damage, release of protein and loss of viability in *Escherichia coli* exposed to high hydrostatic pressure. Int. J. Food Microbiol. 137:214-220.

Lado BH, Yousef AE. 2002. Alternative food-preservation technologies: efficacy and mechanisms. Microbes Infect. 4:433-440.

Lou Y, Yousef AE. 1997. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. Appl. Environ. Microbiol. 63:1252-1255.

Lushchak VI. 2011. Adaptive response to oxidative stress: bacteria, fungi, plants and animals. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153:175-190.

Mackey B. 2000. Injured bacteria, p 315-341. *In* Lund B, Baird-Parker,TC., Gould, GW. Eds. (ed), The microbiological safety and quality of food, vol 1. Springer.

Mackey BM, Mañas P. 2008. Inactivation of *Escherichia coli* by high pressure, p 53-85, High-pressure microbiology. American Society of Microbiology.

Malone AS, Chung YK, Yousef AE. 2006. Genes of *Escherichia coli* O157: H7 that are involved in high-pressure resistance. Appl. Environ. Microbiol. 72:2661-2671

Mañas P, Pagán R. 2005. Microbial inactivation by new technologies of food preservation. J. Appl. Microbiol. 98:1387- 1399.

Marcén M, Ruiz V, Serrano MJ, Condón S, Mañas P. 2017. Oxidative stress in *E. coli* cells upon exposure to heat treatments. Int. J. Food Microbiol. 241:198-205.

Miles CA. 2006. Relating cell killing to inactivation of critical components. Appl. Environ. Microbiol.72:914-917.

Mols M, Pier I, Zwietering MH, Abee T. 2009. The impact of oxygen availability on stress survival and radical formation of *Bacillus cereus*. Int. J. Food Microbiol. 135:303-311.

Mols M, Van Kranenburg R, Van Melis CC, Moezelaar R, Abee T. 2010. Analysis of acid-stressed *Bacillus cereus* reveals a major oxidative response and inactivation-associated radical formation. Environ. Microbiol. 12:873-885.

Mols M, Abee T. 2011. Primary and secondary oxidative stress in *Bacillus*. Environ. Microbiol. 13:1387-1394.

Pakhomova ON, Khorokhorina VA, Bowman AM, Rodaitė-Riševičienė R, Saulis G, Xiao S, Pakhomov AG. 2012. Oxidative effects of nanosecond pulsed electric field exposure in cells and cell-free media. Arch. Biochem. Biophys. 527:55-64.

Park S, Imlay JA. 2003. High levels of intracellular cysteine promote oxidative DNA damage by driving the fenton reaction. Bacteriol. 185:1942-1950.

Patsoukis N, Papapostolou I, Georgiou CD. 2005. Interference of non-specific peroxidases in the fluorescence detection of superoxide radical by hydroethidine oxidation: a new assay for H_2O_2. Anal. Bioanal. Chem. 381:1065-1072.

Ramos SJ., Chiquirrín M., García S., Condón S., Pérez MD. 2015. Effect of high pressure treatment on inactivation of vegetative pathogens and on denaturation of whey proteins in different media. LWT-Food Sci. Technol. 63:732-738.

Richard H, Foster JW. 2004. *Escherichia coli* glutamate-and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. J. Bacteriol. 186:6032-6041.

Saldaña G, Puértolas E, Álvarez I, Meneses N, Knorr D, Raso J. 2010. Evaluation of a static treatment chamber to investigate kinetics of microbial inactivation by pulsed electric fields at different temperatures at quasi-isothermal conditions. J. Food Eng. 100:349-356.

San Martin M, Barbosa-Cánovas G, Swanson B. 2002. Food processing by high hydrostatic pressure. Crit. Rev. Food Sci. and Nutr. 42:627-645.

Sharma V, Sakai Y, Smythe KA, Yokobayashi Y. 2013. Knockdown of *recA* gene expression by artificial small RNAs in *Escherichia coli*. Biochem. Biophys. Res. Commun. 430:256-259.

Stanier RY, Ingraham JL, Wheelis M L, Painter PR. 1992. Microbiología, 2nd ed. Reverté S.A, Barcelona.

Suh JH, Knabel SJ. 2000. Comparison of different reducing agents for enhanced detection of heat-injured *Listeria monocytogenes*. J. Food Prot. 63:1058-1063.

Van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E. 2002. Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek 82:187-216.

Wuytack EY, Phuong LDT, Aertsen A, Reyns K, Opstal I, Diels A, Michiels C. 2003. Comparison of sublethal injury induced in *Salmonella enteric* serovar Typhimurium by heat and by different nonthermal treatments. J. Food Prot. 66:31-37.

Zielonka, J., Kalyanaraman, B., 2010. Hydroethidine-and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. Free Radic. Biol. Med. 48(8), 983-1001.

Table 1. Resistance parameters (S_l, k_{max}, N_{res}, D and $2D$) obtained from the fitting of Geeraerd model, and area under the curve (AUC) corresponding to survival curves of *E. coli* treated by different agents (heat, HHP, PEF and acidity).

	Heat		
	TSAYE	**TSAYE + NaCl**	**MM-cys**
S_l	1.41 ± 0.34	0.80 ± 0.13	2.01 ± 0.14
k_{max}	3.40 ± 0.48	7.43 ± 0.79	1.84 ± 0.15
D	0.68 ± 0.09	0.31 ± 0.03	1.26 ± 0.20
$2D$	2.78 ± 0.16	1.43 ± 0.12	4.52 ± 0.10
RMSE	0.137	0.434	0.125
R^2	0.97	0.98	0.98
AUC	1.79	5.07	0.596
	HHP		
	TSAYE	**TSAYE + NaCl**	**MM-cys**
S_l	2.90 ± 0.26	0.21 ± 0.19	6.52 ± 1.88
k_{max}	0.57 ± 0.02	0.81 ± 0.03	0.17 ± 0.02
D	4.04 ± 0.15	2.84 ± 0.13	13.37 ± 1.96
$2D$	10.98 ± 0.22	5.90 ± 0.20	33.27 ± 2.04
RMSE	0.058	0.070	0.058
R^2	0.99	0.99	0.99
AUC	9.08	19.5	2.55
	PEF		
	TSAYE	**TSAYE + NaCl**	**MM-cys**
k_{max}	0.03 ± 0.01	0.05 ± 0.01	0.02 ± 0.00
N_{res}	-2.96 ± 0.29	-3.06 ± 0.12	
D	76.25 ± 2.50	49.82 ± 2.24	139.16 ± 2.37
$2D$	152.51 ± 3.31	99.64 ± 4.27	278.33 ± 4.74
RMSE	0.204	0.224	0.149
R^2	0.97	0.98	0.97
AUC	161	243	84.5
	Acidity		
	TSAYE	**TSAYE + NaCl**	**MM-cys**
S_l	65.10 ± 7.97	5.67 ± 1.51	
k_{max}	0.09 ± 0.02	0.13 ± 0.01	
D	24.32 ± 2.53	17.64 ± 0.94	
$2D$	113.75 ± 3.33	40.96 ± 2.44	
RMSE	0.086	0.037	
R^2	0.97	0.99	
AUC	68.15	307.6	

S_l (shoulder length): min; k_{max} (inactivation rate): 1/min (heat, HHP and acidity) and 1/µs (PEF); N_{res} (residual population density): (Log10 CFU/mL); D (decimal reduction time value) and $2D$ (time for inactivation of the first two Log cycles): min (heat, HHP and acidity) and µs (PEF); AUC (Area Under the Curve): min x $\log_{10}$ CFU (heat, HHP and acidity) and µs x $\log_{10}$ CFU (PEF). Goodness of fit is indicated by maximum RMSE (root mean squared error) and minimum R^2 (determination coefficient).

FIGURE CAPTIONS

Fig. 1. Survival curves of *E. coli* BW25113 obtained in TSAYE (●), TSAYE-NaCl (■) and MM-cys (▲) after treating cells by heat (58ºC) (A), HHP (300 MPa) (B), PEF (20 kV/cm) (C) and acidity (pH 3.0) (D).

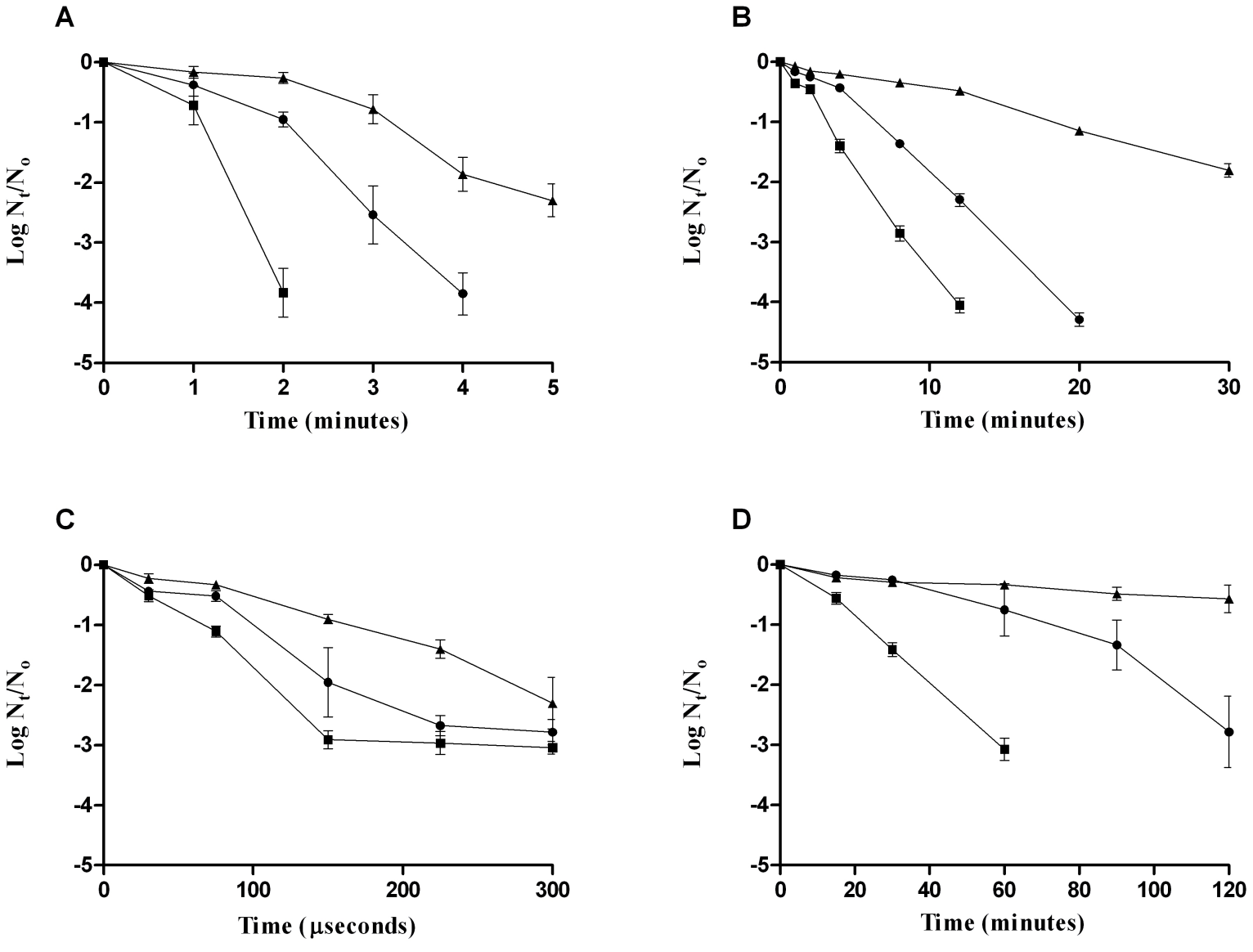
Fig. 2. Percentage of inactivated (●) and stained cells with DHE (○), HPF (Δ) and PI (□) of *E. coli* BW25113 along time for each treatment applied: heat (58ºC) (A), HHP (300 MPa) (B), PEF (20 kV/cm) (C) and acidity (pH 3.0) (D).

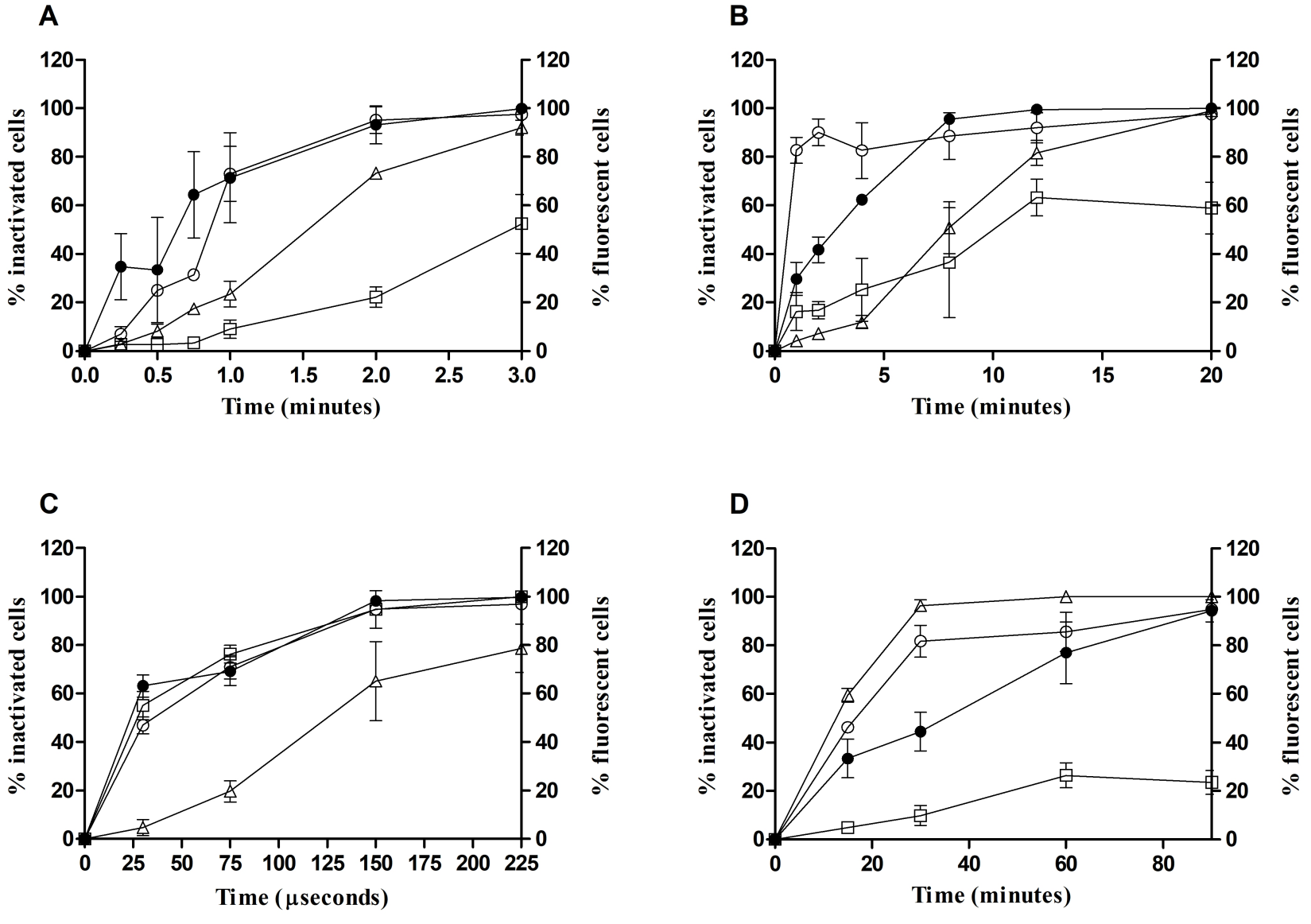
Fig. 3. Single (DHE, HPF, PI) and double staining (DHE-HPF and HPF-PI) of *E. coli* cells treated by heat (58ºC/1 min) (A), HHP (300 MPa/8 min) (B), PEF (20 kV/cm/30 μs) and acidity (pH 3.0/30 min) (D).

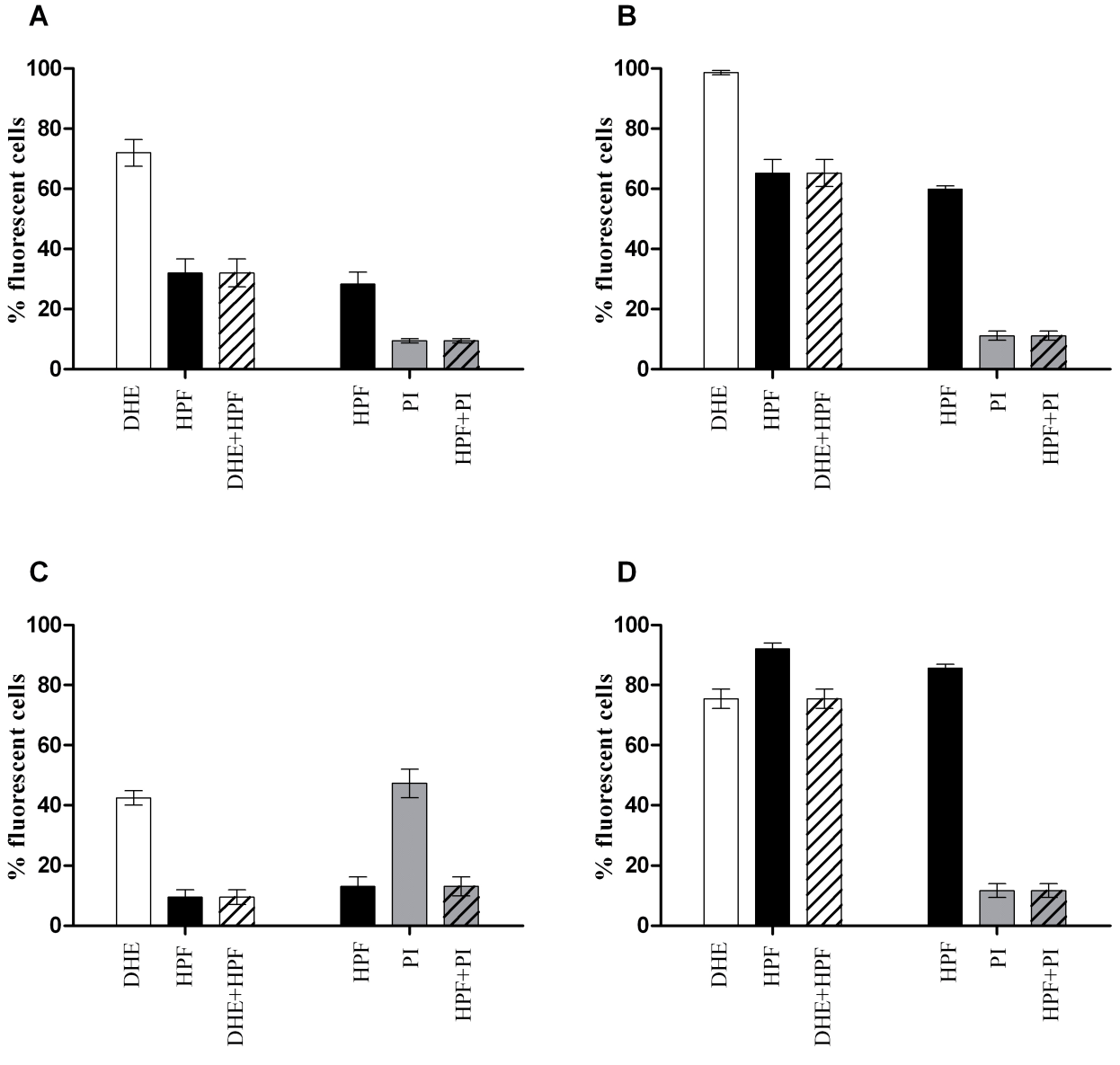
Fig. 4. Comparison between the percentage of inactivated cells recovered in TSAYE, TSAYE-NaCl and MM-cys and the percentage of cells stained with DHE, HPF and PI after treating *E. coli* BW25113 with different technologies: heat (58ºC/1 min) (white bars), HHP (300 MPa/4 min) (soft grey bars), PEF (20 kV/cm/30 μs) (dark grey bars) and acidity (pH 3.0/ 60min) (black bars).

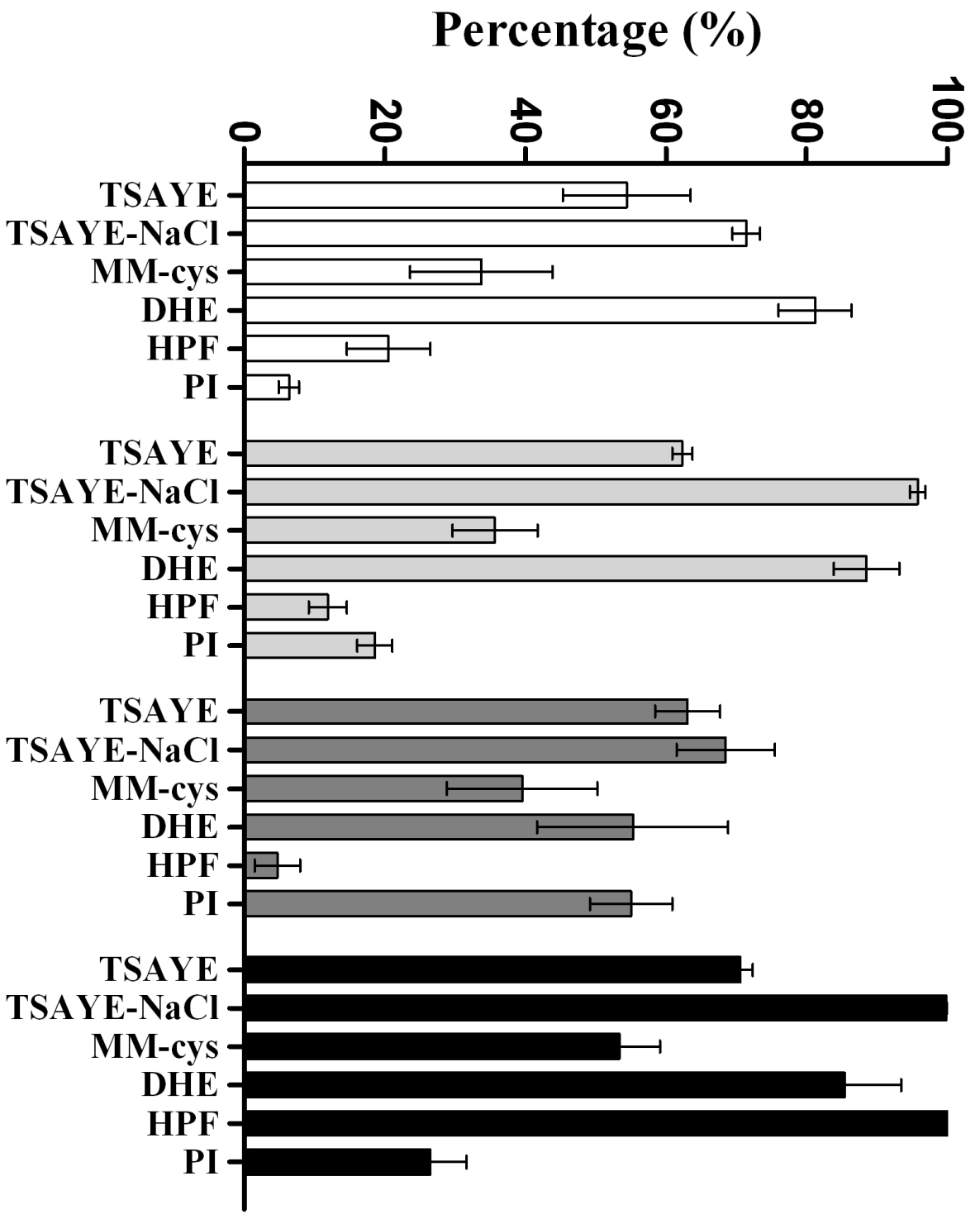
Fig. 5. Difference in Log cycles of inactivation of the mutant strains Δ*DnaK* (grey bars) and Δ*RecA* (black bars) in comparison to the parental strain for the treatments with each technology: heat (58ºC/3 min), HHP (300 MPa/8 min), PEF (20 kV/cm/225 μs) and acidity (pH 3.0/60 min). Cells were recovered in MM-cys.

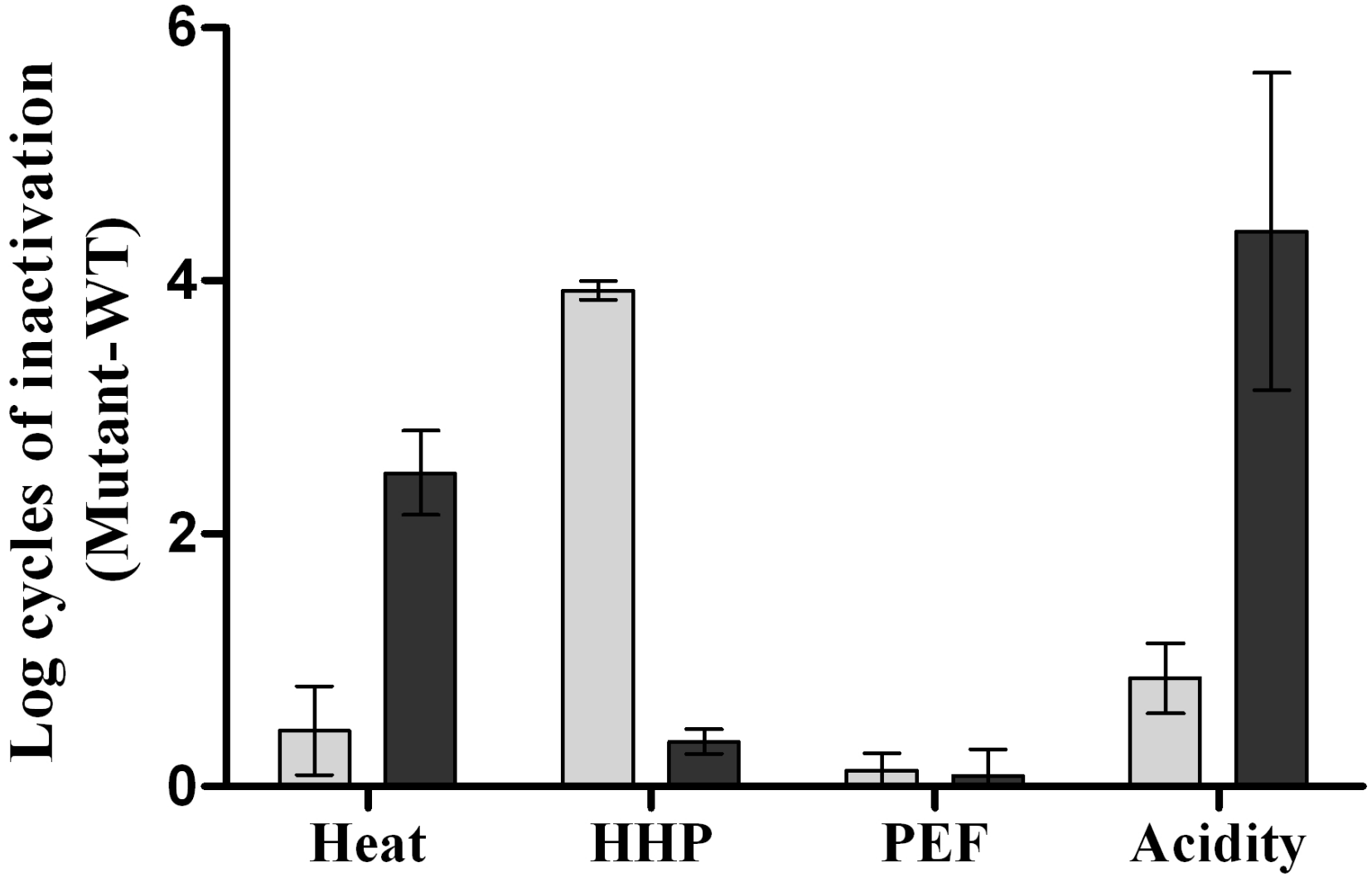
Fig. 6. DNA electrophoresis of an 1860pb fragment amplified from DNA extracted from *E. coli* BW25113 native cells and cells treated by heat (58ºC/3 min), HHP (300 MPa/8 min), PEF (20 kV/cm/225 μs) and acidity (pH 3.0/60 min) at equivalent lethality (1.7±0.3 log cycles). Samples appear in the following order from left to right: DNA ladder, native cells, heat, HHP, PEF and acidity.

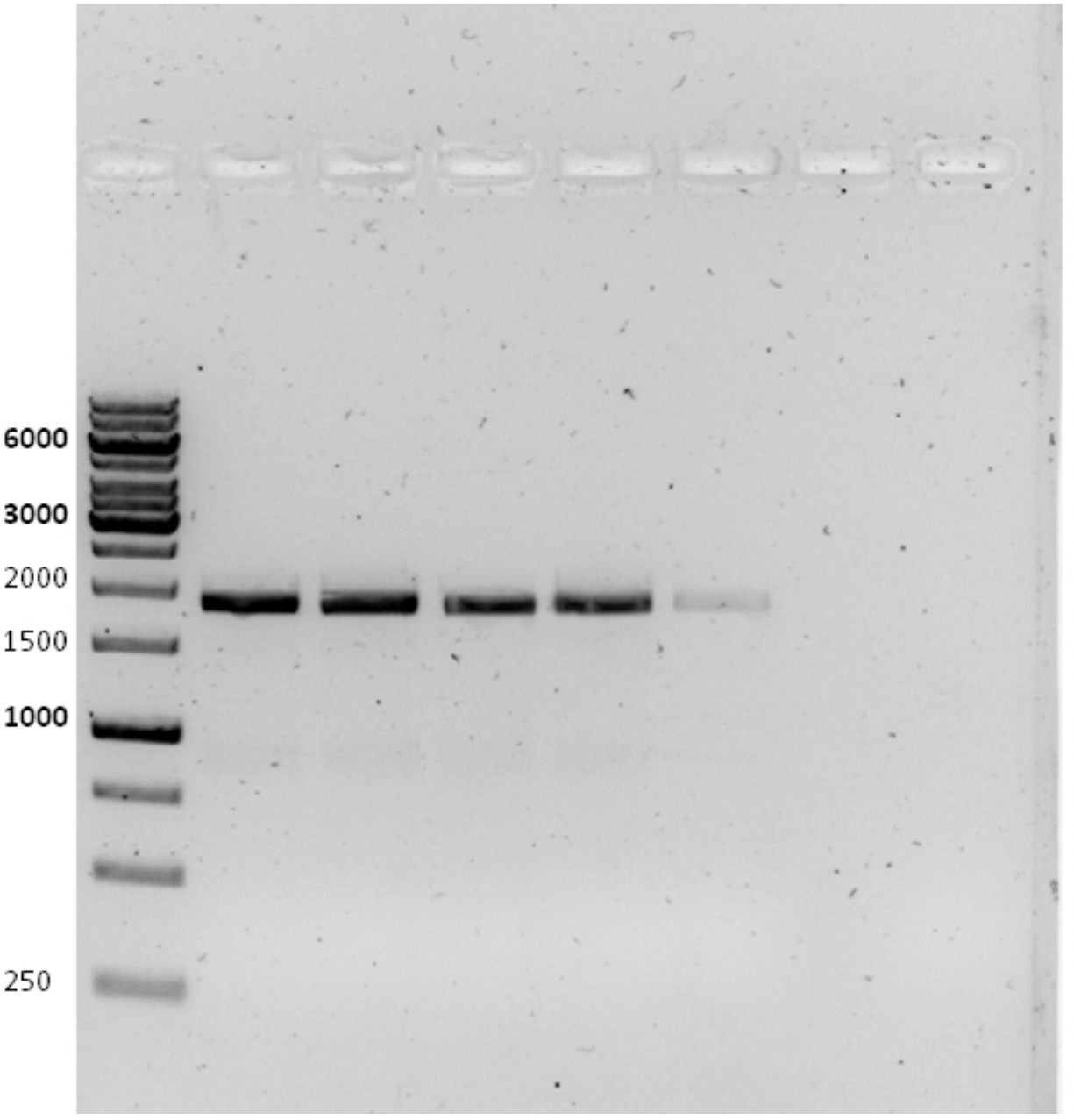

A
B
C
D
Log N_t/N_o
Time (minutes)
Time (μseconds)
0
-1
-2
-3
-4
-5
0
1
2
3
4
5
10
20
30
100
200
300
40
60
80
120


A
% inactivated cells
% fluorescent cells
Time (minutes)
B
% inactivated cells
% fluorescent cells
Time (minutes)
C
% inactivated cells
% fluorescent cells
Time (μseconds)
D
% inactivated cells
% fluorescent cells
Time (minutes)


A
B
C
D
% fluorescent cells
100
80
60
40
20
0
DHE
HPF
DHE+HPF
HPF
PI
HPF+PI


Percentage (%)
0
20
40
60
80
100
TSAYE
TSAYE-NaCl
MM-cys
DHE
HPF
PI
TSAYE
TSAYE-NaCl
MM-cys
DHE
HPF
PI
TSAYE
TSAYE-NaCl
MM-cys
DHE
HPF
PI
TSAYE
TSAYE-NaCl
MM-cys
DHE
HPF
PI


Log cycles of inactivation
(Mutant-WT)
6
4
2
0
Heat
HHP
PEF
Acidity


6000
3000
2000
1500
1000
250