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Exploring the mechanisms of *Staphylococcus aureus* pulsed electric fields resistance acquisition after exposure to heat and alkaline shocks

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ABSTRACT

This study aimed to acquire a deeper knowledge of the mechanisms of PEF resistance development after the exposure of *Staphylococcus aureus* to sublethal alkaline and heat shocks, with a particular focus on the modifications of cell envelope properties and their impact on electroporation and its reversion. Both shocks significantly ($p < 0.05$) increased the surface negative charge but they barely affected surface hydrophobicity or membrane fluidity. This resulted in an increased electroporation threshold (≈ 2 kV/cm) for alkaline-shocked but not for heat-shocked cells. Heat and alkaline shock-dependent development of PEF resistance did not require *de novo* RNA, protein, or lipid synthesis. Addition of nisin (100 UI/mL) to the treatment medium not only counteracted the protective effect of sublethal shocks against PEF, but even increased the lethality of PEF treatments (up to 8.9-fold increase in Log cycles of inactivation) against heat-shocked and alkaline-shocked cells.

Industrial relevance: This work contributed to a deeper understanding of the mechanisms leading to the development of PEF resistance, which is essential for PEF process optimization and for the design of PEF-based combined processes for food decontamination or pasteurization.

1. Introduction

Thermal treatment is the most widely used procedure for microbial inactivation in foods. Nevertheless, it is known that it causes some undesirable effects on foods, leading to quality losses. Therefore, the food industry is interested in exploring different alternatives to heat for microbial inactivation (Mañas & Pagán, 2005). Among these alternatives, the use of Pulsed Electric Fields (PEF), is remarkable, since is capable of causing bacterial inactivation while preserving the sensory and nutritional quality of foodstuffs (Raso & Barbosa-Cánovas, 2003). This technology consists of the application of short duration (1–100 μs) high electric field pulses (10–50 kV/cm) to a sample placed between two electrodes (Heinz, Álvarez, Angersbach, & Knorr, 2001). Several studies have been conducted to determine the resistance to PEF of different foodborne pathogens, for instance, *Listeria monocytogenes*, various serotypes of *Salmonella*, *Escherichia coli*, *Cronobacter sakazakii* and *Staphylococcus aureus*, among others (Álvarez, Mañas, Condón, & Raso, 2003; Arroyo, Cebrián, Pagán, & Condón, 2010; Liu, Zeng, Ngadi, & Han, 2017; Rodríguez-Calleja, Cebrián, Condón, & Mañas, 2006; Saldaña et al., 2009; L-H. Wang, Wang, Zeng, & Liu, 2016; R. Wang, Ou, Zeng, & Guo, 2019; Yun, Zeng, Brennan, & Zhi-wei, 2017), varying the treatment parameters and environmental conditions, with the aim of optimizing the protocol established for this technology to be effective against each microorganism.

The cellular envelopes are considered to be the main target structure of PEF (Mañas & Pagán, 2005), as it is believed that the electroporation generated in the cytoplasmic membrane is the principal cause leading to the different phenomena produced in the bacterial cell (Pagán & Mañas, 2006). Several theories have been proposed to explain the mechanism of electroporation and one of the most accepted ones is the electromechanical instability theory (Barbosa-Cánovas, Pothakamury, Góngora-Nieto, & Swanson, 1999; Ho & Mittal, 1996; Toepfl, Siemer, Saldaña-Navarro, & Heinz, 2014; Weaver & Chizmadzhev, 1996; Zimmermann, 1986; Zimmermann, Pilwat, & Riemann, 1974). This theory considers that the application of an external electrical field increases the accumulation of free charges at both membrane surfaces, and thus, leads to an increase in the transmembrane potential. This rise of opposite charges generates electrostatic attraction forces, resulting in membrane compression, reduction of membrane thickness and pore formation. The number and size of pores largely depend on the electric field strength and the treatment time. At this point, it is important to highlight that it is necessary to reach a certain threshold of transmembrane potential,

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which is called critical electric field, to produce a compression of the membrane strong enough to generate the pores. Hence, if the electric field applied is close to the critical electric field or the treatment time is short, the number and size of the generated pores will be low, and they will reseal once the PEF treatment had finished. Nevertheless, if more intense PEF treatments are applied, the number and size of the pores will increase and, in this case, permeabilization will be irreversible, even leading to the mechanical disruption of the cell (Zimmermann, 1986). Although several theories have been proposed to explain the electroporation phenomenon, until the moment there is no clear evidence on the underlying mechanism of membrane permeabilization at the molecular level.

In this context, results obtained in a study conducted in our laboratory (Cebrián, Raso, Condón, & Mañas, 2012) demonstrated that a previous exposure of *S. aureus* cells to heat and alkali leads to an increase in their resistance to PEF. The protective effect generated after these shocks was proven at different electric field strengths, for cells in different physiological states, for different strains and for different treatment conditions. By contrast, exposure to other stresses such as acid, hydrogen peroxide, hyperosmotic medium and cold temperatures did not result in an increase in PEF resistance in *S. aureus* cells. It was also reported that the higher PEF resistance after the heat and alkaline shocks was correlated with an increased ability to repair or withstand sublethal injuries, and it was suggested that this increase in resistance could be due to a higher concentration or activity of Heat Shock Proteins (HSPs). Be that as it may, further work is required to verify that hypothesis and to elucidate the mechanisms leading to the development of PEF resistance in *S. aureus* after heat and alkaline shocks. Hence, the aim of this investigation was to deepen on the mechanisms of PEF resistance acquisition after the exposure of *S. aureus* cells to sublethal alkaline and heat shocks, with a particular focus on the modifications of certain *S. aureus* envelope properties, such as fluidity, surface charge and surface hydrophobicity, induced by those shocks. In addition, the impact of these sublethal shocks on the electroporation process and its reversion was assessed.

2. Materials and methods

2.1. Bacterial cultures and media

The strain of *S. aureus* CECT 4459 was provided by the Spanish Type Culture Collection. Bacterial suspensions were obtained as indicated in Cebrián et al. (2012). Hence, the bacterial culture was maintained frozen at −80 °C in cryovials. Stationary-phase cultures were prepared by inoculating 10 mL of tryptone soya broth (Biolife, Milan, Italy) supplemented with 0.6% (*w/v*) yeast extract (Biolife; TSB-YE) with a loopful of growth from tryptone soy agar supplemented with 0.6% (w/v) yeast extract (Biolife; TSA-YE). The resulting culture was incubated at 37 °C for 6 h, in a shaking incubator. 50 μL of this culture was inoculated into 50 mL of fresh TSB-YE and incubated for 24 h under the same conditions, which resulted in a stationary-phase culture containing approximately 7×10^8 cells/mL. Exponential-phase cells were prepared by inoculating 50 μL of the stationary-phase culture into 50 mL of fresh TSB-YE and incubating for 3.0–3.5 h, until the optical density at 600 nm reached 0.8, which corresponded to approximately 8×10^7 cells/mL.

2.2. Adaptation to sublethal stresses

Exponential and stationary growth phase cells were exposed to the following sublethal stresses (shocks), that were previously chosen as conditions that caused the highest increase in homologous resistance (Cebrián, Sagarzazu, Pagán, Condón, & Mañas, 2010): acid shock at pH 4.5 (adjusted with hydrochloric acid (HCl), Panreac S. A., Barcelona, Spain); alkaline shock at pH 9.5 (adjusted with sodium hydroxide, Panreac); oxidative shock with 50 μM hydrogen peroxide (Sigma-Aldrich, S. Louis, U.S.A.) and heat shock at 45 °C. They were also exposed to an osmotic shock with 10% (*w/v*) sodium chloride (NaCl; Panreac) ($a_w = 0.94$) and to a cold shock at 4 °C. During acid, alkaline, osmotic and hydrogen peroxide shocks, the temperature of the cell suspension was kept at 25 °C. It was checked that increasing this temperature up to 37 °C did not influence the results obtained (data not shown). *S. aureus* cultures were centrifuged (6000 *g*; 5 min), resuspended in the same volume of TSB-YE in the presence of the stress factor and then incubated for 2 h. Colony Forming Units (CFU) in TSA-YE were determined before and after exposure to the sublethal stresses and it was checked that no loss of viability or growth had occurred. Before PEF treatments, cells from both the adapted and non-adapted cultures were centrifuged (6000 *g*; 5 min) and resuspended in McIlvaine citrate phosphate buffer (pH 7.0), whose conductivity was adjusted with distilled water to 2 mS/cm.

In certain experiments, sublethal alkaline and heat shocks were applied in the presence of certain inhibitors, in order to study the biosynthetic requirements for the development of PEF resistance. Hence, these shocks were applied in the presence of chloramphenicol (Sigma-Aldrich; 500 μg/mL), rifampicin (Sigma-Aldrich; 0.001 μg/mL) or cerulenin (Sigma-Aldrich; 100 μg/mL). Previous experiments showed that those were the minimum growth inhibitory concentrations for native cells (data not shown).

2.3. PEF treatments

PEF treatments were carried out in an exponential waveform pulse equipment (Cebrián, Mañas, & Condón, 2015). High electric field pulses were produced by discharging a set of 10 capacitors *via* a thyristor switch (Behlke, Kronberg, Germany) in a batch treatment chamber. The capacitors were charged using a high voltage DC power supply (FUG, Rosenhein, Germany), and a function generator (Tektronix, Wilsonville, Oregon, U.S.A.) delivered the on-time signal to the switch. The treatment chamber was made of a cylindrical plastic tube closed with two polished stainless-steel electrodes. The gap between electrodes was 0.25 cm and the electrode area was 2.01 cm^2. The actual electric field strength and electrical intensity applied were measured in the treatment chamber with a high voltage probe and a current probe, respectively connected to an oscilloscope (Tektronix). The energy associated to pulses at electric field strengths of 10, 12, 14, 16, 18, 20, 22, 24, 26 28 and 30 kV/cm was 0.13, 0.74, 1.35, 1.86, 2.33, 2.96, 3.49, 4.11 4.73, 5.30 and 5.96 kJ/kg respectively. Treatments of up to 100 pulses (exponential decay; pulse width ≈ 3 μs) were applied. The equipment includes provisions for measuring sample temperature (Raso, Álvarez, Condón, & Sala, 2000). In all the experiments the temperature of the samples never exceeded 35 °C during the treatment. In order to perform the PEF treatments, microbial suspensions at a concentration of approximately 10^8 CFU/mL, were placed into the treatment chamber with a sterile syringe. After each treatment, the appropriate serial dilutions were prepared in sterile TSB-YE and pour plated. In certain experiments, lysozyme (Sigma Aldrich; 50 μg/mL) or Nisin (Danisco; 100 UI/mL) were added to the treatment medium.

2.4. Incubation of treated samples and survival counting

The recovery medium was TSA-YE and, when required, NaCl was added (TSA-YE-NaCl) to estimate the percentage of sublethally injured cells (Mackey, 2000). The lack of tolerance to the presence of NaCl is attributed to a loss in the functionality and/or integrity of the cytoplasmic membrane (Mackey, 2000). The NaCl concentration employed was established in previous experiments as the Maximum Non-Inhibitory Concentration (MNIC) for untreated cells, and corresponded to 2.39 M (Cebrián, Arroyo, Mañas, & Condón, 2014). It was verified that heat and alkaline shocks did not change the MNIC of *S. aureus* cells (data not shown). Plates were incubated for 24 h at 37 °C unless NaCl was added to the agar; in such case incubation times of 48 h were needed. After incubation, CFU were counted.

2.5. Recovery of PEF-treated cells in liquid media (TSB-YE and TSB-YE-NaCl)

After PEF treatments, cells were diluted (1/10) in TSB-YE or TSB-YE + 2.39 M NaCl (TSB-YE-NaCl) and incubated at room temperature. Samples were collected at preset times and plated onto TSA-YE and TSA-YE-NaCl, in order to determine the rate of recovery of tolerance to NaCl. It should be noted that the PEF treatments applied in these experiments were those required to permeabilise 90% of the cell population. Hence, 5 pulses (26 kV/cm) were applied to non-adapted and heat-shocked exponential growth phase cells; 10 pulses were applied to non-adapted and heat-shocked stationary growth phase cells, and to alkaline-shocked exponential growth phase cells; and 20 pulses were applied to alkaline-shocked stationary growth phase cells.

2.6. Assessment of the percentage of cells with permeabilized membranes by PI staining

The fluorescent dye propidium iodide (PI; Sigma-Aldrich) was used to evaluate cell membrane permeabilization by PEF treatments, following the protocol described in Cebrián et al. (2015). PI is commonly used as a marker for membrane permeabilization, since membranes of healthy cells prevent its entry inside the cell, where it bounds to nucleic acids rendering a strong red fluorescence. A stock solution of PI in distilled water (1 mg/mL) was prepared. Samples of cell suspensions were centrifuged (6000 *g*; 5 min), resuspended in pH 7.0 McIlvaine citrate-phosphate buffer at a concentration of approximately 10^8 CFU/mL, and mixed with the PI solution (PI final concentration of 1.5 μM before PEF treatments). After the treatments, cells were incubated for 10 min at room temperature. Previous experiments showed that the presence of PI in the treatment medium did not modify the treatment conditions or microbial PEF resistance (data not shown). The percentage of permeabilized cells was determined by microscopic examination using a Nikon Eclipse E400 microscope (Nippon Kogaku KK, Japan) equipped with phase-contrast optics and an epifluorescence unit. In all cases, a × 100 objective was used with immersion oil, giving a total magnification of ×1000. Cell counts were performed on at least five microscopic fields with high cellular concentration (>50 cells per field). The percentage of permeabilized cells was calculated by comparing the total number of cells, determined by using phase-contrast optics, with the number of cells showing fluorescence. Data were normalized by subtracting the percentage of untreated cells showing fluorescence, which was always lower than 2%. The normalized data were plotted as percentages of PI-stained cells after the different PEF treatments. It should be noted that in this investigation, PI was added to the bacterial suspension before the PEF treatment, and thus, the percentage of fluorescent cells corresponds to the percentage of cells permeabilized during the treatment.

2.7. Measurement of fluorescence anisotropy

Fluorescence anisotropy of the probe DPH (1, 6-diphenyl 1,3,5-hexatriene; Sigma-Aldrich) was used to monitor changes in membrane fluidity (Aricha et al., 2004). Anisotropy values (*r*) are inversely related to membrane fluidity (Shinitzky, 1984). Briefly, samples of bacterial cultures were washed twice with PBS (Sigma-Aldrich) containing 0.25% formaldehyde (pH 7.4) for fixation, and then incubated for 45 min at 37 °C with 5×10^{-7} M DPH (added as a 10^{-4} M solution in tetrahydrofuran) for probe insertion in the membrane. Steady-state fluorescence anisotropy was measured at 30 °C with a Cary-Eclipse spectrofluorometer provided with a manual polarizer accessory (Varian Inc., Mulgrave, Victoria, Australia), with excitation at 355 nm and emission at 425 nm, 5- and 5-nm slits, respectively, and a 3-s integration time.

Anisotropy values (*r*) were calculated according to Shinitzky (1984), as follows:

$$r = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + 2 \times G \times I_{VH}} \text{ with } G = \frac{I_{HV}}{I_{HH}}$$

$_V$ and $_H$ stand for polarisation direction (vertical and horizontal directions); *I* is the corrected fluorescence intensity obtained by:

$$I_{HH} = I_{(L)HH} - I_{(BUFFER+CELL)HH} - I_{(BUFFER+PROBE)HH} + I_{(BUFFER)HH}$$

$I_{(L)}$ is the fluorescence intensity of probe-labelled cell suspension; $I_{(BUFFER+CELL)}$ is the fluorescence intensity of non-labelled cell suspension; $I_{(BUFFER+PROBE)}$ is the fluorescence intensity of the buffer incubated with the DPH probe; $I_{(BUFFER)}$ is the fluorescence intensity of the sole buffer. This calculation was repeated for the other three signals (I_{HV}, I_{VV}, I_{VH}).

2.8. Cytochrome c binding assay

The cytochrome *c* binding assay was performed according to the protocol previously described by Bayer et al. (2006) to determine cell surface charge. Hence, cells were centrifuged (6700 *g*; 5 min) and washed twice with 20 mM morpholinepropanesulfonic acid (MOPS) buffer pH 7.0. Cells were suspended in the same buffer and adjusted to an optical density of 7.0, measured at 578 nm ($OD_{578} = 7.0$). Afterwards, cytochrome *c*, which is positively charged at pH 7.0, (Sigma-Aldrich) was added (0.5 mg/mL) to the bacterial suspension and the mixture was incubated at 25 °C for 10 min. Once incubation had finished, cells were centrifuged (6700 *g*; 5 min), the pellet was discarded, and the amount of cytochrome *c* present in the supernatant was determined spectrophotometrically, measuring the OD_{530} (Bayer et al., 2006). The higher the OD_{530} obtained, the lower the interaction of cytochrome *c* with cell surfaces, and therefore, the more positively charged the *S. aureus* cell envelope.

2.9. Microbial adhesion to hydrocarbons (MATH) test

Cell surface hydrophobicity was evaluated by means of the microbial adhesion to hydrocarbons (MATH) test, which was performed according to the protocol followed by Nachtigall, Weber, Rothenburger, Jaros, and Rohm (2019), with some modifications. Thus, cells were centrifuged (6700 *g*; 5 min; 4 °C), washed twice with 9 g/L NaCl and resuspended in PBS (pH 7.4). 3 mL of the bacterial suspension was then adjusted to an OD of 0.4, measured at 577 nm ($OD_{577} = 0.4$; A_0), and subsequently mixed (vortex, 60 s) with 0.3 mL of n-hexadecane (Sigma-Aldrich) in glass test tubes (previously washed with 37% HCl for 10 min in an ultrasonic bath, as proposed by Rosenberg (1984)). The mixture rested in the glass tubes for 30 min at room temperature, to allow phase separation. Afterwards, the two-phase system was transferred into polystyrene cuvettes and the OD_{577} was measured (A_1). To exclude effects from remaining hydrocarbon droplets in the aqueous phase, 3.0 mL of PBS buffer was used instead of cell suspension as blank (A_{blank}) in the spectrophotometer. Cell surface hydrophobicity [%] was then calculated as follows:

$$CSH = \frac{A_0 - (A_1 - A_{blank})}{A_0} \text{ X } 100$$

2.10. Statistical analysis

Results obtained after conducting the experiments were statistically analysed (Student *t*-tests, Analysis of Variance (ANOVA)) using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, California, U.S.A.). All the experiments included in this document were carried out at least by triplicate -unless specifically stated- and the error bars indicate the standard deviation of the means.

3. Results

3.1. Influence of the exposure to sublethal alkaline and heat shocks on the properties of the cellular envelopes of S. aureus cells

As mentioned above, in the study conducted by Cebrián et al. (2012), it was demonstrated that the exposure to an alkaline and heat shock caused an increase in the PEF resistance of *S. aureus* cells. Data on the effect of these shocks on a given PEF treatment (50 pulses, 26 kV/cm, pH 7.0) in *S. aureus* stationary and exponential phase cells is included in Supplementary Table 1. As an example, heat shock decreased the inactivation level of stationary phase cells by a factor of 1.5, whereas for alkaline shock a 2.5-fold increase in survival was observed. Ample information about these and other sublethal shocks in *S. aureus* PEF survival is included in the study of Cebrián et al. (2012), although the mechanisms that led to this increase in resistance to PEF were not completely elucidated.

On the other hand, it is widely known that cell envelopes are the main target in bacterial inactivation by PEF treatments. Considering all these aspects, the aim of this investigation was to acquire a deeper understanding of the mechanisms that lead to an increase in PEF resistance after the exposure to an alkaline and heat shock, and the first step was to assess the influence of these sublethal shocks on different properties of the cell envelopes (membrane fluidity, surface hydrophobicity and surface charge) in *S. aureus* cells.

In this context, Fig. 1A depicts the anisotropy values (r) obtained after the exposure of *S. aureus* CECT 4459 cells in stationary and exponential growth phase to an alkaline and a heat shock. Anisotropy values of control cells (cells without previous exposure to sublethal stress) were determined as well. As can be observed in this figure, results obtained indicate that only heat-shocked exponential growth phase cells displayed a significant change ($p < 0.05$) in membrane fluidity as compared to control (non-shocked) cells. Thus, the r value of these cells, which is inversely correlated to membrane fluidity, increased by approx. 0.05 units. It should also be noted that the application of none of the other stresses included in this study (acid, oxidative, cold, and osmotic) resulted in a change in membrane fluidity ($p > 0.05$), regardless of the growth phase (Supplementary Table 1).

As far as cell surface hydrophobicity (CSH) is concerned (Fig. 1B), neither the alkaline nor the heat shock provoked a significant change in this membrane property ($p > 0.05$). By contrast, the exposure to acid and oxidative shocks resulted in an increase in CSH of *S. aureus* cells, although this increase was only significant ($p < 0.05$) for the exponential growth phase cells (Supplementary Table 1).

As can be observed in Fig. 1C, heat and alkaline shocks resulted in a significant increase ($p < 0.05$) in the surface negative charge of *S. aureus* cells, both in stationary and exponential growth phase, which was evidenced by a remarkable decrease in the supernatant absorbance after the incubation with cytochrome C (up to 23%). In this case, the application of none of the other stresses included in this study resulted in a change in cell surface charge ($p > 0.05$), regardless of the growth phase (Supplementary Table 1).

3.2. Influence of the exposure to sublethal alkaline and heat shocks on the electroporation process of S. aureus cells and its possible reversion

As pointed out for other stress resistance responses (Cebrián, Condón, & Mañas, 2009), the observed increase in PEF resistance after exposure to heat and alkaline shocks could be due either to a stabilization of cellular structures or to the development of a higher ability to repair or withstand the sublethal damage caused by PEF. Thus, the possible changes in the electroporation process of *S. aureus* cells and its reversion, due to the exposure to sublethal alkaline and heat shocks, were evaluated. For this purpose, the influence of the application of these shocks on the permeabilization and cellular recovery capacity of *S. aureus* cells was studied in depth.

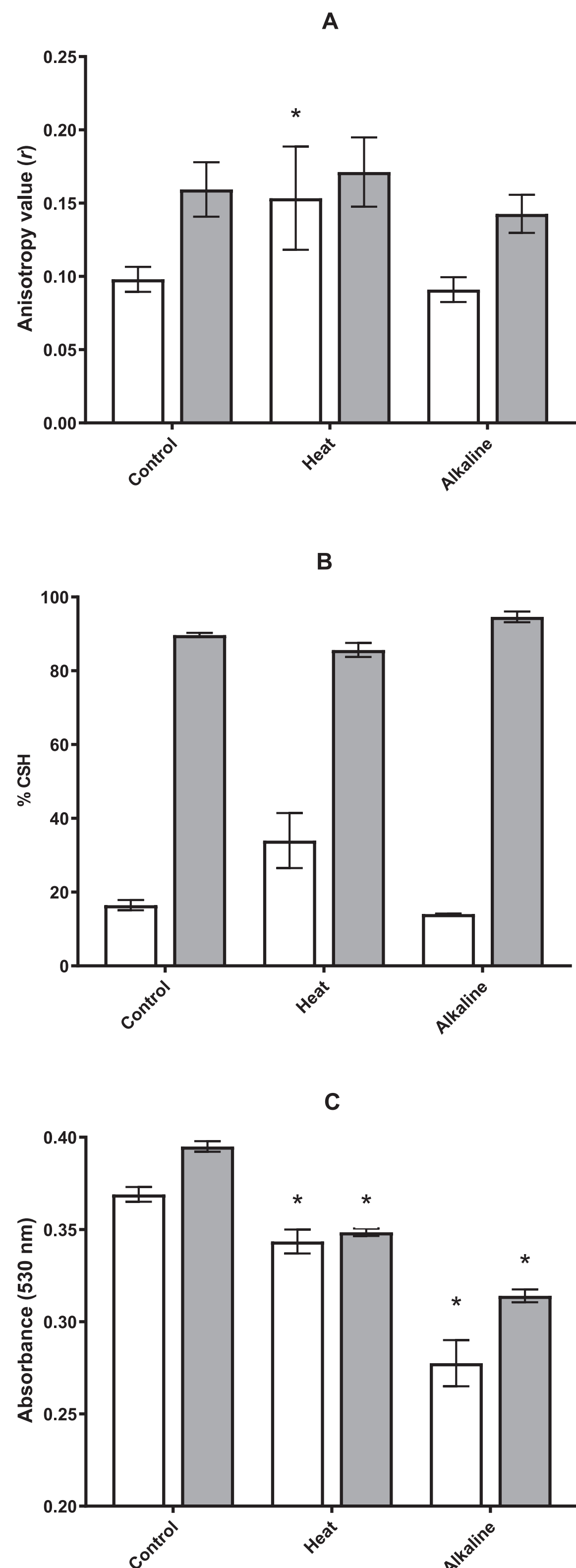

Fig. 1. Anisotropy (r) values (A), Percentage of cell surface hydrophobicity (B) and Absorbance (530 nm) of the supernatant after incubation with cytochrome C (C) of non-adapted (Control), alkaline- (pH 9.5; 2 h) shocked, and heat- (45 °C; 2 h) shocked exponential (white bars) and stationary (grey bars) growth phase *S. aureus* CECT 4459 cells. Asterisks (*) indicate values statistically different from control values ($p < 0.05$).

Fig. 2 displays the percentage of *S. aureus* stationary and exponential growth phase cells permeabilized to PI after treatments of different number of pulses at 18 kV/cm (2 A and C) and after treatments of 25 pulses at different electric field strengths (2B and D). As can be deduced from this figure, heat shock exposure barely modified the permeabilization of the cells to PI in comparison to the control cells. This effect can be observed in all the scenarios tested; hence, results were similar for the stationary and exponential growth phase cells, and both, in the treatments of different number of pulses and at the different electric field strengths applied. By contrast, exposure of cells to an alkaline shock caused a remarkable decrease in the percentage of cells permeabilized to PI during the PEF treatment. On the one hand, in the treatments consisting of the application of different number of pulses at 18 kV/cm (2 A and C), this percentage was always lower for the alkaline-shocked cells, up to 100 pulses, which was the maximum number of pulses applied. On the other hand, in the case of treatments that varied the electric field strength (2B and D), the percentage of permeabilization was also lower in the case of the alkaline-shocked cells, although until a certain extent. Hence, when the electric field strength applied reached 22 kV/cm, no significant differences ($p > 0.05$) were found between any of the conditions assessed. However, it should be noted that the percentage of PI-permeabilized cells was close to 100% under these experimental conditions for all the cells studied.

Afterwards, the cellular recovery capacity in two different liquid media (TSB-YE and TSB-YE-NaCl) of *S. aureus* CECT 4459 control, heat-shocked and alkaline-shocked cells after PEF treatments was evaluated. As indicated in the methodology section, the PEF treatment conditions established for these experiments were those required to permeabilise 90% of the cell population. Samples from the bacterial cultures incubated in both media were collected at preset times and plated onto TSA-YE and also onto TSA-YE-NaCl in the case of incubation in TSB-YE, to determine the rate of recovery of tolerance to NaCl and therefore, quantify the level of damage in the cytoplasmic membrane. Fig. 3A and B include data obtained for cells incubated in TSB-YE, considered as an adequate medium for cell recovery. In these graphs, it can be observed that the difference in plate counts between TSA-YE and TSA-YE-NaCl, which indicates the percentage of cells unable to tolerate NaCl, was progressively reduced along incubation time, up to 60–120 min. Moreover, as can be observed in these figures, alkaline-shocked cells displayed a slightly increased ability to repair their injuries, in comparison to control and to heat-shocked cells. This effect was observed for stationary (3A) and for exponential (3B) cells.

On the other hand, when PEF-treated cells were incubated in TSB-YE-NaCl (Fig. 4A and B), the presence of this latter compound prevented damage recovery, and led to a progressive further loss of cellular viability instead. In this case, results obtained indicate that the loss of viability concerning heat- and, particularly, alkaline-shocked cells, was slower than that of non-adapted cells (Fig. 4A and B). As can be deduced from the comparison of both figures, the magnitude of this effect was greater for stationary growth phase cells (Fig. 4A), than for exponential

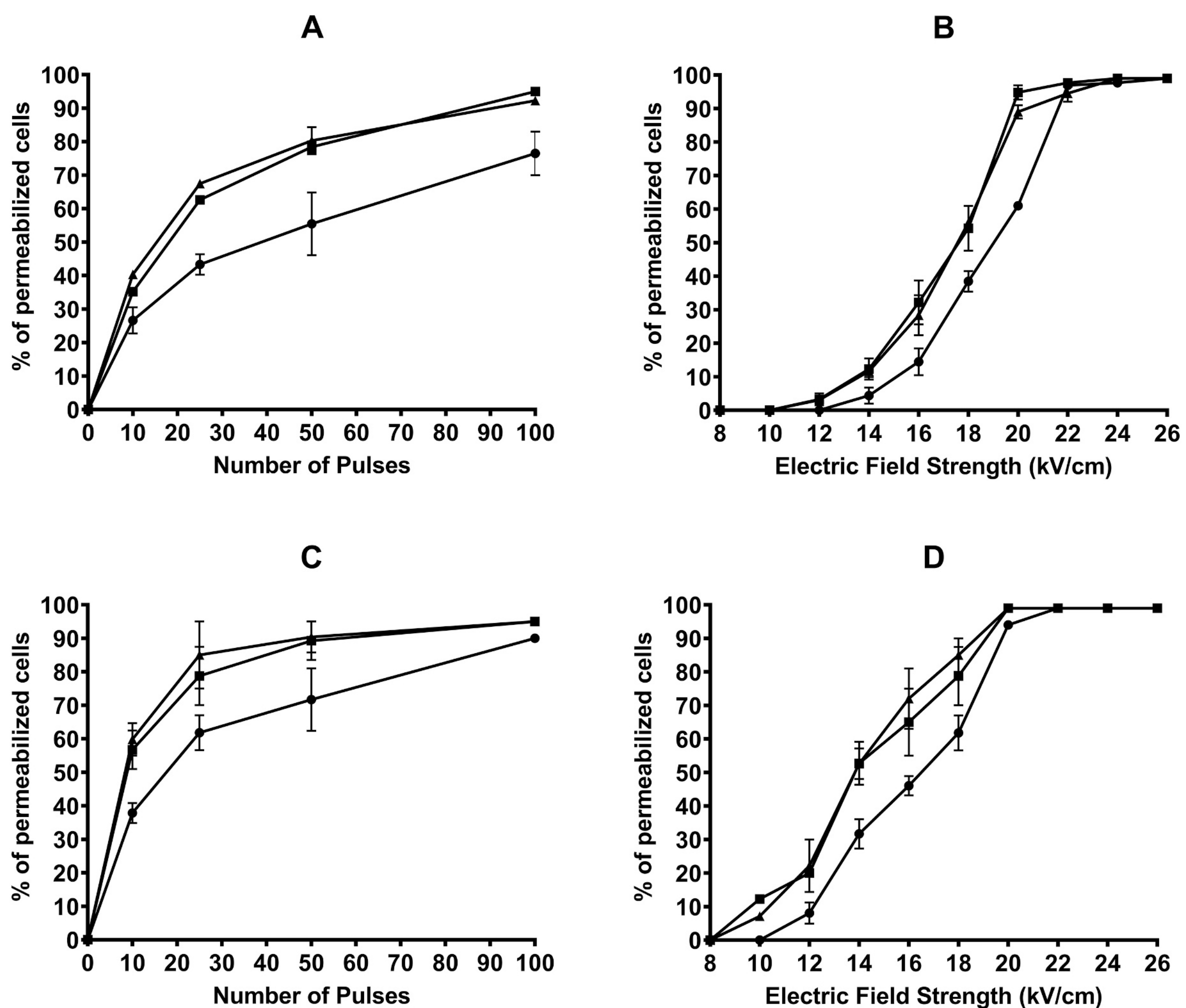

Fig. 2. Percentage of *S. aureus* CECT 4459 non-adapted (square), heat-shocked (triangle) and alkaline-shocked (circle) cells, in stationary (A, B) and exponential (C, D) phase of growth, permeabilized to PI during treatments of different number of pulses at 18 kV/cm (A and C) and treatments of 25 pulses (exponential decay; pulse width ≈3 μs) at different electric field strengths (B and D).

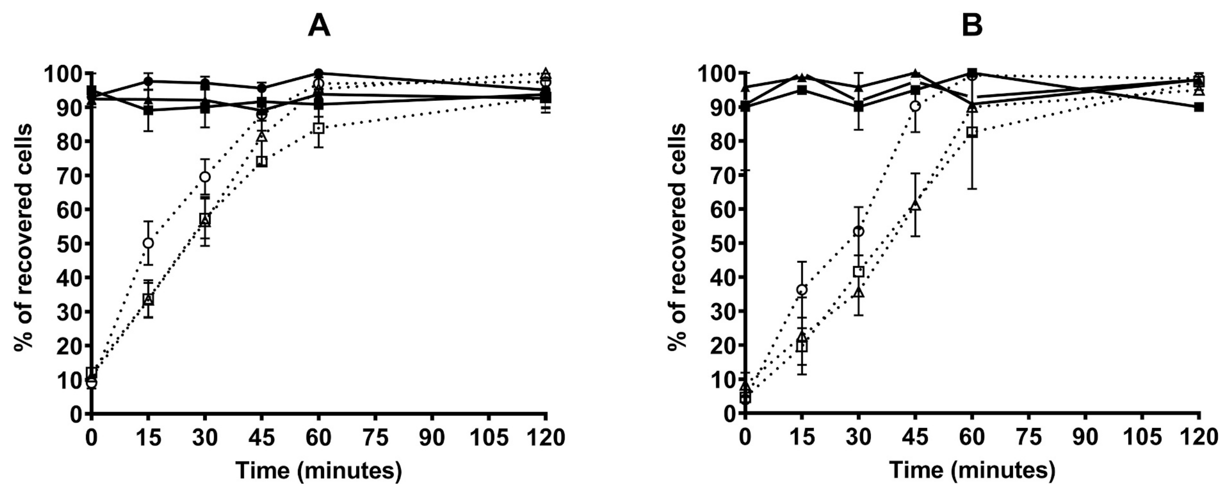

Fig. 3. Percentage of recovered cells in TSB-YE of *S. aureus* CECT 4459 non-adapted (square), heat-shocked (triangle) and alkaline-shocked (circle) cells, in stationary (A) and exponential (B) phase of growth, and plated onto TSA-YE (continuous lines, closed symbols) and TSA-YE-NaCl (discontinuous lines, open symbols).

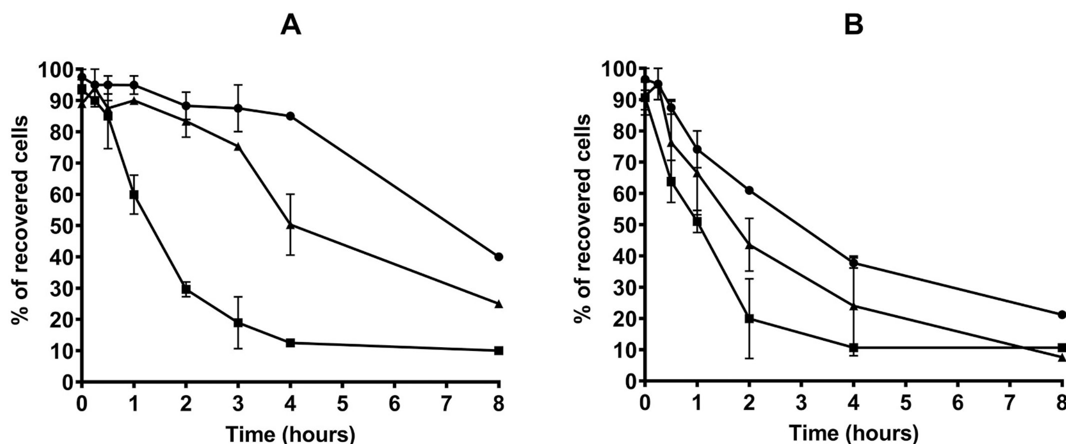

Fig. 4. Percentage of recovered cells in TSB-YE-NaCl of *S. aureus* CECT 4459 non-adapted (square), heat-shocked (triangle) and alkaline-shocked (circle) cells, in stationary (A) and exponential (B) phase of growth, and plated onto TSA-YE (continuous lines, closed symbols).

growth phase cells (Fig. 4B).

3.3. Biosynthetic requirements for the heat and alkaline shock-dependent development of PEF resistance

It is widely acknowledged that stress tolerance is generally acquired by means of *de novo* protein synthesis (Scheyhing, Hormann, Ehrmann, & Vogel, 2004). In fact, Cebrián et al. (2012) hypothesized that the increase in PEF resistance observed for the heat- and alkaline-shocked cells could be due to a higher concentration or activity of HSPs. Therefore, in this study, sublethal alkaline and heat shocks were also applied in presence of protein (chloramphenicol), RNA (rifampicin) and lipid (cerulenin) synthesis inhibitors, to study the biosynthetic requirements for the heat and alkaline shock-dependent development of PEF resistance. As can be observed in Table 1, no significant differences ($p > 0.05$) in *S. aureus* PEF inactivation were observed, regardless of the addition of any of the three inhibitors. These results suggest that the heat and alkaline shock-dependent development of PEF resistance did not require *de novo* RNA, protein, or lipid synthesis.

3.4. Combined processes

Given the cationic nature of lysozyme and nisin, and the change in surface charge previously observed after the application of a heat and an

Table 1
Log cycles of inactivation after 50 pulses (exponential decay; pulse width ≈3 μs; 26 kV/cm) of stationary and exponential growth phase cells of *S. aureus* CECT 4459 before (Control) and after a 120 min alkaline (pH 9.5) shock and heat (45 °C) shock with or without (W/I) the addition of inhibitors. Standard deviations are included in parentheses.

	Adaptation medium	Control cells	Alkaline Shock	Heat Shock
Stationary phase	W/I	2.29 (0.21)	0.90 (0.03)	1.57 (0.35)
	Chloramphenicol	–	0.90 (0.04)	1.56 (0.27)
	Rifampicin	–	1.01 (0.03)	1.77 (0.24)
	Cerulenin	–	0.96 (0.08)	1.67 (0.16)
Exponential phase	W/I	3.57 (0.42)	1.97 (0.44)	2.60 (0.31)
	Chloramphenicol	–	2.04 (0.34)	2.40 (0.11)
	Rifampicin	–	2.15 (0.21)	2.40 (0.07)
	Cerulenin	–	1.95 (0.01)	2.85 (0.07)

alkaline shock (Fig. 1), the effect of the combination of these compounds with PEF for the inactivation of non-shocked, heat-shocked, and alkaline-shocked cells was evaluated. Cells were subjected to a PEF treatment (26 kV/cm, 25 pulses, pH 7.0) with or without lysozyme (50 μg/mL) or nisin (100 UI/mL) in the treatment medium. Fig. 5 includes the Log cycles of inactivation obtained. As can be observed in this figure, heat-and alkaline-shocked cells were more resistant to PEF than non-shocked cells (control) ($p < 0.05$), as it had been demonstrated before (Cebrián et al., 2012). When PEF were applied in the presence of lysozyme or nisin, control cells were inactivated to the same level and no additional lethality was observed. By contrast, heat-shocked cells were sensitized to PEF in the presence of either lysozyme or nisin, in such a way that the protective effect exerted by the previous heat shock was counteracted, and resistance of these cells to the treatment was similar to that of control cells. Alkaline-shocked cells were sensitized by the presence of lysozyme, and 0.5 additional log cycles of inactivation were observed, as compared to PEF alone. However, these cells still remained significantly more resistant than control cells ($p < 0.05$) to the combined process PEF + Lysozyme. Finally, the combination of PEF and nisin was particularly effective for the inactivation of alkaline-shocked cells. Under these conditions, these cells were the most sensitive ($p < 0.05$) to PEF treatments.

4. Discussion

The presence of microbial resistance responses to environmental stresses is a phenomenon of concern for the food industry due to the uncertainty in the final number of survivors, to the treatments applied and, in certain cases, to the decrease in the safety level of the final products. Therefore, deeper basic knowledge about this phenomenon is required. As it has been suggested for other stress resistance responses (Cebrián et al., 2009), the observed increase in PEF resistance of *S. aureus* cells after an exposure to heat and alkaline shocks could be due either to a stabilization of cellular structures or to the development of a higher ability to repair or withstand the sublethal injuries caused by the treatments. In this context, it should be remarked that cell envelopes constitute the main target structure in bacterial inactivation by PEF treatments, and therefore, their structure, composition and properties could play an essential role in the maintenance of their functionality, which is essential for cellular repair and growth following treatments.

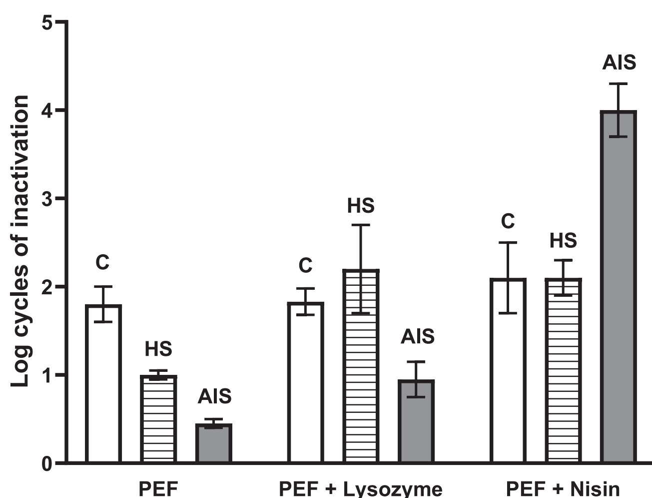

Fig. 5. Log cycles of inactivation of *S. aureus* non-shocked (control; C), heat-shocked (HS), and alkaline-shocked (AlS) stationary growth phase cells after a PEF treatment of 25 pulses (exponential decay; pulse width ≈3 μs; 26 kV/cm) in McIlvaine buffer pH 7.0 (PEF), McIlvaine buffer pH 7.0 + 50 μg/mL lysozyme (PEF + Lysozyme), and McIlvaine buffer pH 7.0 + 100 UI/mL nisin (PEF + Nisin).

Results obtained suggest that changes in membrane fluidity would not be responsible for the heat and alkaline shock-dependent development of PEF resistance, since only heat-shocked cells in exponential growth phase showed a different membrane fluidity (Fig. 1; Supplementary Table 1), whereas heat-shocked cells in stationary growth phase and alkaline-shocked cells were able to acquire PEF resistance as well. These results are in line with the ones obtained by Cebrián, Condón, and Mañas (2016), which suggest that, at least for *S. aureus*, membrane fluidity does not seem to be related to PEF resistance. By contrast, it seems reasonable to consider that the increase in PEF resistance of cells that have been previously exposed to a heat and alkaline shock might be caused by modifications of the cell surface charge, since our results indicate that only the application of these shocks (and especially alkaline shock) led to an increase in the surface negative charge of *S. aureus* cells (Fig. 1; Supplementary Table 1), which has been demonstrated to be correlated with an increased PEF resistance. In this context, it has been widely reported that the stability of the D-alanyl substituents of teichoic acid is extremely low under alkaline conditions (Hyyrylainen, 2000; Neuhaus & Baddiley, 2003; Perego et al., 1995). In addition, Golberg, Rae, and Rubinsky (2012) demonstrated that increasing the cell surface electronegativity through inhibition of D-ala esterification of lipoteichoic acid (LTA) caused a significant increase in the resistance of *L. monocytogenes* to PEF treatments. Thus, these authors suggested that the charge on the bacterial cell wall affects electroporation and therefore, can affect its PEF resistance. On the other hand, modifications of CSH would not seem to be involved in the increase in PEF resistance of heat- and alkaline-shocked cells, given that exposure to these shocks had no effect on the CSH of *S. aureus* cells (Fig. 1). By contrast, the exposure to acid and oxidative shocks resulted in an increase in CSH of *S. aureus* cells, although this increase was only significant ($p < 0.05$) for the exponential growth phase cells (Supplementary Table 1). However, it has already been demonstrated that the application of these shocks did not have an impact on the PEF resistance of this microorganism (Cebrián et al., 2012).

Changes in these properties of the cell envelopes after the exposure to alkaline and heat shocks could lead to modifications of the electroporation process and/or its reversion, which are directly correlated with PEF resistance (Delso, Martínez, Cebrián, Álvarez, & Raso, 2020; Golberg et al., 2012). Results obtained regarding the permeabilization to PI (Fig. 2), and cellular recovery capacity of *S. aureus* cells (Figs. 3 and 4) show remarkable differences between the behaviour of non-stressed, heat-shocked, and alkaline-shocked cells. Hence, the heat shock application contributed neither to changes in cell permeabilization nor in the cellular recovery rate in TSB-YE. Nevertheless, heat-shocked cells displayed a higher capacity to withstand an additional sublethal NaCl stress, as evidenced by their slower loss of viability in TSB-YE-NaCl (Fig. 4). On the other hand, the exposure to an alkaline shock led to a decrease in the percentage of cell permeabilization, to an increase in the cellular recovery capacity, and to an increased capacity to withstand an additional sublethal NaCl stress. These observations are partially in line with the hypothesis proposed by Cebrián et al. (2012), who suggested that whereas the higher resistance to PEF of alkaline-shocked cells might be due to a higher resilience of their membranes together with an increased ability to repair or withstand sublethal damage, the increased resistance of heat-shocked cells would be mainly due to an increased ability to repair or withstand sublethal injuries. Thus, results here reported indicate, on the one hand, that alkaline-shocked cells displayed a faster recovery capacity and a higher threshold for electroporation. Nevertheless, the decreased loss of viability rate observed in medium containing NaCl would suggest that the magnitude of the injuries suffered by the alkaline-shocked cells could be lower than that of the control cells. In this context, it has previously been suggested that pore size might determine the reversibility of *S. aureus* cells electroporation (Freire, Lattanzio, Orera, Mañas, & Cebrián, 2021). Results described in that work suggest that pores would need to acquire a particular size to lead to irreversible electroporation, since it is suggested that *S. aureus*

inactivation might be related to the exit of proteins of a molecular weight higher than 6 kDa, approx. Moreover, the fact that two different treatment conditions (25 kV/cm - 20 μs and 18 kV/cm - 400 μs) could lead to the same degree of cell permeabilization (around 90%) but extremely different degrees of cell inactivation (10 *vs* 90%), would also support that hypothesis (Freire et al., 2021). Hence, alkaline-shocked cells would display less amount of pores or pores of smaller size in comparison to control cells, and that would be the reason why they were able to survive for a longer period of time in a NaCl containing medium. In the case of heat-shocked cells, results obtained in this study lead to similar conclusions. Hence, data from recovery in liquid media also suggest that the degree of damage would be lower than for control cells, given that loss of viability in liquid medium with NaCl (Fig. 4) was notably slower. Moreover, the fact that heat-shocked cells presented less severe injuries than control cells would explain why, as described in Cebrián et al. (2012), differences in PEF resistance between the non-adapted and heat-shocked cells were not observed until treatments of >10 pulses were applied, moment at which control cells would not be able to recover from their injuries whereas heat-shocked cells would be able to do it (or to a higher extent). However, further work will be required to fully elucidate whether alkaline- and, particularly, heat-shocked cells are more PEF resistant due to more stable structures of the envelopes or to a higher damage repair capacity.

On the other hand, and regarding the biosynthetic requirements for the acquisition of PEF resistance after exposure to heat and alkaline shocks, it should be noted that, as pointed out above, *de novo* protein synthesis is generally acknowledged as the main way to achieve stress tolerance (Scheyhing et al., 2004) and that, for instance, HSPs are induced not only by temperature upshifts but also by several stressing conditions, including alkaline shocks (Flahaut, Hartke, Giard, & Auffray, 1997; Taglicht, Padan, Oppenheim, & Schuldiner, 1987). Nevertheless, it has also been reported that in certain cases the acquisition of stress resistance can occur without *de novo* protein synthesis as well (Cebrián et al., 2010; Flahaut et al., 1997). In this sense, results obtained in this investigation indicate that the heat and alkaline shock-dependent development of PEF resistance did not require *de novo* protein synthesis. Thus, these results would also imply that, although HSPs might play a role in *S. aureus* PEF resistance (either by stabilising the envelopes or by increasing the damage repair capacity of cells), they would not be involved in the acquisition of PEF resistance after the exposure to heat and alkaline shocks. Results obtained also indicate that neither *de novo* RNA nor lipid synthesis was required for the heat and alkaline shock-dependent development of PEF resistance, what supports the aforementioned hypothesis, *i.e.*, that the change in surface charge (*e.g.* by means of teichoic acid de-d-alanylation) might be the major responsible for the increase in PEF resistance observed in *S. aureus* cells. To the best of our knowledge, the biosynthetic requirements for the development of bacterial PEF resistance had not been explored before, although our results somehow resemble those of Somolinos, Espina, Pagán, and García (2010), who observed that the repair of sublethal damage caused by PEF to *L. monocytogenes* was not prevented by the addition of chloramphenicol, cerulenin or rifampicin to the recovery medium.

Finally, results obtained when nisin or lysozyme (cationic peptide/protein) was added to the treatment medium demonstrate how an adequately designed combined process might help to counteract the protective responses that could be developed depending on the environmental or processing conditions. Thus, results here reported suggest that changes in cell surface charge provoked by the alkaline and heat shocks, that involved an increase in the negative surface charge, would sensitize *S. aureus* cells to treatments consisting of the application of PEF in presence of nisin, compound that presents a positive charge. In this sense, Peschel et al. (1999) had already suggested that *S. aureus* resistance to nisin was related to the amount of positively charged D-alanine esters in its cell wall teichoic acids and hypothesized that the increased positive charge at the bacterial cell walls would play an important role in the resistance mechanisms of this microorganism against cationic peptides, such as nisin.

It should be noted that differences observed between the effect caused by the addition of nisin and lysozyme could be due to various factors, including not only the concentrations tested but also their differences in size, charge and antimicrobial activity. The combination of PEF with nisin or lysozyme for microbial inactivation has already been explored before, with wide differences in the outcome of such combinations. Thus, Saldaña, Monfort, Condón, Raso, and Álvarez (2012) observed that the addition of nisin to the treatment medium did not influence the PEF lethality of *Salmonella* Typhimurium and *E. coli* cells, independently of the temperature of study (≤ 50 °C). By contrast, Calderón-Miranda, Barbosa-Cánovas, and Swanson (1999) observed a synergistic effect of PEF treatments and the addition of nisin on *Listeria innocua* inactivation. Discrepancies found among the different studies might be easily explained on the basis of the different microorganisms studied (*e.g.* Gram negatives *vs* Gram positives) and treatments applied. Moreover, results here reported demonstrate that environmental factors might also significantly affect the outcome obtained since, for the same strain of *S. aureus*, the effect of the combined process was completely different for control, heat shocked and alkaline shocked cells.

5. Conclusions

The increase in PEF resistance observed in *S. aureus* cells that had been previously exposed to a heat and alkaline shock might be caused by modifications of the cell surface charge, which would lead in turn to modifications of the electroporation process and/or its reversion. It should be noted that this acquisition of PEF resistance did not require *de novo* RNA, protein, or lipid synthesis. The present work also demonstrates that addition of nisin to the treatment medium could not only counteract the protective effect of heat and alkaline shocks against PEF in *S. aureus* cells, but also greatly increase the lethality on alkaline-shocked cells. Further work would be required to fully elucidate whether alkaline- and, particularly, heat-shocked *S. aureus* cells were more PEF resistant due to more stable structures of the envelopes or to a higher damage repair capacity, and to determine whether the conclusions of this study could be generalized to other microorganisms and/or to which groups of them. On the other hand, the study of the influence that surface charge might exert on the process of electroporation and/or microbial inactivation by PEF might be highly relevant for the future design and/or optimization of treatments using this technology, applied individually or in a combined process.

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CRediT authorship contribution statement

Laura Nadal: Investigation, Methodology, Formal analysis, Writing – original draft. **Guillermo Cebrián:** Conceptualization, Writing – review & editing, Supervision. **Pilar Mañas:** Conceptualization, Writing – review & editing, Supervision.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2024.103707.

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