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Pulsed electric fields cause sublethal injury in *Escherichia coli*

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ABSTRACT

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Aims: The objective was to investigate the occurrence of sublethal injury in *Escherichia coli* by pulsed electric fields (PEF) at different pH values.

Methods and Results: The occurrence of sublethal injury in PEF-treated *E. coli* cells depended on the pH of the treatment medium. Whereas a slight sublethal injury was detected at pH 7, 99·95% of survivors were injured when cells were treated at pH 4 for 400 μs at 19 kV. The PEF-injured cells were progressively inactivated by a subsequent holding at pH 4.

Conclusions: PEF cause sublethal injury in *E. coli*. The measurement of sublethal injury using a selective medium plating technique allowed prediction of the number of cells that would be inactivated by subsequent storage in acidic conditions.

Significance and Impact of the Study: This work could be useful for improving food preservation by PEF technology and contributes to the knowledge of the mechanism of microbial inactivation by PEF.

Keywords: *Escherichia coli*, pulsed electric fields, pH, sublethal injury.

INTRODUCTION

Pulsed electric fields (PEF) are one of the most relevant non-thermal processes for food preservation because of their potential to inactivate microorganisms without altering organoleptic and nutritional properties of foods (Barbosa-Cánovas *et al.* 1999).

Knowledge of the mechanisms of microbial inactivation is required to design effective PEF treatments that, alone or in combination with other physical treatments or antimicrobial agents, could form alternatives to traditional heat preservation.

Microbial inactivation by PEF is believed to be caused by the effects of PEF on the cell envelopes. PEF can cause formation of pores affecting the integrity and functionality of the membrane. These pores can be reversible or irreversible, depending on the degree of membrane damage (Weaver and Chizmadzhev 1996). A proportion of cells would become leaky during PEF but reseal to a greater or lesser extent after it. Therefore, microorganisms that survive PEF treatments may be sublethally injured.

Since sublethal injury is supposed to be related to the higher sensitivity of survivors to stress conditions after treatment, from a practical point of view the occurrence of sublethally injured cells would prove valuable in developing appropriate combination processes. Moreover, under suitable conditions, sublethally injured cells might be repaired, therefore their occurrence is a very important aspect to be taken into account in food safety. A few cells being able to repair damage after PEF treatment could result in infective concentrations.

Most published data (Simpson *et al.* 1999; Russell *et al.* 2000; Dutreux *et al.* 2000a, 2000b; Ulmer *et al.* 2002) has not demonstrated the occurrence of sublethal injury after PEF treatments using a selective medium plating technique. Only Liang *et al.* (2002) and Unal *et al.* (2001) have observed sublethally injured cells when PEF were combined with

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ozone and heat, respectively. It is not clear whether sublethal injury is caused by PEF or is a consequence of the combined treatment. In most studies microorganisms have been PEF-treated under neutral pH. As cell membrane is possibly the main target under PEF treatments and cell envelopes are deeply affected by the reduction of the surrounding pH (Neidhardt *et al.* 1990), the occurrence of sublethal injury after PEF should be evaluated at low pHs. Moreover, it would be very convenient as PEF technology has been proposed to preserve liquid acid foods.

The aim of this work was to investigate the occurrence of sublethal injury in *Escherichia coli* after PEF treatments at different pHs.

MATERIAL AND METHODS

Microorganism and growth conditions

The strain of *E. coli* (STCC 471) used in this investigation was supplied by the Spanish type culture collection (Valencia, Spain). During this investigation it was maintained on slants of tryptic soy agar (Biolife, Milan, Italy) with 0·6% of yeast extract added (Biolife) (TSAYE).

A broth subculture was prepared by inoculating, with one single colony from a plate, a test tube containing 5 ml of sterile tryptic soy broth (Biolife, Milan, Italy) with 0·6% of yeast extract added (Biolife) (TSBYE). After inoculation this tube was incubated at 37 °C for 18 h. With this subculture, 250 ml Erlenmeyer flasks containing 50 ml of TSBYE were inoculated to a final concentration of 10^4 cells per ml. These flasks were incubated under agitation (130 rpm; Selecta, mod. Rotabit, Barcelona, Spain) at 37 °C until the stationary growth phase was reached.

PEF equipment

The PEF equipment used in this investigation has been previously described by Raso *et al.* (2000). The circuit configuration generated square waveform pulses at different frequencies and pulse widths. A pulse width of 2 μs and a pulse repetition rate of 2 Hz was used in this study.

PEF treatments

Before treatment, microorganisms were centrifuged at $6000 \times g$ for 5 min at 4 °C and resuspended in citrate-phosphate McIlvaine buffer of pH 7, 6, 5, 4 and 3 (Dawson *et al.* 1974) whose concentration was adjusted to an electrical conductivity of 2 mS cm^{-1}. Next, 0·5 ml of the microbial suspension, at a concentration of 10^9 CFU per ml, was placed into the treatment chamber with a sterile syringe. In all experiments the temperature of the samples after treatment was lower than 35 °C.

Measurement of acid sensitivity

After PEF-treatment, appropriate serial dilutions were prepared in sterile TSB and McIlvaine buffer of pH 4 and maintained at room temperature for 125 min. Samples were taken at preset intervals.

Viable counts

After dilutions, 0·1 ml samples were removed and plated onto TSAYE, or in this medium with 4% sodium chloride (Probus, Barcelona, Spain) added (TSAYE-SC). Previous experiments showed that TSAYE-SC did not affect untreated cells. Plates with TSAYE medium were incubated for 24 h at 37 °C and those with TSAYE-SC medium for 48 h at the same temperature. Previous experiments showed that longer incubation times did not influence survival counts. After incubation, colony forming units (CFU) were counted with an improved image analyser automatic counter (Protos, Analytical Measuring Systems, Cambridge, UK) as previously described (Condón *et al.* 1996).

Sublethally injured cells were estimated by the difference in the number of CFU obtained after plating heated cells in TSAYE and TSAYE-SC.

Survival curves were based on mean values obtained from at least three independent experiments.

RESULTS

Figure 1 shows survival curves at 19 kV cm^{-1} of PEF-treated *E. coli* cells in citrate-phosphate buffer at pH 7 and 4 and incubated for survival counting in TSAYE and TSAYE-SC medium. *Escherichia coli* cells recovered in TSAYE showed higher PEF resistance when treated at

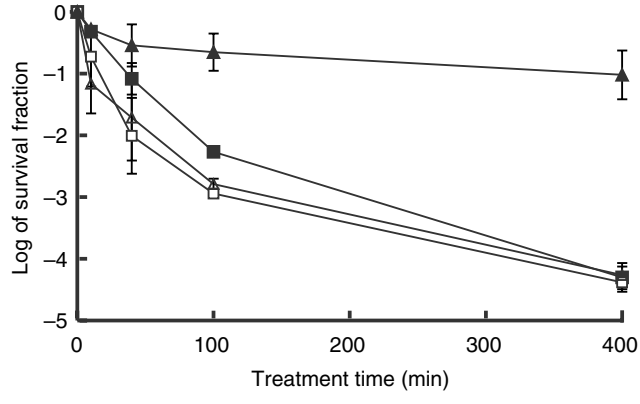

Fig. 1 Survival curves of *Escherichia coli* cells after PEF treatments at 19 kV cm^{-1} in citrate-phosphate buffer of pH 4 (▲) and 7 (■) and recovered in TSAYE (closed symbol) and TSAYE-SC medium (opened symbol). Data are means ± standard deviations (errors bars).

pH 4. Whereas a slight sublethal injury was detected at pH 7, 99·95% of survivors were injured when cells were treated at pH 4 for 400 μs. Similar results were observed after PEF treatment at 25 kV cm^{-1} (data not shown). As seen in this figure, the proportion of injured cells increased with the duration of PEF treatment. On the other hand, this figure shows there is a coincidence between the number of survivors to the PEF treatment at pH 7 and that at pH 4 when cells were recovered in the selective medium.

Figure 2 shows the influence of the pH of the treatment medium on the PEF resistance and on the occurrence of sublethal injury in PEF-treated *E. coli* cells. PEF-treated *E. coli* cells at 19 kV cm^{-1} for 100 μs showed the maximum resistance at pH 4 when recovered in TSAYE. However, the PEF resistance of *E. coli* was similar at any pH investigated when TSAYE-SC was used as recovery media. This figure illustrates that the magnitude of sublethal injury depended on the pH of treatment medium. Whereas sublethal injury was not detected at pH 5 and 6, at pH 3 and 7 a slight sublethal injury was observed, and reached a maximum at pH 4.

Figure 3 shows the survivors determined by counting on TSAYE and TSAYE-SC immediately after PEF in citrate-phosphate buffer at pH 4 at 19 kV cm^{-1} for 400 μs and during subsequent incubation under acid conditions. Survivors remaining after the PEF treatment were progressively inactivated by a subsequent acid incubation at room temperature for 125 min. However, the number of non-damaged cells kept constant independently of the subsequent incubation time. PEF-injured cells were progressively inactivated by a subsequent incubation under acid condition and were not detected after 125 min. The same experiment carried out with PEF-treated cells at pH 7 did not show any sublethal injury nor sensitivity to the subsequent acid incubation at room temperature (data not shown).

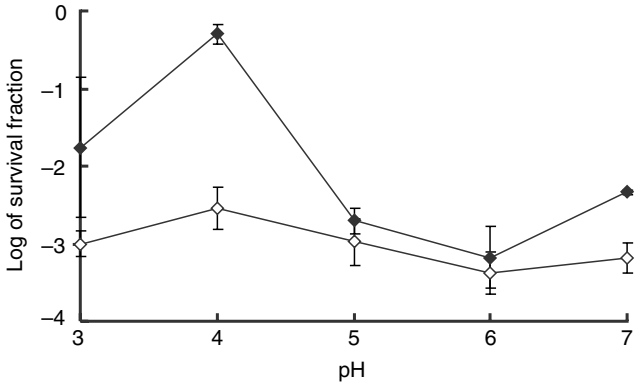

Fig. 2 Survivors of *Escherichia coli* after a PEF treatment at 19 kV for 100 μs in citrate-phosphate buffer at pH 3, 4, 5, 6, 7 and recovered in TSAYE (◆) and TSAYE-SC medium (◇). Data are means ± standard deviations (errors bars)

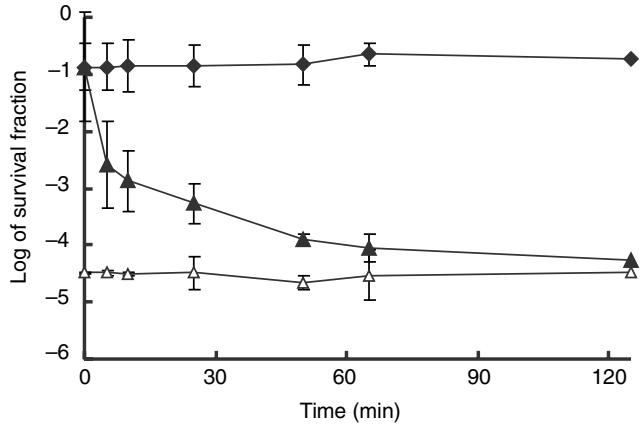

Fig. 3 Survivors of *Escherichia coli* after a PEF treatment at 19 kV cm^{-1} for 400 μs in citrate-phosphate buffer at pH 4 and a subsequent incubation at pH 4 (▲) and recovered in TSAYE (closed symbol) and TSAYE-SC medium (opened symbol). Data are means ± standard deviations (errors bars)

DISCUSSION

Data reported in the literature on the influence of the pH of the treatment medium on microbial PEF resistance are inconclusive. Whereas according to several authors (Sale and Hamilton 1967; Hülsheger *et al.* 1981) the pH of treatment media did not affect microbial inactivation by PEF, Vega-Mercado *et al.* (1996) observed that *E. coli* was more PEF-sensitive under acidic conditions, and Alvarez *et al.* (2000) that *Salmonella senftenberg* was more resistant at acid pH. As shown in Fig. 1, our strain of *E. coli* was also more PEF-resistant at low pHs. The higher PEF resistance observed in *E. coli* at pH 4 seems to be related to the capacity of these cells to repair sublethal injury. PEF-treated *E. coli* cells at pH 7 seem to be more sensitive because of the lack of this repair capacity as the survival curve obtained after PEF treatment at pH 7 is very similar to that obtained after PEF treatment at pH 4 and recovery on TSAYE-SC. Either the mechanisms of sublethal injury repair are directly affected by PEF at pH 7 and are not at pH 4 or the intrinsic resistance of *E. coli* cells to PEF is different depending on the pH of the treatment medium and the lesions inflicted by PEF at neutral pHs are not repairable.

The number of survivors after a PEF treatment at pH 7 or pH 4 is practically the same when TSAYE-SC is used as recovery medium (Fig. 1). Thereby, the number of micro-organisms inactivated at pH 7 is equal to the sum of the number of cells inactivated and injured at pH 4. The same happened at any treatment medium pH. As shown by Fig. 2, the number of survivors recovered in the selective medium remained constant independently of the pH of treatment medium. Therefore, when the possibility of repairing

sublethal injury was avoided the maximum microbial inactivation could be reached independently of the influence of the pH of the treatment medium.

Data reported in the literature on the occurrence of sublethal injury after PEF treatments measured using a selective medium plating technique (Simpson *et al.* 1999; Russell *et al.* 2000; Dutreux *et al*. 2000a, 2000b; Ulmer *et al.* 2002) had not shown any PEF-injury, concluding that bacterial inactivation by PEF may be an 'all or nothing' event. Our results have demonstrated the occurrence of sublethal injury after PEF treatment depending on the pH of treatment medium. Whereas injury was detected in PEF-treated cells at pH 4, at neutral pHs it was hardly detected. Perhaps the higher treatment medium pH used by the authors (Simpson *et al.* 1999; Russell *et al.* 2000; Dutreux *et al.* 2000a, 2000b) was the cause for the non-detection of sublethally injured cells after PEF. According to our results we can conclude that microbial inactivation by PEF is not always an 'all or nothing' event and would depend on the treatment conditions investigated.

Sublethal injury measured using a selective medium plating technique is not a valuable tool unless it shows the sensitivity of these cells to other stress conditions used in practice. As PEF technology has been proposed to preserve fruit juices, the sensitivity of PEF-treated cells to acid conditions was evaluated.

As shown by Fig. 3, PEF-treated *E. coli* cells at pH 4 were sensitive to a subsequent incubation under acid conditions. The sensitivity of these cells seemed to be related to the occurrence of sublethal injury as the number of survivors recovered in the selective medium and the number of survivors after the subsequent incubation under acid conditions for 125 min is practically the same. Therefore, the lesions inflicted by PEF seems to be unspecific since the number of cells sensitive to the sodium chloride and the low pH was the same. On the other hand, when sublethal injury was not detected, as happens when *E. coli* cells are PEF-treated at neutral pHs, the subsequent incubation under acidic conditions had no effect (data not shown).

As also shown in Fig. 3, incubation under acidic conditions after PEF did not injure more cells because the number of non-damaged cells kept constant independently of the incubation time. Non-sublethally injured cells, obtained either after a PEF treatment at pH 7 or at pH 4 were not sensitive to a subsequent holding in acidic conditions.

The mechanisms by which PEF sensitizes bacteria to a subsequent acid challenge is unknown. Damage to the bacterial cell membrane is believed to be an important event, leading to the inactivation of cells by PEF. Impaired ability to maintain a transmembrane pH gradient (ΔpH) has been described for *Listeria monocytogenes* (Simpson *et al*. 1999; Russell *et al.* 2000) and *Salmonella typhimurium* (Simpson *et al.* 1999) and membrane leakiness following PEF has been reported for several other microorganisms (Hamilton and Sale, 1967; Simpson *et al.* 1999). Loss of such membrane functions would be expected to impair pH homeostasis, and this might account for the increased sensitivity to acid conditions.

The sensitization of cells to acid by a prior PEF treatment would be an important consideration because it would allow pathogens to be inactivated by much milder treatments than would be necessary. The measurement of sublethal injury using a selective medium plating technique would allow to predict the level of microbial inactivation after storage of acid foods.

Moreover, the fact that sublethal injury has been detected after PEF treatments would also indicate that other antimicrobial substances added to the food might be able to act even after PEF treatment increasing their bactericidal action and their action spectrum, allowing to achieve the desired level of microbial inactivation while retaining the essential organoleptic properties and nutritive value of the food.

These studies support the view that membrane damage is an important event in the inactivation of bacteria by PEF. Further investigation is needed in order to clearly define the nature of membrane damage and its relation to cell death depending on species, growth conditions, experimental conditions, etc.

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REFERENCES

Alvarez, I., Raso, J., Palop, A. and Sala F.J. (2000) Influence of different factors on the inactivation of *Salmonella senftenberg* by pulsed electric fields. *International Journal of Food Microbiology* **55**, 143–146.

Barbosa-Cánovas, G.V., Góngora, M.M., Pothakamury, U.R. and Swanson, B.G. (1999) *Preservation of Foods with Pulsed Electric Fields*. San Diego: Academic Press.

Condón, S., Palop, A., Raso, J. and Sala, F.J. (1996) Influence of the incubation temperature after heat treatment upon the estimated heat resistance values of spores of *Bacillus subtilis*. *Letters in Applied Microbiology* **22**, 149–152.

Dawson, R.M.C., Elliot, D.C., Elliot, W.H. and Jones, K.M. (1974) *Data for Biochemical Research*. Oxford: Oxford at Clarendon Press.

Dutreux, N., Notermans, S., Góngora-Nieto, M.M., Barbosa-Cánovas, G.V. and Swanson, B.G. (2000a) Effects of combined exposure of *Micrococcus luteus* to nisin and pulsed electric fields. *International Journal of Food Microbiology* **60**, 147–152.

Dutreux, N., Notermans, S., Wijtzes, T., Góngora-Nieto, M.M, Barbosa-Cánovas, G.V. and Swanson, B.G. (2000b) Pulsed electric

fields inactivation of attached and free-living *Escherichia coli* and *Listeria innocua* under several conditions. *International Journal of Food Microbiology* **54**, 91–98.

Hamilton, W.A. and Sale, A.J.H. (1967) Effects of high electric fields on microorganisms: II. Killing of bacteria and yeasts. *Biochimica and Biophysica Acta* **148**, 789–800.

Hülsheger, H., Potel, J. and Niemann, E.G. (1981) Killing of bacteria with electric pulses of high field strength. *Radiation and Environmental Biophysics* **20**, 53–65.

Liang, Z., Mittal, G.S. and Griffiths, M.W. (2002) Inactivation of *Salmonella Typhimurium* in orange juice containig antimicrobial agents by pulsed electric fields. *Journal of Food Protection* **65**, 1081–1087.

Neidhardt, F.C., Ingraham, J.L. and Schaechter, M. (1990) *Physiology of the Bacterial Cell: A Molecular Approach*. Sunderland, MA, USA: Sinauer Associates, Inc. Publishers.

Raso, J., Alvarez, I., Condón, S. and Sala, F.J. (2000) Predicting inactivation of *Salmonella senftenberg* by pulsed electric fields. *Innovative Food Science & Emerging Technologies* **1**, 21–29.

Russell, N.J., Colley, M., Simpson, R.K., Trivett, A.J. and Evans, R.I. (2000) Mechanim of action of pulsed high electric field (PHEF) on the membranes of food-poisoning bacteria is an "all-or-nothing" effect. *International Journal of Food Microbiology* **55**, 133–136.

Sale, A.J.H. and Hamilton, W.A. (1967) Effects of high electric fields on microorganisms: I. Killing of bacteria and yeast. *Biochimica and Biophysica Acta* **148**, 781–788.

Simpson, R.K., Whittington, R., Earnshaw, R.G. and Russell, N.J. (1999) Pulsed high electric field causes all or nothing membrane damage in *Listeria monocytogenes* and *Salmonella typhimurium*, but membrane H^+-ATPase is not a primary target. *International Journal of Food Microbiology* **48**, 1–10.

Ulmer, H.M., Heinz, V., Gänzle, M.G., Knorr, D. and Vogel, R.F. (2002) Effects of pulsed electric fields on inactivation and metabolic activity of *Lactobacillus plantarum* in model beer. *Journal of Applied Microbiology* **93**, 326–335.

Unal, R., Kim, J-G. and Yousef, A.E. (2001) Inactivation of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Lactobacillus leichmannii* by combinations of ozone and pulsed electric field. *Journal of Food Protection* **64**, 777–782.

Vega-Mercado, H., Pothakamury, U.R., Chang, F-J., Barbosa-Cánovas, G.V. and Swanson, B.G. (1996) Inactivation of *Escherichia coli* by combining pH, ionic strength and pulsed electric fields hurdles. *Food Research International* **29**, 117–121.

Weaver, J.C., and Chizmadzhev, Y.A. (1996) Theory of electroporation: a review. *Bioelectrochemistry and Bioenergetics* **41**, 135–160.