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Inactivation of *Salmonella* Typhimurium and *Staphylococcus aureus* by pulsed electric fields in liquid whole egg

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ABSTRACT

In this study, the lethal effectiveness of pulsed electric fields (PEF) on the inactivation of *Salmonella enterica* subs. *enterica* ser. Typhimurium and *Staphylococcus aureus* in liquid whole egg (LWE) has been investigated. Maximum inactivation levels of 4 and 3 Log_{10} cycles of the population of *Salmonella* Typhimurium and *S. aureus* were achieved with treatments of 45 kV/cm, 30 μs and 419 kJ/kg, and 40 kV/cm for 15 μs and 166 kJ/kg, respectively. The non-linear kinetics of inactivation observed for both microorganisms at all the investigated electric field strengths were described by mathematical equations based on the Weibull distribution. The developed equations enabled to compare the microbial resistance to PEF and to establish the most suitable treatment conditions to achieve a determined level of microbial inactivation. PEF treatments varying from 30 kV/cm, 67 μs and 393 kJ/kg to 45 kV/cm, 19 μs and 285 kJ/kg allow to reduce 3 Log_{10} cycles the population of the microorganism of concern in PEF food processing of LWE, *Salmonella* Typhimurium.

Industrial relevance: The data presented in this investigation in terms of electric field strength, specific energy and treatment time result of relevance to evaluate the possibilities of PEF technology to pasteurize LWE with this technology. The models developed in this study can be applied to engineering design, and for the evaluation and optimization of the PEF technology as a new technique to obtain *Salmonella* free LWE.

Based on our results it is not recommended to apply treatments of energy levels higher than 250 kJ/kg, since PEF lethality hardly increased but markedly augmented the energetic costs. For these energy values, PEF technology by itself is not sufficient (3 Log10 cycles in the best case scenario) to assure the safe security of LWE. Therefore, intelligent combinations of PEF with other preservation technologies have to be developed in order to use pulsed electric fields as an alternative to heat pasteurization of LWE.

1. Introduction

Egg and egg products are a nutritious part of our diet and a useful ingredient in foods due to their functional properties (foaming, emulsifying and gelling). Unfortunately, egg and egg products are responsible for a large number of foodborne illnesses each year, constituting a serious obstacle to the well-being of populations, and being the source of tremendous economic losses. In 2006, from a total of 5710 outbreaks in Europe, egg and egg products were the most common food vehicle, responsible for 11.7% of these outbreaks. *Salmonella*, and mainly serovars *Salmonella* Enteritidis and *Salmonella* Typhimurium, was the microorganism responsible in most of the cases (European Food Safety Authority, 2007).

Currently, the egg industry primary intervention to improve the microbiological safety of liquid egg is thermal treatment. However, the thermal sensitivity of liquid egg components limits the temperature at which the product can be heated; some soluble proteins begin to precipitate at temperatures as low as 57 °C (Hamid-Samimi, Swartzel, & Ball, 1984; Herald & Smith, 1989). The temperatures required for pasteurization of LWE can induce changes in egg quality, including coagulation of egg proteins and changes in other functional properties. Therefore, new nonthermal technologies are being investigated for inactivating *Salmonella* and other microorganisms isolated from LWE such as *Staphylococcus aureus*, *Listeria monocytogenes* (Foegeding & Stanley, 1987; Erickson & Jenkins, 1992) in LWE with a minimum impact on the freshness properties (Jeantet, Baron, Nau, Roignant, & Brulé, 1999; Lee, Heinz, & Knorr, 2003; Mañas, Pagán, Álvarez, & Condón, 2003; Álvarez, Niemira, Fan, & Sommers, 2006).

Pulsed electric fields (PEF) could be a possible technology to hygienize LWE since it has been observed in other products that PEF can inactivate vegetative cells of spoiling and pathogenic bacteria at ambient temperature diminishing consequently the negative impact of heat on the quality properties (Barbosa-Cánovas, Pierson, Zhang, & Schaffner, 2001). The process involves the application of short duration pulses of high electric field strengths (1–50 kV/cm) to foods placed between 2 electrodes at ambient temperature. These

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field strengths create pores on cell membranes (electroporation) leading to the cellular death without affecting other cellular structures or food components such as proteins or enzymes (Wouters, Álvarez, & Raso, 2001). The design of effective PEF treatments that assure safety and stability of foods requires the identification of those factors (electric field strength, treatment time and specific energy) influencing microbial PEF resistance and the knowledge of the inactivation kinetics of microorganisms by PEF.

Results published in literature concerning the effectiveness of PEF to pasteurize LWE are not completely conclusive, probably due to the narrow range of treatment conditions and microorganisms investigated. Most of the studies on microbial inactivation by PEF in LWE have been evaluated on *Escherichia coli* (Martin-Belloso et al., 1997; Amiali, Ngadi, Raghavan, & Smith, 2004; Bazhal, Ngadi, Raghavan, & Smith, 2006). However, there is hardly any data related to the PEF lethality on *Salmonella* serotypes and *S. aureus*. On the other hand, contradictory inactivation levels have been reported in literature, probably due to that the PEF treatment conditions have not been adequately indicated. Thus, while some authors hardly inactivated 2 Log_{10} cycles of the microbial population at treatments of 30–50 kV/cm and 100 μs (Hermawan, Evrendilek, Dantzer, Zhang, & Richter, 2004; Bazhal, et al., 2006; Huang, Mittal, & Griffiths, 2006; Amiali et al., 2004), other authors achieved between 3 and 6 Log_{10} reductions (Martin-Belloso et al. 1997; Jeantet et al., 1999). Finally, in most of the studies, microbial inactivation is related to the field strength and the treatment time but not to the specific energy applied. Few inactivation studies report the energy delivered by PEF treatment and even fewer correlate the energy applied with the efficacy of PEF (Martin-Belloso, et al., 1997; Heinz, Phillips, Zenker, & Knorr, 1999; Gongora-Nieto, Pedrow, Swanson, & Barbosa-Canovas, 2003; Korolczuk et al., 2006. The inclusion of the specific energy as a PEF parameter would result of interest in a product like LWE with a high value of electrical conductivity in order to compare results and to determine the energy consumption of the process.

According to the published studies, currently it is difficult to properly evaluate the viability of PEF as a LWE hygienization system. The high number of electric parameters influencing the process, and the absence of kinetics data related to microbial inactivation in LWE by PEF, make necessary new investigations to evaluate the possibilities of PEF as a pasteurization system of LWE. The research objectives for this study were to investigate the effects of PEF process parameters (electric field strength, treatment time and specific energy) on inactivating *Salmonella* Typhimurium and *S. aureus* in liquid whole egg, and to determinate the process conditions to maximize the microbial inactivation by PEF in LWE. The microorganisms used in this investigation are the most PEF resistant strains of five different strains of *Salmonella* Typhimurium and *S. aureus* (Saldaña et al., 2009).

2. Materials and methods

Extra-large grade A eggs were purchased from a local supermarket. Eggshells were thoroughly washed with 70% ethanol and allowed to dry air. Sanitized eggs were aseptically broken and transformed into a sterile stomacher bag (Tekmar Co. Cincinnati, Ohio, U.S.A) and homogenized for 2 min at 230 r.p.m. in a stomacher laboratory blender 400 (Tekmar Co. Cincinnati, Ohio, U.S.A). The obtained LWE was centrifuged at 102 × g for 2 min (centrifuge Heraeus, Model Megafuge 1.0 R) in order to eliminate the air, and maintained at 2 to 4 °C until ready for use. The pH of LWE was 7.5 ± 0.3 and its electric conductivity was 0.69 ± 0.02 S/m.

2.1. Microorganisms and growth conditions

The strains of *Salmonella* Typhimurium (STCC 878) and *S. aureus* (STCC 4459) used in this investigation were supplied by the Spanish Type Culture Collection (STCC). Broth subcultures were prepared by inoculating test tubes containing 5 ml of tryptic soy broth (Biolife, Milan Italy) plus 0.6% (w/v) of yeast extract (Biolife) (TSBYE) with a single colony and incubated for 18 h at 37 °C. With this subculture, flasks containing 50 ml of prewarmed TSBYE were inoculated to a final concentration of approximately 10^6 CFU/ml and incubated at 37 °C. The culture was incubated under agitation (135 r.p.m.) (mod. Rotabit, Selecta, Spain) at 37 °C for 24 h until the stationary growth phase was reached (Saldaña, et al., 2009; Cebrián, Sagarzazu, Pagán, Condón, & Mañas, 2007).

2.2. PEF equipment

PEF equipment used in this investigation was supply by ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden). The apparatus generates 3 μs-square waveform pulses with a pulse frequency up to 300 Hz. The maximum output voltage and current were 30 kV and 200 A, respectively. The equipment consists of a direct-current power supply (DCPS) which converts the 3-phase line voltage to a regulated DC voltage. It charges up 6 IGBT switching modules (high-power solid-state switches) to a primary voltage around 1000 V. An external trigger pulse gates all the modules and controls its discharge to a primary pulsed signal of around 1000 V. Finally, a pulse transformer transforms this primary 1000 V pulse to a high voltage pulse of desired high voltage.

Microorganisms were treated in a batch parallel-electrode treatment chamber. The chamber consists of two polished stainless steel cylinders separated 0.4 cm by an insulating material tube. The area of the treatment zone was 0.79 cm^2. In order to temper the sample previously to PEF treatments, an ethylenglicol solution was pumped through the ground electrode. With an electrical conductivity of the sample of 0.69 ± 0.02 S/m at 20 °C the ohmic resistance of the treatment chamber is 76 Ω. Actual electric field strength applied and pulse width were measured in the treatment chamber with a high voltage probe connected to an oscilloscope (Tektronix, TDS 220, Wilsonville, OR). Treatment time was calculated by multiplying the pulse width (τ) by the number of pulses applied. The frequency of the pulses was controlled by an external function generator (Tektronix, AFG 320, Wilsonville, OR). The energy per pulse (W') was calculated by the following equation:

$$W' = \int_0^{\infty} k \cdot E(t)^2 dt \quad (1)$$

where k (S/m) is the electrical conductivity of LWE; E (V/m) is the electric field strength; and $t(s)$ is the duration of the pulse. The total energy (kJ) applied (W) was calculated by multiplying the energy per pulse (W') by the number of pulses. The total specific energy (kJ/kg) applied (W) was determined by dividing the total energy by the mass of treated LWE. The temperature of the treatment medium was measured as previously described (Raso, Álvarez, Condón, & Sala, 2000).

2.3. Microbial inactivation experiments

Before treatment, microorganisms were centrifuged at 6000 × g for 5 min at 4 °C and re-suspended in 1 ml of LWE. The microbial suspension (0.3 g) at a concentration of approximately 10^9 CFU/ml was placed into the treatment chamber with a sterile syringe, as it has been previously described (Raso et al., 2000). The temperature of the LWE was tempered at an initial value of 15.0 ± 0.1 °C. Cumulative treatment times ranged from 3 to 150 μs and electric field strengths were set at 20, 25, 30, 35, 40 and 45 kV/cm which corresponded to specific energies per pulse of 8.3, 12.9, 18.6, 25.4, 33.1 and 41.9 kJ/kg, respectively. A pulse frequency of 0.5 Hz was used. Under these conditions, the final temperature of the treatment media was always below 35 °C.

After PEF treatments, the sample was extracted with a sterile syringe and appropriate serial dilutions were prepared in sterile 0.1% peptone water (Biolife) and plated into tryptic soy agar (Biolife, Milan Italy) plus 0.6% (w/v) of yeast extract (Biolife) (TSAYE). Plates were incubated at 37 °C for 24 h. After incubation, colonies were counted with an image analyzer automatic counter (Protos, Analytical Measuring Systems, Cambridge, UK) as previously described Condón, Palop, Raso, and Sala (1996). Survival curves were obtained by plotting Log_{10} of the survival fraction (the number of survivors divided by the number of viable cells in the untreated control sample) against treatment time or specific energy for each electric field strength. Each experiment was performed twice on separate days and the average results are presented.

2.4. Membrane damage of Salmonella Typhimurium and S. aureus cells following PEF treatments in LWE

After PEF treatments, the number of injured *Salmonella* Typhimurium and *S. aureus* cells was determined by pour plating samples on TSAYE containing 3% and 11% NaCl, respectively (TSAYE + NaCl), which are the maximum non-inhibitory concentration (MNIC) for both microorganisms (Saldaña et al, 2009; Cebrián et al., 2007). Plates with TSAYE + NaCl were incubated at 37 °C for 48 h to allow repairing and recovering of the injured cells before enumeration. All the experiments were replicated two times.

2.5. Mathematical model

A model based on the Weibull distribution was used to describe the survival curves. If the microbial PEF resistance follows a Weibull distribution, the equation is as follows (Mafart, Couvert, Gaillard, & Leguerinel, 2002):

$$Log_{10}S(x) = -\left(\frac{x}{\delta}\right)^{p} \quad (2)$$

where $S(x)$ is the survival fraction (CFU/ml); x is the treatment time (μs) or the specific energy (kJ/kg); δ and p are scale and shape parameters, respectively. The δ value represents the time (μs) or the specific energy (kJ/kg) necessary to inactivate the first Log_{10} cycle of the microbial population. The p parameter accounts for upward

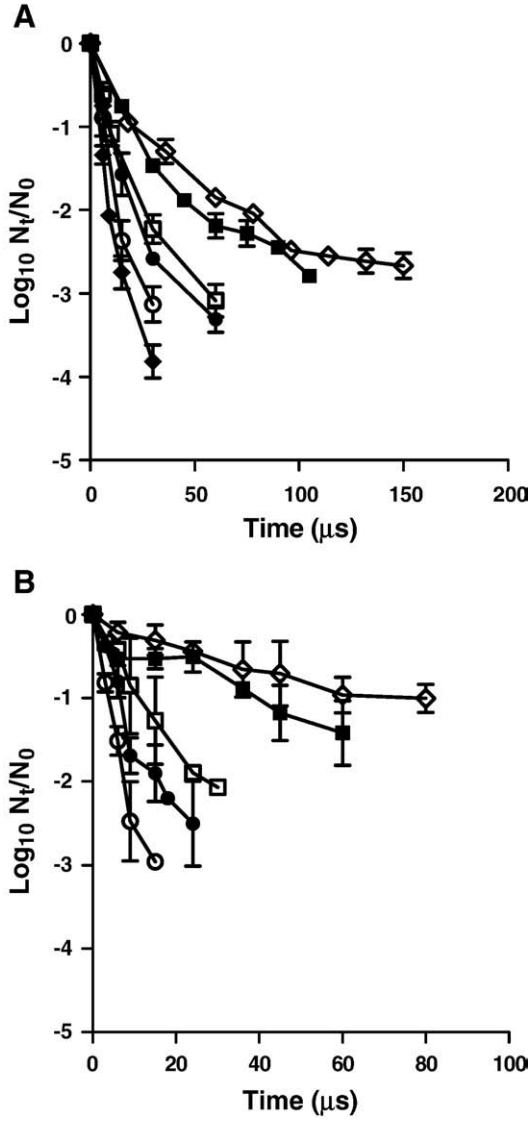

Fig. 1. Influence of the treatment time and the electric field strength on the inactivation of *Salmonella* Typhimurium (A) and *S. aureus* (B) by PEF in LWE: 20 kV/cm (◊); 25 kV/cm (■); 30 kV/cm (□); 35 kV/cm (●); 40 kV/cm (○); 45 kV/cm (♦).

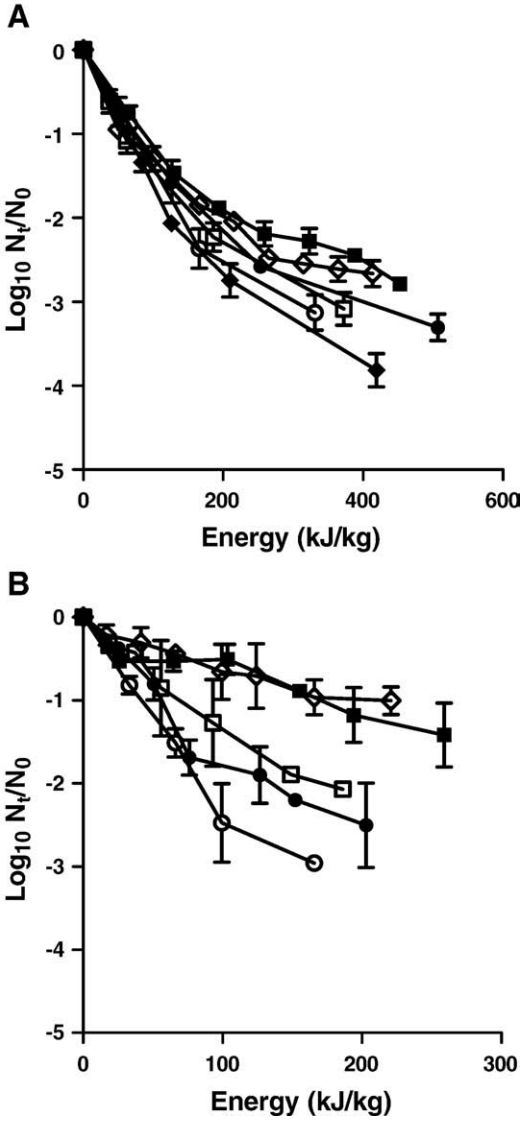

Fig. 2. Influence of the specific energy and the electric field strength on the inactivation of *Salmonella* Typhimurium (A) and *S. aureus* (B) by PEF in LWE: 20 kV/cm (◊); 25 kV/cm (■); 30 kV/cm (□); 35 kV/cm (●); 40 kV/cm (○); 45 kV/cm (♦).

concavity of a survival curve ($p<1$), a linear survival curve ($p=1$), and downward concavity ($p>1$).

To determine the δ and p values, the least-squares criterion by the Solver function of the Excel 5.0 package (Microsoft, Seattle, WA) and the GraphPad PRISM® (Graph Software, San Diego, CA) was used. Correlation coefficient (R^2) and root mean square error (RMSE) between predicted and observed data were used to test the fitness of the models. In order to determine the accuracy of the final models, the RMSE (root mean square error), the bias (B_f) and the accuracy factors (A_f) were used (Baranyi, Pin, & Ross, 1999). The bias factor indicates by how much on average, a model overpredicts (bias factor>1) or underpredicts (bias factor<1) the observed data. On the other hand, the accuracy factor indicates how much the estimated data differ from the observed ones. R^2, RMSE, standard deviation, B_f and A_f, and 95% confidence limits were calculated by Excel 5.0 (Microsoft) and GraphPad PRISM® (GraphPad Software, Inc., San Diego, California, EE.UU.).

3. Results

3.1. Influence of the electric field strength, treatment time and specific energy on the inactivation of Salmonella Typhimurium and S. aureus in LWE by PEF

Figs. 1 and 2 show the influence of the treatment time (Figs. 1A and B) and the specific energy (Figs. 2A and B) on the inactivation of *Salmonella* Typhimurium (Figs. 1A and 2A) and *S. aureus* (Figs. 1B and 2B) after PEF treatments at different electric field strengths (20–45 kV/cm) in LWE. As observed, the inactivation of both microorganisms increased with the treatment time, the specific energy and the electric field strength. However, the increment on the microbial lethality did not follow an exponential relationship with the treatment time and specific energy at the electric field strengths investigated. Survival curves were concave upwards at all electric field strengths; the microbial inactivation was faster at the first moments of the treatment, and then progressively declined. In order to describe the microbial inactivation and to compare the bacterial PEF-resistance, survival curves were fitted to Eq. (2).

The estimated parameters δ and p from the model are shown in Table 1. RMSE and R^2 values of the fits range from 0.034 to 0.187 and from 0.971 to 0.994, respectively, indicating that Eq. (2) adequately described the survival curves showed in Figs. 1 and 2. The time or the specific energy required to reduce 1 Log_{10} cycle the population of *Salmonella* Typhimurium and *S. aureus* (δ value) by PEF decreased by increasing the electric field strength. The shape parameters (p value) were lower than 1, which indicates that the survival curves were concave upward. Equations describing how the parameters of the primary model change with changes in environmental factors correspond to the secondary level of modeling. ANOVA analysis confirmed that there were non-significant differences ($p>0.05$) among the p values obtained at different electric field strengths. As non-significant differences among p values were observed, survival curves were refitted with p set at their mean values at 0.574 and 0.740 for *Salmonella* Typhimurium and *S. aureus* when survival curves were plotted against the treatment time and of 0.585 and 0.718 for the specific energy, respectively (Table 2).

Linear relationships were observed between the Log_{10} of the new scale parameters (δ value) obtained after the second fitting and the electric field strength for both, treatment time (Eq. 3 and 4) and specific energy (Eq. 5 and 6), in *Salmonella* Typhimurium and *S. aureus* (Table 2).

In order to determine the accuracy of the models, observed data were correlated with the estimated ones. The estimated data were calculated from the tertiary equations re-defined from Eq. (2) by substituting the p and δ values by their secondary models showed in Table 2. The R^2, RMSE, B_f and A_f values for each microorganism were the following when the Log_{10} of the survival fraction was plotted against the treatment time:

Salmonella Typhimurium: $R^2=0.971$; RMSE = 0.173; $B_f=1.023$; $A_f=1.119$.

Staphylococcus aureus: $R^2=0.948$; RMSE = 0.185; $B_f=0.998$; $A_f=1.220$.

Table 1
δ and p values estimated with the mathematical model based on the Weibull distribution after the first fitting of the observed values of *Salmonella* Typhimurium and *S. aureus*, at different electric field strengths in terms of the treatment time (A) and the specific energy (B).

A					
	Field strength (kV/cm)	δ (μs) (IC 95%)	p (IC 95%)	R^2	RMSE
Salmonella Typhimurium	20	18.03 (11.79–24.28)	0.490 (0.393–0.587)	0.986	0.094
	25	16.23 (10.48–21.99)	0.550 (0.423–0.677)	0.984	0.098
	30	9.17 (4.93–13.41)	0.613 (0.437–0.789)	0.991	0.058
	35	6.76 (5.17–8.36)	0.564 (0.493–0.637)	0.991	0.055
	40	4.97 (3.23–6.72)	0.654 (0.507–0.802)	0.986	0.082
	45	2.80 (1.12–4.48)	0.572 (0.399–0.745)	0.991	0.079
S. aureus	20	73.03 (63.52–82.62)	0.666 (0.522–0.810)	0.982	0.052
	25	39.01 (33.65–44.36)	0.805 (0.551–1.010)	0.945	0.034
	30	11.77 (10.55–12.73)	0.802 (0.781–0.992)	0.986	0.075
	35	6.13 (5.04–7.49)	0.708 (0.632–0.911)	0.971	0.185
	40	3.13 (2.45–3.82)	0.721 (0.599–0.843)	0.986	0.072
B					
	Field strength (kV/cm)	δ (kJ/kg) (IC 95%)	p (IC 95%)	R^2	RMSE
Salmonella Typhimurium	20	49.78 (32.55–67.01)	0.490 (0.393–0.587)	0.986	0.094
	25	70.01 (45.19–94.83)	0.550 (0.423–0.677)	0.984	0.098
	30	56.95 (30.63–83.28)	0.680 (0.437–0.789)	0.991	0.057
	35	57.16 (14.23–100.1)	0.565 (0.335–0.795)	0.983	0.123
	40	54.91 (34.58–72.06)	0.654 (0.507–0.801)	0.979	0.165
	45	39.13 (15.61–62.65)	0.572 (0.399–0.745)	0.991	0.079
S. aureus	20	201.7 (175.3–228.0)	0.666 (0.522–0.810)	0.982	0.092
	25	160.6 (143.7–177.4)	0.677 (0.758–0.885)	0.955	0.132
	30	70.94 (66.70–75.19)	0.821 (0.758–0.885)	0.994	0.080
	35	50.28 (41.45–59.12)	0.707 (0.593–0.822)	0.977	0.187
	40	33.56 (26.22–4.89)	0.721 (0.600–0.843)	0.986	0.160

Table 2
Secondary models for the shape and scale parameters for *Salmonella* Typhimurium and *S. aureus* treated by PEF in LWE in terms of the treatment time and the specific energy (E is the electric field strength expressed in kV/cm).

		δ value	R^2	p value (SD)[a]
Treatment time	*Salmonella* Typhimurium	$\text{Log}\,\delta = -0.037^{*}E + 2.140$ (Eq. 3)	0.948	0.574 (0.056)
	S. aureus	$\text{Log}\,\delta = -0.069^{*}E + 3.582$ (Eq. 4)	0.963	0.740 (0.061)
Specific energy	*Salmonella* Typhimurium	$\text{Log}\,\delta = -0.0093^{*}E + 2.059$ (Eq. 5)	0.990	0.585 (0.070)
	S. aureus	$\text{Log}\,\delta = -0.0403^{*}E + 3.129$ (Eq. 6)	0.951	0.718 (0.055)

[a] SD: standard deviation.

When the Log_{10} of the survival fraction was plotted against the specific energy, the values were:

Salmonella Typhimurium: $R^2 = 0.967$; RMSE = 0.185; $B_f = 1.015$; $A_f = 1.127$.

Staphylococcus aureus: $R^2 = 0.954$; RMSE = 0.172; $B_f = 1.010$; $A_f = 1.219$.

Based on the values obtained for the R^2, RMSE, B_f and A_f the developed tertiary models accurately predict, in the range investigated, the PEF inactivation of both bacteria treated in LWE.

The developed equations permitted to compare the PEF resistance and to determinate the influence of the electric field strength, the treatment time and the specific energy on the PEF sensitivity of both microorganisms in LWE. Fig. 3 shows the PEF treatments (electric field strength, treatment time and specific energy) necessary to achieve an inactivation of 3 Log_{10} cycles of *Salmonella* Typhimurium (thin lines) and *S. aureus* (thick lines) in LWE. The continuous lines correspond to PEF treatments at different field strengths and treatment times, and the dotted lines to combinations of field strength and specific energy. As observed, both microorganisms would have a similar PEF resistance at 30 kV/cm. Under 30 kV/cm, *S. aureus* resulted more PEF resistant than *Salmonella* Typhimurium. However, at electric field strengths higher than 30 kV/cm, *Salmonella* Typhimurium would be the most PEF resistant bacteria of the two investigated in LWE. Based on the results, treatments varying from 30 kV/cm, 61 μs and 386 kJ/kg to 45 kV/cm, 20 μs and 259 kJ/kg would be necessary to inactivate 3 Log_{10} cycles of both microorganisms in LWE.

3.2. Membrane damage of *Salmonella* Typhimurium and *S. aureus* cells following PEF treatments in LWE

After applying any inactivation treatment to a microbial population, some cells die, others survive and a certain percentage of the cells are sublethally injured. The induced damage in a microbial population after a treatment can be due to the loss of integrity and/or functionality of their cellular envelopments (Pagán & Mañas, 2006). The existence of sublethal injury after the treatment is usually determined by pour plating samples on two different media; a non-selective media with suitable conditions, where cells may return to its initial native state by repairing the cellular damage; and a selective media, where injured cells cannot be repaired, as a consequence, those cells die (Mackey, 2000). The membrane damage detected by this methodology can be considered as "sublethal damage". The proportion of sublethally damaged cells is determined by the difference on counts between cells recovered in a non-selective media and a selective media after the treatment. The selective media more commonly used to detect the membrane damage is agar with sodium chloride added (Mackey & Derrick, 1982).

Fig. 4 shows the Log_{10} cycles of sublethally damaged cells of *Salmonella* Typhimurium (Fig. 4A) and *S. aureus* (Fig. 4B) after PEF

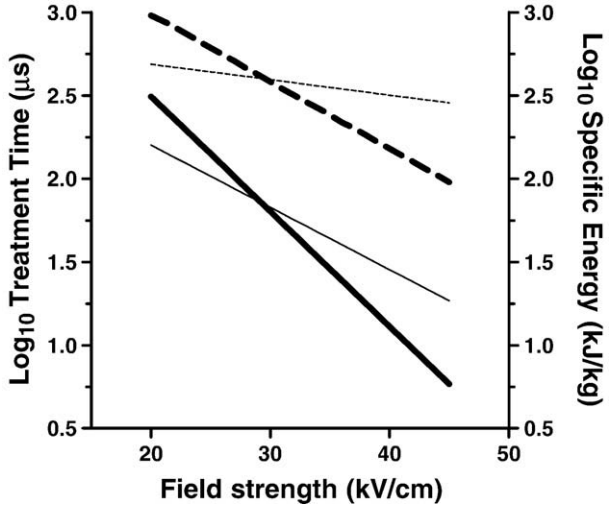

Fig. 3. Relationship between the electric field strength, the Log_{10} of the treatment time (continuous lines) and the specific energy (dotted line) necessary to inactivate 3 Log_{10} cycles of *Salmonella* Typhimurium (thin lines) and *S. aureus* (thick lines) by PEF treatments in LWE.

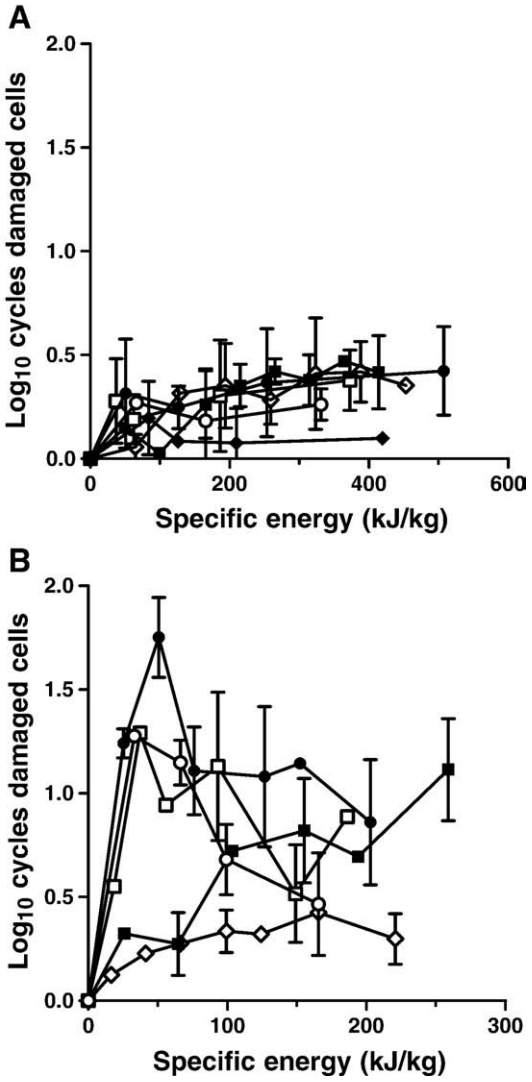

Fig. 4. Log_{10} cycles of sublethally damaged cells of *Salmonella* Typhimurium (A) and *S. aureus* (B) after PEF treatments at different field strengths and specific energies. 20 kV/cm (◊); 25 kV/cm (■); 30 kV/cm (□); 35 kV/cm (●); 40 kV/cm (○); 45 kV/cm (♦).

treatments at different electric field strengths and specific energies applied in LWE. As observed, hardly any damaged cells of the population of *Salmonella* Typhimurium were detected after the application of PEF treatments. In the best case scenario, the highest proportion of damaged cells observed was 0.5 Log_{10} cycles. In the case of *S. aureus*, approximately 1 Log_{10} cycle of sublethally injured cells was determined. At 20 and 25 kV/cm, the proportion of damaged cells increased with the specific energy applied and consequently with the treatment time. At higher field strengths, the level of cell damage also increases with the energy up to maximum values of around 50 kJ/kg. Over these values, the proportion of damaged cells decreased due to microbial inactivation increased (Fig. 2). The existence of sublethal damage indicates that a percentage of the *S. aureus* population treated in LWE shows alterations, probably reparable pores in the membrane, which induce its death under non suitable growth conditions.

4. Discussion

In this investigation, the lethal effectiveness of pulsed electric fields as a non-thermal technology to eliminate *Salmonella* Typhimurium and *S. aureus* in LWE has been investigated. In order to achieve this objective, a systematic study of the influence of the main PEF process parameters (electric field strength, treatment time and specific energy) on the inactivation of two pathogenic microorganisms present in LWE was carried out. Hardly any data related to the PEF sensitivity of *Salmonella* Typhimurium and *S. aureus* in LWE have been reported in literature.

As it is observed in Figs. 1 and 2, the inactivation of both microorganisms increased with the treatment time, the specific energy and the electric field strength. Maximum inactivation levels of 4 and 3 Log_{10} cycle of the population of *Salmonella* Typhimurium and *S. aureus* were achieved with treatments of 45 kV/cm, 30 μs and 419 kJ/kg, and 40 kV/cm for 15 μs and 166 kJ/kg, respectively. Based on published data, PEF treatments at intensities ranging from 30 to 56.7 kV/cm and 100 μs permitted to achieve a maximum inactivation level of 2 Log_{10} cycles of the population of *E. coli*, *L. innocua*, *Pseudomonas fluorescens*, and in the best situation 1 Log_{10} cycles of *Salmonella* Enteritidis treated in liquid egg (Calderon-Miranda, Barbosa-Canovas, & Swanson, 1999; Wouters, Dutreux, Smelt, & Lelieveld, 1999; Dutreux et al., 2000; McDonald, Lloyd, Vitale, Petersson, & Innings, 2000; Aronsson & Ronner, 2001; Gongora-Nieto et al., 2001; Amiali et al., 2004; Hermawan et al., 2004; Bazhal et al., 2006; Huang et al., 2006; San Martín et al., 2007). The apparent higher effectiveness of the treatments applied in this investigation could be due to square-wave-pulses were applied and the actual treatment conditions were measured in the treatment chamber. Since LWE has a relatively high electrical conductivity, it has a low electrical resistivity that reduces the pulse width and the peak voltage applied (Ho, Mittal, & Griffiths, 1995; Barbosa-Canovas, Gongora-Nieto, Pothakamury, & Swanson, 1999). Therefore, for the same PEF treatment, a higher voltage has to be selected, which increases the current, and more pulses have to be applied. Therefore, the use of exponential decay pulses could not provide a sufficient electrical potential gradient for the required length of time to cause ion migration to accumulate at the cell membrane (Huang, et al., 2006).

Since several factors have been considered in this study, it is essential to develop mathematical models which can help to evaluate the influence of each parameter and their interactions on the lethality of the investigated microorganisms (van Schothorst, 1998; McMeekin & Ross, 2002). In this investigation, it has been developed mathematical equations based on the Weibull distribution which have permitted to accurately fit all the survival curves, to compare the microbial PEF resistance in a wide range of treatment intensities, and to establish the treatment conditions (electric field strength, treatment time and specific energy) in order to achieve a certain level of safety in LWE. Although the mathematical equation based on the Weibull distribution has been widely used to described the non-exponential microbial inactivation by PEF in buffers and foods (Rodrigo, Ruíz, Barbosa-Cánovas, Martínez, & Rodrigo, 2003; Gomez, García, Álvarez, Raso, & Condón, 2005; Álvarez, Condón, & Raso, 2006; Rivas, Sampedro, Rodrigo, Martinez, & Rodrigo, 2006; Sampedro, Rivas, Rodrigo, Martinez, & Rodrigo, 2007; Pina-Pérez, Rodrigo-Aliaga, Ferrer-Bernat, Rodrigo-Enguidanos, & Martinez-López, 2007; Puértolas, López, Condón, Raso, & Álvarez, 2009), the use of this model to describe survival curves in LWE has not been reported. On the other hand, the inclusion of the specific energy as a parameter, together with the electric field strength, on the mathematical equations is of great interest in a product like LWE with a high value of its electrical conductivity. Specific energy is an integrated parameter that involves the influence of the electric field strength, the treatment time and the resistance of the treatment chamber (Heinz, et al., 1999). Therefore, the developed equations could be also useful to compare results obtained in different laboratories concerning inactivation of *Salmonella* Typhimurium and *S. aureus* in LWE by PEF.

Based on the results and the model predictions, *Salmonella* Typhimurium resulted more PEF resistant than *S. aureus* in LWE at electric field strengths higher than 30 kV/cm. Under this electric field strength, *S. aureus* should also be taken into consideration. The variation of the microorganism of reference to which refer the PEF treatments in LWE makes necessary more studies in order to compare the PEF resistance of other microorganisms, including other serotypes of *Salmonella* mainly *Salmonella* Enteritidis or the heat resistant *Salmonella* Senftenberg 775 W (Mañas, et al., 2003). This will permit to determine a reference microorganism for the design of possible hygienization industrial treatments of LWE by PEF. In any case, in order to achieve 3- Log_{10}-cycles-reduction of *Salmonella* Typhimurium in LWE treatments of 30 kV/cm–67 μs–393 kJ/kg and 45 kV/cm–19 μs–285 kJ/kg would be necessary to be applied (Fig. 3). Since the rate of the microbial inactivation increases with the electric field strength, the treatment of 45 kV/cm, 285 kJ/kg and 19 μs would be the most suitable from an energetic and time consuming point of view. According to Góngora-Nieto, Pedrow, Swanson and Barbosa-Cánovas (2003), a treatment of 30 kV/cm for 55 μs and 357 kJ/kg, which is in the range to those estimated in this investigation, enabled to extend the shelf-life of the LWE stabilized with 0.5% citric acid at 4 °C to 26 days. Although the treatment indicated would offer an adequate shelf life, the lethal effectiveness is insufficient to achieve a sanitary safe LWE.

Current thermal treatments (60 °C/3.5 min.; 64.4 °C/2.5 min.; 71 °C/1.5 min.) should provide 5- to 9-Log_{10} reductions in the populations of the most frequently isolated *Salmonella* serovars, such as Typhimurium and Enteritidis, but would attain less than a 4-Log_{10} reduction in the population of heat-resistant *Salmonella* strains such as Senftenberg 775 W (Shah, Bradshaw, & Peeler, 1991; Doyle & Mazzotta, 2000; Mañas et al., 2003; Álvarez, Niemira et al., 2006). To achieve the recommended levels of inactivation, more intensive PEF treatments than those used in this investigation would be necessary to be applied. However, based on our results and those of the literature, those more intensive treatments are not technological and energetically viable for the industry. More intense treatments by increasing the electric field strength or the number of pulses could produce the dielectric rupture in the LWE. On the other hand, due to the non-linear inactivation kinetics (Figs. 1 and 2), longer treatment times would require excessively high energy costs. According to our results and as it can be observed in Fig. 2A, treatments higher than 200–250 kJ/kg hardly increased the lethal effectiveness of the process but markedly augmented the energetic costs.

In order to increase the viability of this technology as a LWE hygienization system, it is necessary to improve the lethal effectiveness of PEF by combining it with other preservation technologies. The occurrence of sublethal damage after PEF treatments in LWE (Fig. 4) makes possible the combination of this technology with other

preservation methods, such as the addition of antimicrobial compounds, which could inactivate the injured cells, increasing the lethal effectiveness of PEF (Raso & Barbosa-Cánovas, 2003; Álvarez & Heinz, 2007). This combination would be of interest in the case of *S. aureus*, increasing the effectiveness of the process at least 1 Log_{10} cycle. However, in the case of *Salmonella* Typhimurium, this combination would be less efficient since a maximum value of 0.5 Log_{10} cycles of cell damage has been determined. In this case, the combination of PEF with antimicrobial compounds with activity on Gram negative bacteria or the application of thermal treatments at mild temperatures may be more interesting (Zhang & Mittal, 2005; Nguyen & Mittal, 2007; Mosqueda-Melgar, Raybaudi-Massilia, & Martin-Belloso, 2008; Somolinos, Garcia, Mañas, Condón, & Pagan, 2008; Pina-Pérez, Silva-Angulo, Rodrigo-Enguidanos, & Martínez-López, 2009). According to existing studies, the application of PEF treatments at temperatures of 50 °C increased the lethal effectiveness of PEF around 2 and 3 Log_{10} cycles (Hermawan et al., 2004; Bazhal et al., 2006). This increment of PEF lethality could be enough to guarantee the safe security of LWE treated by this technology. However, this point has to be evaluated.

In conclusion, the results obtained in this investigation indicate that pulsed electric fields is a promising technology to inactivate microorganisms in LWE. Based on the described kinetics of inactivation, treatments up to 250 kJ/kg applied at different electric field strengths resulted energetically suitable for PEF processing. However, the level of microbial inactivation achieved, 4 Log_{10} cycles of *Salmonella* Typhimurium after the most intensive treatments, is not sufficient to assure the safe security of the product. Therefore, intelligent combined processes based on the PEF technology are necessary to be established in order to increase the microbial PEF lethality or to reduce the intensity of PEF treatments to achieve a certain level of pathogenic bacteria inactivation.

Finally, the models developed in this study can be applied to engineering design, and for the evaluation and optimization of the PEF technology as a new technique to obtain *Salmonella* free LWE. Further studies are necessary to determine if the process and the models developed are also valid for other *Salmonella* serovars, to evaluate the impact of the process on the quality and functional properties of LWE, and to determine the possibility of extending the use of the hurdle technology in the LWE industry based on PEF-induced cell injury.

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References

Álvarez, I., & Heinz, V. (2007). Hurdle technology and the preservation of food by pulsed electric fields. In H. L. M. Lelieveld, S. Notermans, & S. W. H. de Haan (Eds.), *Food preservation by pulsed electric fieldsFrom research to application*. (pp. 165—177) Boca Raton: CRC Press.

Álvarez, I., Condón, S., & Raso, J. (2006). Microbial inactivation by pulsed electric fields. In J. Raso, & V. Heinz (Eds.), *Pulsed electric fields technology for the food industry: Fundamentals and applications* (pp. 97—129). New York: Springer.

Álvarez, I., Niemira, B. A., Fan, X., & Sommers, C. H. (2006). Inactivation of *Salmonella* serovars in liquid whole egg by heat following irradiation treatments. *Journal of Food Protection*, *69*(9), 2066—2074.

Amiali, M., Ngadi, M. O., Raghavan, V. G. S., & Smith, J. P. (2004). Inactivation of *Escherichia coli* O157:H7 in liquid dialyzed egg using pulsed electric fields. *Food and Bioproducts Processing*, *82*(C2), 151—156.

Aronsson, K., & Ronner, U. (2001). Influence of pH, water activity and temperature on the inactivation of *Escherichia coli* and *Saccharomyces cerevisiae* by pulsed electric fields. *Innovative Food Science & Emerging Technologies*, *2*, 105—112.

Baranyi, J., Pin, C., & Ross, T. (1999). Validating and comparing predictive models. *International Journal of Food Microbiology*, *48*, 159—166.

Barbosa-Canovas, G. V., Pierson, M. D., Zhang, Q. H., & Schaffner, D. W. (2001). Pulsed electric fields.*Journal of Food Science*, 65—79 Supplement.

Barbosa-Canovas, G. V., Gongora-Nieto, M., Pothakamury, U., & Swanson, B. (1999). Food processing by PEF. *Preservation of Foods with Pulsed Electric Fields (first ed.)* (pp. 156—171). San Diego, California: Academic Press.

Bazhal, M. I., Ngadi, M. O., Raghavan, G. S. V., & Smith, J. P. (2006). Inactivation of *Escherichia coli* O157:H7 in liquid whole egg using combined pulsed electric field and thermal treatments. *LWT — Food Science and Technology*, *39*, 420—426.

Calderon-Miranda, M. L., Barbosa-Canovas, G. V., & Swanson, B. G. (1999). Inactivation of *Listria innocua* in liquid whole egg by pulsed electric fields and nisin. *International Journal of Food Microbiology*, *51*, 7—17.

Cebrián, G., Sagarzazu, N., Pagán, 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. *International Journal of Food Microbiology*, *118*, 304—311.

Condón, S., Palop, A., Raso, J., & 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.

Doyle, M. E., & Mazzotta, S. (2000). Review of studies on the thermal resistance of *Salmonellae*. *Journal of Food Protection*, *63*(6), 779—795.

Dutreux, N., Notermans, S., Wijtzes, T., Gongora-Nieto, M. M., Barbosa-Canovas, G. V., & Swanson, B. G. (2000). 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.

Erickson, J. P., & Jenkins, P. (1992). Behaviour of psychrotropic pathogens *Listeria monocytogenes*, *Yersinia enterocolitica* and *Aeromonas hydrophila* in commercially pasteurized eggs held at 2, 6.7 and 12.8 °C. *Journal of Food Protection*, *55*, 8—12.

European Food Safety Authority, & European Centre for Diseases and Control (2009). The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents in the European Union in 2007.*The EFSA Journal*, *223* http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902269834.htm (Website last accessed: October 21, 2009).

Foegeding, P. M., & Stanley, N. W. (1987). Growth and inactivation of microorganisms isolated from ultrapasteurized egg. *Journal of Food Protection*, *52*(5), 1219—1227.

Gomez, N., García, D., Álvarez, I., Raso, J., & Condón, S. (2005). A model describing the kinetics of inactivation of *Lactobacillus plantarum* in a buffer system of different pH and in orange and apple juice. *International Journal Food Engineering*, *70*, 7—14.

Gongora-Nieto, M. M., Pedrow, P. D., Swanson, B. G., & Barbosa-Canovas, G. V. (2003). Energy analysis of liquid whole egg pasteurized by pulsed electric fields. *Journal of Food Engineering*, *57*, 209—216.

Gongora-Nieto, M. M., Seignour, L., Riquet, P., Davidson, P. M., Barbosa-Canovas, G. V., & Swanson, B. G. (2001). Nonthermal inactivation of *Pseudomonas fluorescens* in liquid whole egg. In G. V. Barbosa-Cánovas, & H. Zhang (Eds.), *Pulsed electric fields in food processing: fundamental aspects and applications* (pp. 193—210). Lancaster, P.A: Technomic Publishing.

Hamid-Samimi, M., Swartzel, K. R., & Ball, H. R. (1984). Flow behaviour of liquid whole egg during thermal treatments. *Journal of Food Science*, *49*, 132—136.

Heinz, V., Phillips, S. T., Zenker, M., & Knorr, D. (1999). Inactivation of *Bacillus subtilis* by high intensity pulsed electric fields under close to isothermal conditions. *Food Biotechnology*, *13*, 155—168.

Herald, T. J., & Smith, D. M. (1989). Functional properties and composition of liquid whole egg proteins as influenced by pasteurization and frozen storage. *Poultry Science*, *68*, 1461—1469.

Hermawan, N., Evrendilek, G. A., Dantzer, W. R., Zhang, Q. H., & Richter, E. R. (2004). Pulsed electric field treatment of liquid whole egg inoculated with *Salmonella* Enteritidis. *Journal of Food Safety*, *24*, 71—85.

Ho, S. Y., Mittal, G. S., & Griffiths, M. W. (1995). Inactivation of *Pseudomonas fluorescens* by high voltage electric pulses. *Journal of Food Science*, *60*(6), 1337—1343.

Huang, E., Mittal, G. S., & Griffiths, M. W. (2006). Inactivation of *Salmonella enteritidis* in liquid whole egg using combination treatments of pulsed electric field, high pressure and ultrasound. *Biosystems Engineering*, *94*, 403—413.

Jeantet, R., Baron, F., Nau, F., Roignant, M., & Brulé, G. (1999). High intensity pulsed electric fields applied to egg white: effect on *Salmonella enteritidis* inactivation and protein denaturation. *Journal of Food Protection*, *62*(12), 1381—1386.

Korolczuk, J., Mc Keag, J. R., Fernandez, J. C., Baron, F., Grosset, N., & Jeantet, R. (2006). Effect of pulsed electric field processing parameters on *Salmonella enteritidis* inactivation. *Journal of Food Engineering*, *75*(1), 11—20.

Lee, D. U., Heinz, V., & Knorr, D. (2003). Effects of combination treatments of nisin and high-intensity ultrasound with high pressure on the microbial inactivation in liquid whole egg. *Innovative Food Science and Emerging Technologies*, *4*, 387—393.

Mackey, B. M. (2000). Injured bacteria. In M. L. Lund, T. C. Baird-Parker, & G. W. Gould (Eds.), *The Microbiological Safety and Quality of Foods* (pp. 315—341). Gaithersburg: Aspen Publisher.

Mackey, B. M., & Derrick, C. M. (1982). The effect of sub-lethal injury by heating, freezing, drying and gamma-radiation on the duration of the lag phase of *Salmonella typhimurium*. *Journal of Applied Bacteriology*, *53*, 243—251.

Mafart, P., Couvert, O., Gaillard, S., & Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. *International Journal of Food Microbiology*, *72*, 107—113.

Mañas, P., Pagán, R., Álvarez, I., & Condón, S. (2003). Survival of *Salmonella senftenberg* 775 W to current liquid whole egg pasteurization treatments. *Food Microbiology*, *20*, 593—600.

Martin-Belloso, O., Vega-Mercado, H., Qin, B. L., Chang, F. J., Barbosa-Canovas, G. V., & Swanson, B. G. (1997). Inactivation of *Escherichia coli* suspended in liquid egg using pulsed electric fields. *Journal of Food Processing and Preservation*, *21*, 193—208.

McDonald, C. J., Lloyd, S. W., Vitale, M. A., Petersson, K., & Innings, F. (2000). Effects of pulsed electric fields on microorganisms in orange juice using electric field strengths of 30 and 50 kV/cm. *Journal of Food Science*, *65*, 984−989.

McMeekin, T. A., & Ross, T. (2002). Predictive microbiology: providing a knowledge-based framework for change management. *International Journal of Food Microbiology*, *78*, 133−153.

Mosqueda-Melgar, J., Raybaudi-Massilia, R. M., & Martin-Belloso, O. (2008). Combination of high-intensity pulsed electric fields with natural antimicrobials to inactivate pathogenic microorganisms and extend the shelf-life of melon and watermelon juices. *Food Microbiology*, *25*, 479−491.

Nguyen, P., & Mittal, G. S. (2007). Inactivation of naturally occurring microorganisms in tomato juice using pulsed electric field (PEF) with and without antimicrobials. *Chemical Engineering and Processing*, *46*, 360−365.

Pagán, R., & Mañas, P. (2006). Fundamental aspects of cell membrane electroporation. In J. Raso, & V. Heinz (Eds.), *Pulsed Electric Field Technology for the Food Industry: Fundamentals and Applications* (pp. 73−94). New York: Springer.

Pina-Pérez, M. C., Rodrigo-Aliaga, D., Ferrer-Bernat, C., Rodrigo-Enguidanos, M., & Martinez-López, A. (2007). Inactivation of *Enterobacter sakazakii* by pulsed electric field in buffered peptone water and infant formula milk. *International Dairy Journal*, *17*, 1441−1449.

Pina-Pérez, M. C., Silva-Angulo, A. B., Rodrigo-Enguidanos, D., & Martínez-López, A. (2009). Synergistic effect of Pulsed Electric Fields and CocoanOX 12% on the inactivation kinetics of *Bacillus cereus* in a mixed beverage of liquid whole egg and skim milk. *International Journal of Food Microbiology*, doi:10.1016/j.ijfoodmicro.2009.01.021.

Puértolas, E., López, N., Condón, S., Raso, J., & Álvarez, I. (2009). Pulsed electric fields inactivation of wine spoilage yeast and bacteria. *International Journal of Food Microbiology*, *130*(1), 49−55.

Raso, J., Álvarez, I., Condón, S., & Sala, F. J. (2000). Predicting inactivation of *Salmonella senftenberg* by pulsed electric fields. *Innovative Food Science & Emerging Technologies*, *1*, 21−30.

Raso, J., & Barbosa-Cánovas, G. V. (2003). Nonthermal preservation of foods using combined processing techniques. *Critical Reviews in Food Science and Nutrition (Critical Reviews in Food Science)*, *43*(3), 265−285.

Rivas, A., Sampedro, F., Rodrigo, D., Martinez, A., & Rodrigo, M. (2006). Nature of the inactivation of *Escherichia coli* suspended in an orange juice and milk beverage. *European Food Research and Technology*, *223*, 541−545.

Rodrigo, D., Ruíz, P., Barbosa-Cánovas, G. V., Martínez, A., & Rodrigo, M. (2003). Kinetic model for the inactivation of *Lactobacillus plantarum* by pulsed electric fields. *International Journal of Food Microbiology*, *81*, 223−229.

Saldaña, G., Puértolas, E., López, N., García, D., Álvarez, I., & Raso, J. (2009). Comparing the PEF resistance and occurrence of sublethal injury on different strains of *Escherichia coli*, *Salmonella* Typhimurium, *Listeria monocytogenes* and *Staphylococcus aureus* in media of pH 4 and 7. *Innovative Food Science and Emerging Technologies*, *10*, 160−165.

Sampedro, F., Rivas, A., Rodrigo, D., Martinez, A., & Rodrigo, M. (2007). Pulsed electric fields inactivation of *Lactobacillus plantarum* in an orange juice-milk based beverage: effect of process parameters. *Journal of Food Engineering*, *80*, 931−938.

San Martín, M. F., Sepúlveda, D. R., Altunakar, B., Góngora-Nieto, M. M., Swanson, B. G., & Barbosa-Cánovas, G. V. (2007). Evaluation of selected mathematical models to predict the inactivation of *Listeria innocua* by pulsed electric fields. *LWT — Food Science and Technology*, *40*(7), 1271−1279.

Shah, D. B., Bradshaw, J. G., & Peeler, J. T. (1991). Thermal resistance of egg-associated epidemic strains of *Salmonella enteritidis*. *Journal of Food Science*, *56*, 391−393.

Somolinos, M., García, D., Mañas, P., Condón, S., & Pagan, R. (2008). Effect of environmental factors and cell physiological state on Pulsed Electric Fields resistance and repair capacity of various strains of *Escherichia coli*. *International Journal of Food Microbiology*, *124*, 260−267.

van Schothorst, M. (1998). Principles for the establishment of microbiological food safety objectives and related control measures. *Food Control*, *9*(6), 379−384.

Wouters, P., Álvarez, I., & Raso, J. (2001). Critical factors determining inactivation kinetics by pulsed electric field food processing. *Trends Food Science & Technology*, *12*, 112−121.

Wouters, P. C., Dutreux, N., Smelt, J. P. P., & Lelieveld, H. L. M. (1999). Effects of pulsed electric fields on inactivation kinetics of *Listeria innocua*. *Applied Environmental Microbiology*, *65*(12), 5354−5371.

Zhang, Y., & Mittal, G. S. (2005). Inactivation of spoilage microorganisms in mango juice using low energy pulsed electric field in combination with antimicrobials. *Italian Journal of Food Science*, *17*, 167−176.