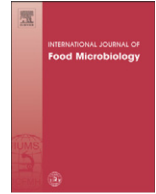
International Journal of Food Microbiology 145 (2011) 476–482

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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



Design of a combined process for the inactivation of *Salmonella* Enteritidis in liquid whole egg at 55 °C

S. Monfort, E. Gayán, S. Condón, J. Raso, I. Álvarez *

Tecnología de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, 50.013 Zaragoza, Spain

ARTICLE INFO

Article history:
Received 28 June 2010
Received in revised form 14 December 2010
Accepted 23 January 2011

Keywords:
Pulsed electric fields
LWE
Salmonella
Heat
Inactivation

ABSTRACT

This paper is an evaluation of the lethal effectiveness of a successive application of pulsed electric fields (PEFs) and heat treatment in liquid whole egg (LWE) in the presence of different additives on the population of *Salmonella* serovar Enteritidis. Synergistic reductions of the *Salmonella* Enteritidis population were observed when LWE samples containing additives were treated with PEF (25 kV/cm; 100 and 200 kJ/kg), heat (55 °C), or PEF followed by heat. The presence of additives, such as 10 mM EDTA or 2% triethyl citrate, increased the PEF lethality 1 $\log_{10}$ cycle and generated around 1.5 $\log_{10}$ cycles of cell damage, resulting in the reduction of undamaged cells of 4.4 and 3.1 $\log_{10}$ cycles, respectively. The application of PEF followed by heat treatment significantly ($p<0.05$) reduced $D_{55°C}$ from 3.9 ± 0.2 min in LWE to 1.40 ± 0.06 min or 0.24 ± 0.02 min in the presence of 10 mM EDTA or 2% triethyl citrate, respectively. A PEF treatment of 25 kV/cm and 200 kJ/kg followed by a heat treatment of 55 °C and 2 min reduced more than 8 $\log_{10}$ cycles of the population of *Salmonella* Enteritidis in LWE combined with 2% triethyl citrate, with a minimal impact on its protein soluble content. The heat sensitizing effect of PEF treatments in the presence of 2% triethyl citrate on the *Salmonella* population could enable LWE producers to reduce the temperature or processing time of thermal treatments (current standards are 60 °C for 3.5 min in the United States), increasing the level of *Salmonella* inactivation and retaining the quality of non-treated LWE.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Eggs and egg products are a nutritious part of our diet and a useful ingredient in foods due to their functional properties. Unfortunately, they are responsible for a large number of foodborne illnesses each year, constituting an obstacle to the well-being of populations and a source of high economic losses (Bufano, 2000).

Currently, the egg industry's primary method of improving the microbiological safety of liquid egg is thermal treatment. According to microbial heat resistance data in the literature, current liquid whole egg (LWE) heat pasteurization at 60 °C for 3.5 min (USA) or at 64 °C for 2.5 min (UK) should provide a reduction of 5–9 $\log_{10}$ cycles in the number of the most frequent *Salmonella* serotypes, *Salmonella* Typhimurium and *Salmonella* Enteritidis (Álvarez et al., 2006; D'Aoust et al., 1987; Doyle and Mazzotta, 2000; Humphrey, 1990; Mañas et al., 2003). However, the high thermal sensitivity of liquid egg components limits the intensity to which the product can be heated, since some soluble proteins begin to precipitate at temperatures as low as 57 °C (Hamid-Samimi et al., 1984; Herald and Smith, 1989). To retain the quality of liquid egg, nonthermal technologies, such as pulsed electric fields (PEFs), are under research for LWE pasteurization (Amiali et al., 2007; Geveke, 2008; Mañas et al., 2000; Ponce et al., 1999). PEF involves the application of short-duration pulses of high electric field strengths (10–50 kV/cm) to foods placed between two electrodes at ambient temperature (Barbosa-Cánovas et al., 2001). Several studies have reported successful PEF inactivation of pathogenic and food spoilage microorganisms, resulting in better retention of flavors and nutrients, as compared with heat-pasteurized products (Charles-Rodriguez et al., 2007; Mañas and Vercet, 2006).

PEF technology has been demonstrated to be effective in inactivating different microorganisms including distinct *Salmonella* serotypes, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, and *Listeria innocua* in LWE (Bazhal et al., 2006; Calderon-Miranda et al., 1999; Hermawan et al., 2004; Huang et al., 2006; Jin et al., 2009; Monfort et al., 2010b; Pina-Perez et al., 2009). However, the lethal effectiveness of PEF treatments at temperatures lower than 35 °C is far from the 5 $\log_{10}$ cycles of inactivation of *Salmonella* recommended by the USDA (USDA, 1969) and could be insufficient to guarantee the absence of *Salmonella* in 25 g or mL of LWE required by European regulation (Directive 89/107/CE). Therefore, PEF technology as a sole preservation method is not suitable for pasteurizing LWE (Monfort et al., 2010a).

The occurrence of cell damage due to PEF (García et al., 2005) has permitted to combine this technology with other preservation hurdles (antimicrobials, low pH, temperature, etc.) to increase the lethality of

* Corresponding author at: Tecnología de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet 177, 50.013 Zaragoza, Spain. Tel.: +34 976 76 26 75; fax: +34 976 76 15 90.
E-mail address: ialvalan@unizar.es (I. Álvarez).

0168-1605/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2011.01.027

treatments (Craven et al., 2008; Gallo et al., 2007; García et al., 2005; Huang et al., 2006; McNamee et al., 2010; Mosqueda-Melgar et al., 2008; Walkling-Ribeiro et al., 2009). The combination of PEF with antimicrobials or with temperature or the successive application of PEF and heat have been evaluated in LWE or egg whites (Amiali et al., 2007; Calderon-Miranda et al., 1999; Hermawan et al., 2004; Jin et al., 2009). Exposure of *L. innocua* to 100 IU/mL nisin following PEF resulted in 5.5 $\log_{10}$ cycles of inactivation for an electric filed intensity of 50 kV/cm and 32 pulses (Calderon-Miranda et al., 1999). At 30 kV/cm and 40 °C, the population of *E. coli* O157:H7 and *Salmonella* Enteritidis were reduced by 5 $\log_{10}$ cycles in liquid egg yolk (Amiali et al., 2006). Hermawan et al. (2004) reduced 4.3 $\log_{10}$ cycles of the *Salmonella* Enteritidis population by combining PEF (25 kV/cm; 250 μs) and heat (55 °C/3.5 min) in LWE. Although additive and synergistic lethal effects have been observed, the achieved level of inactivation could be insufficient to obtain *Salmonella*-free LWE.

The objective of this investigation was to design a more lethal effective combined process based on the application of PEF followed by heat treatment to LWE in the presence of different additives on the population of *Salmonella* serovar Enteritidis, one of the microorganisms responsible for most foodborne illnesses associated with the consumption of eggs and egg products (EFSA and ECDC, 2009).

The chemical compounds used in this investigation are recognized as GRAS (generally recognized as safe) in the United States and in the EU as additives. Some are additives classified as preservatives: potassium sorbate (E202), potassium benzoate (E213), nisin (E234), dimethyl dicarbonate (DMDC; E242), and lysozyme (E1105). Others are additives with supplementary properties. For example, disodium EDTA (E385) is used as a preservative or as an inhibitor of product discoloration (Helander et al., 1997), and triethyl citrate (E1505) is used in egg products to improve their whipping properties (Cho et al., 2009). Finally, the preservative ethyl lauroyl arginate (LAE) has also been used. It is considered GRAS in the United States but is still pending coding with an *E* number in the EU. The concentrations of the additives added to LWE in this investigation were the highest legally permitted in the United States or the EU or those usually used in the literature (Codex STAN-192-1995; Council Directive 89/107/CE; Real Decreto 142/2002; Rodrigez et al., 2004).

2. Material and methods

2.1. Preparation of LWE

Extra large Grade A eggs were purchased from a local supermarket. The eggshells were thoroughly washed with 70% ethanol and allowed to air dry. The sanitized eggs were aseptically broken and transferred to a sterile stomacher bag (Tekmar Co., Cincinnati, Ohio, USA) and homogenized for 2 min at 230 rpm in a stomacher laboratory blender (model 400, Tekmar Co., Cincinnati, Ohio, USA). The obtained LWE was centrifuged at 102 × *g* for 2 min (centrifuge Heraeus, model Megafuge 1.0 R) to eliminate air and maintained at 2–4 °C until ready for use. The pH of LWE was 7.5 ± 0.3, and its electric conductivity, 0.67 ± 0.03 S/m.

2.2. Preparation of LWE with additives

The additives used were nisin, potassium sorbate, potassium benzoate, DMDC, LAE, disodium EDTA, triethyl citrate, and lysozyme. Nisin, DMDC, and lysozyme were supplied by Sigma (Sigma-Aldrich, St. Louis, MO); potassium sorbate, potassium benzoate, and EDTA by PANREAC (Panreac Química Sau, Spain); LAE by LAMIRSA (Miret Laboratories, S.A, Spain); and triethyl citrate by Merck (Merck KGaA, Germany). Additives were added directly to 20 mL of LWE to obtain the corresponding final concentrations. The concentrations used in this study were 1000 IU/mL nisin, 4000 ppm potassium sorbate, 4000 ppm potassium benzoate, 1000 ppm DMDC, 200 ppm LAE, 20 mM EDTA, 4% triethyl citrate, and 4000 ppm lysozyme. The 20 mL-LWE flasks, with additives, were kept at 4 °C until use (maximum storage time, 1 h). The additives did not alter the pH of LWE.

2.3. Microorganisms

The strain of *Salmonella* Enteritidis (STCC 7300) used in this investigation was supplied by Spanish Type Culture Collection (STCC). The broth subculture was prepared as previously described Monfort et al. (2010b), in flasks containing 50 mL of tryptic soy broth (Biolife, Milan, Italy) plus 0.6% (w/v) of yeast extract (Biolife) (TSBYE) inoculated to an initial concentration of approximately 10^6 CFU/mL. The cultures were incubated under agitation (135 rpm) (mod. Rotabit, Selecta, Spain) at 37 °C for 24 h until the stationary growth phase was reached (Álvarez et al., 2003). Before treatment, bacterial suspensions were centrifuged at 6000 × *g* for 5 min at 4 °C and re-suspended in 1 mL of LWE with the corresponding additive at a concentration of approximately 10^9 CFU/mL. LWE with additives and inoculated with *Salmonella* Enteritidis was stored at 4 °C until use (maximum storage time, 1 h). The additives at the added concentrations did not affect the number of survivors nor cell damage was detected during the storage time at 4 °C (data not shown).

2.4. PEF, heat, and PEF followed by heat treatments

0.3 mL of LWE with the corresponding additive and 10^9 CFU/mL of the bacterial suspension was placed in the PEF treatment chamber, as previously described (Monfort et al., 2010b). The PEF equipment used in this investigation was previously described by Saldaña et al. (2010). Microorganisms were treated in a tempered batch parallel-electrode treatment chamber (15.0 ± 0.1 °C) with a distance between electrodes of 0.4 cm and an area of 0.79 cm^2. 3 μs-square waveform pulses were applied at 0.5 Hz. The temperature of the LWE, measured as previously described Raso et al. (2000), was always lower than 35 °C. The energy per pulse (W') was calculated using 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 applied (kJ) was calculated by multiplying the energy per pulse (W') by the number of pulses. The total specific energy (W) applied (expressed in kJ/kg) was determined by dividing the total energy by the mass of treated LWE. Electric field strengths of 25 kV/cm and specific energies of 100 (8 pulses) and 200 kJ/kg (16 pulses) were applied. A previous investigation showed that 25 kV/cm and 200 kJ/kg was the most lethal effective PEF treatment in LWE to inactivate different *Salmonella* serovars with the lowest energetic consumption (Monfort et al., 2010a).

For PEF followed by heat treatments (PEF-heat treatment), 0.07 mL of PEF-treated LWE in the presence of an additive was added to 0.63 mL of LWE in tubes containing the same additive and previously stabilized in an incubator (FX Incubator, A.F. Ingenieria S.L., Valencia, Spain) at 55 ± 0.2 °C. The actual temperature was controlled by using a thermocouple wire introduced in a 0.7 mL LWE test tube inside the incubator. At preset time intervals, 0.1 mL samples were taken for the heat resistance determinations and immediately diluted in 0.1% peptone water (Biolife). The maximum time between PEF and heat treatments was approximately 5 min. This period did not affect the number of survivors of *Salmonella* Enteritidis after PEF treatments (data not shown).

2.5. Sampling

0.1-mL aliquots of samples not treated and treated with PEF, heat, or PEF followed by heat were removed and serially diluted with sterile

0.1% peptone water 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. To quantify the number of injured cells, samples were also plated into TSAYE containing the maximum non-inhibitory concentration of sodium chloride for this *Salmonella* serovar, 3% (TSAYE + NaCl). Plates with TSAYE + NaCl (selective media) were incubated at 37 °C for 48 h. The existence of sublethal injury after the treatment is determined by pour plating samples on two different media; a non-selective media with suitable conditions, where cells may return to their native state by repairing the cellular damage; and a selective media, where injured cells cannot be repaired, as consequence, those cells die (Mackey, 2000). The selective media used to detect membrane damage is agar with sodium chloride added (Mackey and Derrick, 1982).

After incubation, colonies were counted with an image analyzer automatic counter (Protos, Analytical Measuring Systems, Cambridge, UK), as previously described by Condón et al. (1996). Each experiment was performed at least three times on separate days. Average results are presented below.

2.6. Percentage of soluble protein content of LWE

The method described by Hamid-Samimi et al. (1984) was used. 1 mL aliquots of treated and non-treated LWE were diluted in 100 mL of 1% NaCl water solution. The solutions were centrifuged at 27,000 × *g* for 60 min at 0–5 °C (Sorvall, model RC 28S, DuPont Instruments, Wilmington, USA). Filtered supernatant was diluted in water with a 2.5% SDS solution in a proportion of 7 (SDS):3 (water) (v/v). Absorbance was measured at 280 nm in a Unicam UV500 spectrophotometer (Unicam Limited, Cambridge, UK). The percentage of soluble protein content of treated LWE was expressed in terms of the non-treated LWE.

2.7. PEF and heat resistance parameters

Plate counts of the treated samples were divided by the control plate counts to obtain the survival fraction. The $\log_{10}$ of the survival fraction was then used to define the lethality of PEF treatments. The lethality of heat treatments was measured by their decimal reduction time value (D_t value), which is defined as minutes of treatment at a given temperature for the number of survivors to drop 1 $\log_{10}$ cycle. D_t values were calculated from the slope of the regression line of the straight portion of the survival curve obtained at every treatment temperature by plotting the $\log_{10}$ of the survival fraction versus treatment time. PEF lethality and D_t values, and their corresponding 95% confidence limits, were calculated with GraphPad PRISM® (GraphPad Software, Inc., San Diego, CA). Significant differences between PEF lethality and thermal D_t values, obtained respectively from PEF treatments and heating survival curves, were determined by ANOVA tests with GraphPad PRISM®.

3. Results

3.1. PEF treatments

The PEF resistance of *Salmonella* Enteritidis was determined for each additive used in the study after treatment of 25 kV/cm and two energetic levels, 100 and 200 kJ/kg (Fig. 1). Fig. 1 also illustrates the PEF inactivation when microorganisms were recovered in selective media. The presence of additives increased PEF lethality. This increment of the PEF lethal efficiency was higher at 100 (Fig. 1a) than at 200 kJ/kg (Fig. 1b). At 100 kJ/kg, the highest increase of PEF lethality was 1.1 $\log_{10}$ cycles in the presence of 20 mM EDTA, achieving a maximum inactivation of 2.2 $\log_{10}$ cycles. In general, the application of 25 kV/cm at 200 kJ/kg increased the lethality of *Salmonella* Enteritidis 0.5 ± 0.4 $\log_{10}$ cycle when no additives were used and 0.7 ± 0.2 when additives were added to LWE. Specifically, in the presence of additives such as DMDC, nisin, triethyl citrate, and EDTA, the lethality was 2.4, 2.6, 2.7 and 2.6 $\log_{10}$ cycles, respectively. When *Salmonella* Enteritidis was recovered in selective media after PEF treatments at 100 and 200 kJ/kg, significant cell damage ($P < 0.05$) was detected with the addition of triethyl citrate or EDTA to LWE. In the presence of these additives, around 90% of the survivors were damaged after PEF treatments. The application of more intensive PEF treatments (35 kV/cm at 100 or 200 kJ/kg) did not significantly increase PEF lethality or damage suffered by *Salmonella* Enteritidis in LWE with additives (data not shown). Based on the obtained results, the presence of 4% of triethyl citrate or 20 mM of EDTA in LWE increased the PEF lethality of *Salmonella* Enteritidis.

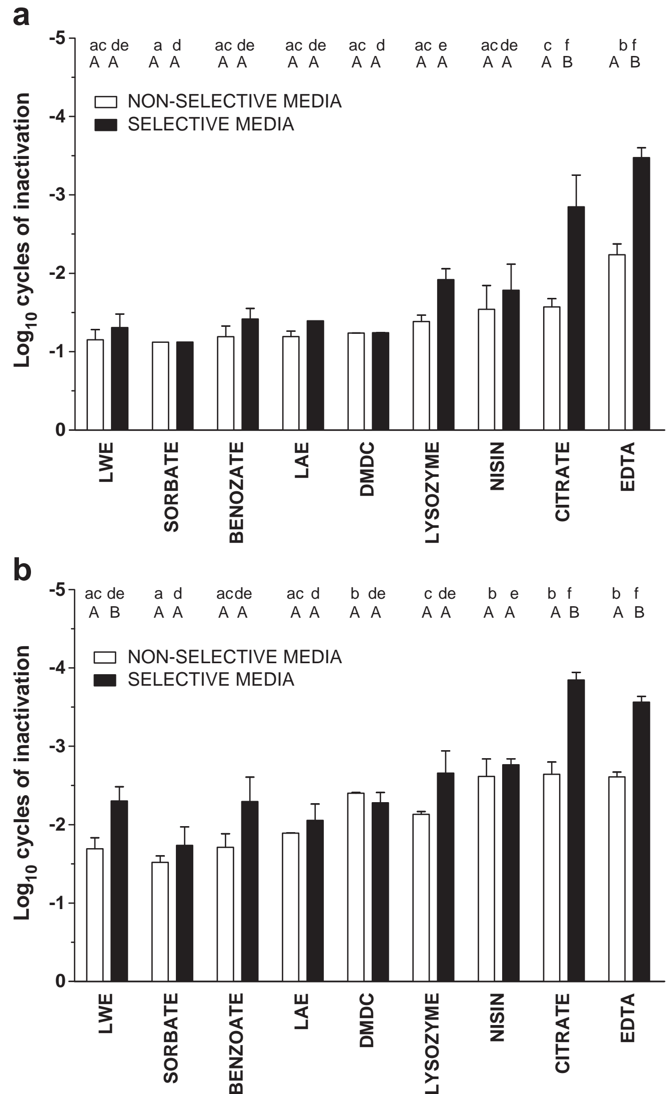


Fig. 1. $\log_{10}$ reductions of *Salmonella* Enteritidis treated in LWE with different additives by PEF treatments of 25 kV/cm and 100 kJ/kg (1a) or 25 kV/cm and 200 kJ/Kg (1b) and recovered in non-selective (white bars) and selective media (black bars). The 95% confidence limits are shown as error bars. Bars with different letters were significantly different ($P < 0.05$). "a, b, c" letters compare significant differences among additives when bacteria are recovered in non-selective media (white bars); "d, e, f" letters compare significant differences among additives when bacteria are recovered in selective media (black bars); "A, B" letters indicate significant differences for the same additive comparing the level of inactivation in selective and non-selective media.

In order to evaluate the influence of the concentration of the additives on PEF lethality, Fig. 2 shows the inactivation and cell damage of *Salmonella* Enteritidis treated at 25 kV/cm and 200 kJ/kg with different concentrations of triethyl citrate and EDTA. In the case

of triethyl citrate (Fig. 2a), PEF lethality increased significantly at concentrations of 2%, and the same level of inactivation was observed at 2% and 4% (around 2.6 $\log_{10}$ cycles). The number of damaged cells due to PEF treatment (around 0.7 $\log_{10}$ cycle) did not vary from 0% to 2% of triethyl citrate. At 4%, 1.2 $\log_{10}$ cycles of damaged cells were observed. Based on these results, concentrations of 4% of triethyl citrate should be used to achieve maximum lethality and cell damage.

When EDTA was added to LWE (Fig. 2b), PEF lethality increased with concentrations up to 5 mM EDTA, achieving 2.7 $\log_{10}$ cycles of inactivation. However, the level of inactivation did not increase significantly. For a concentration of 10 mM EDTA, the number of damaged cells reached a maximum of 1.7 $\log_{10}$ cycles. At this concentration, a 4.4 $\log_{10}$ cycles of reduction of undamaged cells could be achieved. Based on these results, 10 mM EDTA was selected for the following investigation.

3.2. PEF followed by heat treatments

Fig. 3 shows the survival curves corresponding to heat treatments at 55 °C of *Salmonella* Enteritidis in LWE previously treated by PEF (25 kV/cm, 200 kJ/kg) without additives and in presence of 2% triethyl citrate or

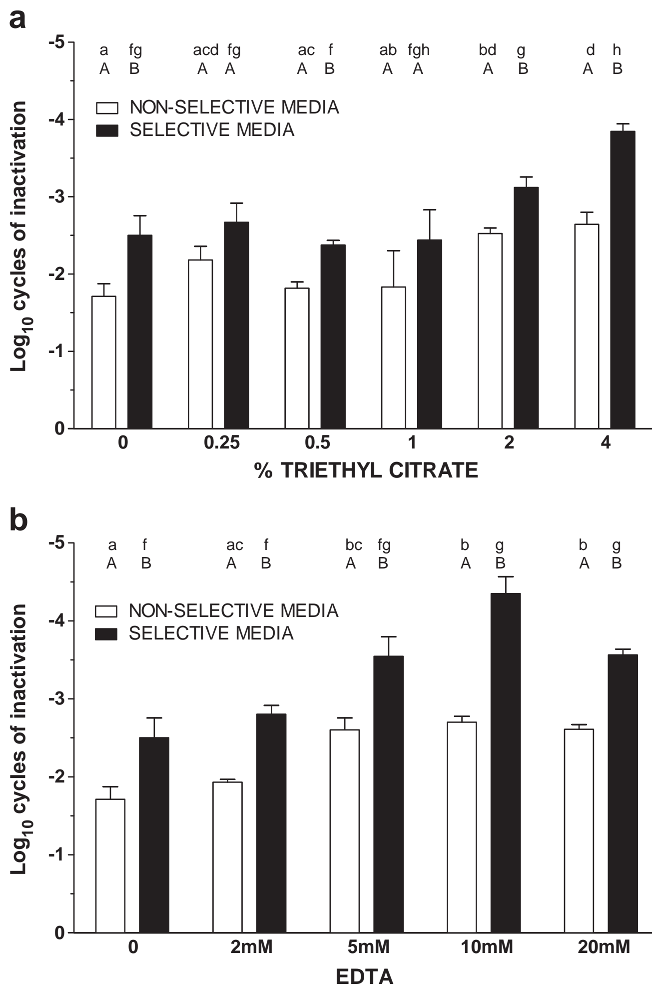


Fig. 2. Log_{10} reductions of *Salmonella* Enteritidis treated in LWE in presence of different concentrations of triethyl citrate (2a) or EDTA (2b) by PEF treatments of 25 kV/cm and 200 kJ/Kg (1b) and recovered in non-selective (white bars) and selective media (black bars). The 95% confidence limits are shown as error bars. Bars with different letters were significantly different ($P<0.05$). "a, b, c, d" letters compare significant differences among additives when bacteria are recovered in non-selective media (white bars); "f, g, h" letters compare significant differences among additives when bacteria are recovered in selective media (black bars); "A, B" letters indicate significant differences for the same additive comparing the level of inactivation in selective and non-selective media.

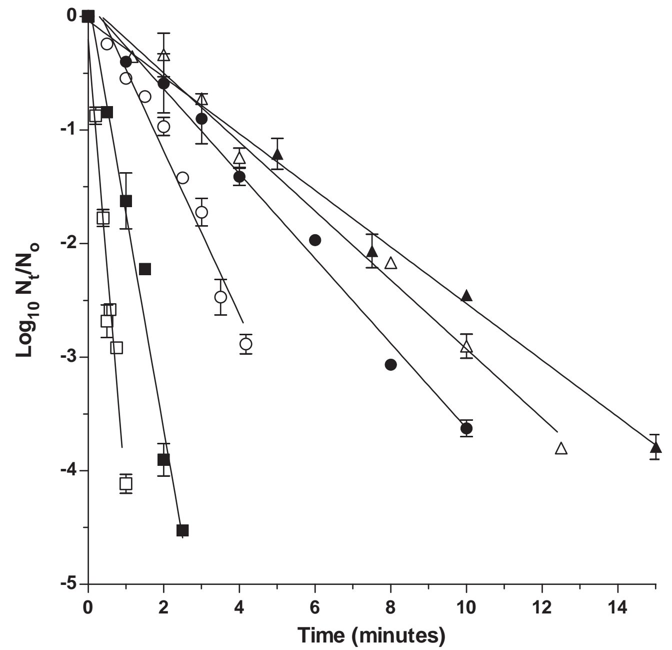


Fig. 3. Survival curves of *Salmonella* Enteritidis in LWE at 55 °C after different additive-PEF-heat treatments. Treatments: (▲) heat; (●) EDTA-heat; (■) citrate-heat; (△) PEF-heat; (○) EDTA-PEF-heat; (□) citrate-PEF-heat. Additives: 10 mM EDTA; 2% triethyl citrate. PEF treatment: 25 kV/cm; 200 kJ/kg.

10 mM EDTA. In order to compare results, survival curves at 55 °C in the presence and absence of these compounds have been included. The concentration of triethyl citrate was reduced from 4% to 2%, since small egg precipitations were observed in LWE after 5 min of treatment at 55 °C. At 2%, no precipitation was observed during the heat treatments. As can be observed in Fig. 3, the survival curves were linear for all the treatments investigated, which allowed the corresponding $D_{55°C}$ values to be calculated to characterize the heat resistance. The application of previous PEF treatments or the presence of additives increased the heat sensitivity of *Salmonella* Enteritidis in LWE. The $D_{55°C}$ values corresponding to the heat treatment in the presence of EDTA or citrate were 2.69 ± 0.01 min and 0.53 ± 0.01 min, respectively. The application of PEF treatments before heating slightly but significantly ($p<0.05$) reduced $D_{55°C}$ from 3.89 ± 0.19 min to 3.28 ± 0.18 min. When 10 mM EDTA or 2% triethyl citrate was added to LWE, the application of PEF followed by the heat treatment reduced the $D_{55°C}$ values to 1.40 ± 0.06 min and 0.24 ± 0.02 min, respectively.

Table 1 shows the $D_{55°C}$ values obtained from the survival curves of *Salmonella* Enteritidis of Fig. 3 treated by heat or heat after a PEF treatment (25 kV/cm; 200 kJ/kg) in LWE with and without additives. Table 1 also includes the $\log_{10}$ reductions of the population of *Salmonella* Enteritidis after PEF (25 kV/cm; 200 kJ/kg), heat (55 °C; 2 min) or PEF followed by heat treatments in presence or absence of additives. As observed, the application of PEF treatments significantly reduced the $D_{55°C}$ values of *Salmonella* Enteritidis independently of the presence of additives in LWE. The increment of heat sensitivity due to PEF was higher when additives were added to LWE, especially in the presence of triethyl citrate. Based on these $D_{55°C}$ values and considering a treatment of 2 min at 55 °C, lethality was always higher when a previous PEF treatment was applied and total inactivation was always larger in the additive-PEF-heat treatments than the sum of the individual treatments. This indicates that there is a synergistic lethal effect when additive-PEF-heat treatment is applied. A total inactivation of 2.3, 4.2 and 10.5 $\log_{10}$ cycles of *Salmonella* Enteritidis would be obtained after PEF followed by heat treatments when no additives, EDTA or citrate were used, respectively (in Table 1, the total inactivation obtained with the PEF followed by heat treatment was calculated as the addition of $\log_{10}$

Table 1
D_t values (expressed in min) of *Salmonella* Enteritidis treated by heat at 55 °C with and without a previous PEF treatment in LWE with and without additives. Log_{10} reductions of *Salmonella* Enteritidis treated by PEF, heat (55 °C; 2 min), and PEF followed by heat treatment (55 °C; 2 min) in LWE with and without additives. Additives: 10 mM and 2% triethyl citrate. PEF treatment: 25 kV/cm; 200 kJ/kg. $D_{55°C}$ values with different letters in the exponent (a, b) were significantly different (P<0.05) (comparisons have been done between treatments: heat *vs* heat after PEF).

	No additive		EDTA		Citrate	
	$D_{55°C}$ values (min)	Log_{10} reductions	$D_{55°C}$ values (min)	Log_{10} reductions	$D_{55°C}$ values (min)	Log_{10} reductions
PEF	–	1.7	–	2.7	–	2.2
Heat	3.89 ± 0.19^a	0.5	2.69 ± 0.01^a	0.7	0.53 ± 0.01^a	3.8
Heat (after PEF)	3.28 ± 0.18^b	0.6	1.40 ± 0.06^b	1.5	0.24 ± 0.02^b	8.3
Total PEF followed by heat	–	2.3	–	4.2	–	10.5

reductions of "PEF" and "heat after PEF" rows). These levels of inactivation were larger than the sum of the lethalities of the individual treatments (addition of log_{10} cycles of inactivation of "PEF" and "heat" rows of Table 1): 2.2 (1.7 log_{10} reductions due to PEF plus 0.5 log_{10} reductions due to heat) when no additives were used, 3.4 (2.7 due to PEF plus 0.7 due to heat) for EDTA, and 6.0 log_{10} reductions (2.2 due to PEF plus 3.8 due to heat) for citrate. The inactivation shown in table 1 for each additive-PEF-heat treatment was validated by applying the corresponding treatment to LWE inoculated with 10^9 CFU/mL of *Salmonella* Enteritidis. A PEF treatment (200 kJ/kg) followed by heat (55 °C, 2 min) allowed to inactivate 2.0 ± 0.3 and 3.8 ± 0.3 log_{10} cycles, in absence of additive or in presence of 10 mM EDTA, respectively. For the citrate-PEF-heat treatment, survivors were not detected afterward (less than 10 CFU/mL).

In order to evaluate the impact of the treatment on the quality of LWE, the soluble protein contents of non-treated LWE, LWE with 2% of triethyl citrate treated by PEF (25 kV/cm; 200 kJ/kg) followed by heat (55 °C; 2 min) and LWE treated with the current heat pasteurization treatments were determined. Soluble protein content is a technique used to evaluate the protein coagulation level due to processing (Herald and Smith, 1989). The absorbance of non-treated and citrate-PEF-heat treated LWE was 0.772 ± 0.025 and 0.763 ± 0.026, respectively. In other words, the soluble protein content was reduced only by 1%, indicating that the quality of LWE was apparently unaffected. Compared to current heat pasteurization treatments, the absorbance of LWE treated at 60 °C/3.5 min or 64 °C/2.5 min was 0.736 ± 0.001 and 0.578 ± 0.001, respectively, that is 6 and 25% reduction of the soluble protein content compared to non-treated LWE.

4. Discussion

PEF technology is under investigation as an alternative to LWE heat pasteurization, since it can inactivate *Salmonella* serovars at room temperature. However, the achieved level of inactivation with PEF is far from that obtained with heat pasteurization (60 °C/3.5 min; 64 °C/2.5 min), a 5–9 log_{10} cycles reduction in the number of the most frequent *Salmonella* serotypes (Monfort et al., 2010a). In order to increase PEF lethality, PEF treatments must be combined with other treatments, or hurdles. The hurdle approach is used to produce minimally processed food by applying several sub-lethal treatments to achieve microbial stability rather than focusing solely on one lethal preservation method (Leistner, 2000). Microbial stability is achieved by combining these hurdles to increase the destruction of microorganisms as well as preventing the cell repair of survivors (McNamee et al., 2010). The total preservation effect of combining several preservation factors can be merely additive, but in terms of food quality and safety, a synergistic effect is preferable (Raso et al., 2005). An additive effect occurs when the combined effect of two or more preservation factors is equal to the sum of the effect of each factor acting individually, whereas a synergistic effect occurs when the combined effect is greater than the sum of the effect of each factor (Raso et al., 2005). This investigation has explored the lethal effectiveness of a successive application of PEF and heat treatment in LWE in the presence of different additives on the population of *Salmonella* serovar Enteritidis.

For LWE, the lethal effect of PEF in combination with antimicrobials (nisin, citric acid, and CocoanOX) has been shown to be very effective (up to 5 log_{10} cycles) in the literature; however, these results are only related to Gram positive bacteria (*L. innocua*, *Micrococcus luteus*, and *Bacillus cereus*). No data have yet been provided on the effect of PEF in the presence of additives in Gram negative bacteria (Calderon-Miranda et al., 1999; Dutreux et al., 2000; Góngora-Nieto et al., 2003; Pina-Perez et al., 2009). In this investigation, the application of PEF treatments to LWE in the presence of different additives has shown an increase in PEF lethality of up to 1 log_{10} cycle in the best-case scenario (1000 IU/mL nisin, 2% triethyl citrate, or 5 mM EDTA), allowing for a total reduction of 2.6 ± 0.3 log_{10} in the population of *Salmonella* Enteritidis (Figs. 1 and 2). The application of PEF treatments in the presence of additives has also resulted in the occurrence of cell damage, mainly in presence of 4% triethyl citrate or 10 mM EDTA. The occurrence of sublethal damage after PEF treatments makes possible the combination of this technology with other preservation methods, such as cold storage of the treated product. García et al. (2005) observed that PEF injured cells died during a subsequent storage under refrigeration in the treatment medium. In LWE treated by PEF in presence of EDTA or triethyl citrate, cold storage could permit the inactivation of damaged cells. Furthermore, LWE contains natural antimicrobials which could contribute to inactivate injured cells. Although the outer membrane of Gram negative bacteria is an effective barrier against external agents, antimicrobial agents can significantly weaken it. This is commonly seen in the example of EDTA, a chelating agent that disintegrates membranes by monopolizing divalent cations (Ca^{2+} and Mg^{2+}) that stabilize the cytoplasmic membrane (Nohynek et al., 2006). Since the cytoplasmic membrane is the target of PEF treatments (Mañas and Pagan, 2005), the presence of EDTA in the treatment medium would explain the higher PEF sensitivity of *Salmonella* Enteritidis and the occurrence of cell damage observed in this investigation (Fig. 2B). In the case of triethyl citrate, researchers have never described the antimicrobial activity of this compound, which is used in egg products to improve their whipping properties (Cho et al., 2009). Since triethyl citrate is an ester of citric acid, its mechanisms of action could be related to that of citric acid, which can bind metal ions like Ca^{2+} and Mg^{2+} (Brul and Coote, 1999). Therefore, triethyl citrate could act similarly to EDTA. However, other mechanisms could be involved, since triethyl citrate decreased the heat resistance to a larger extent than EDTA (Fig. 3). In any case, the mechanism of action of triethyl citrate is a point that would require deeper study based on the potential of this compound as a sensitizing agent against heat or PEF treatments.

Based on the lethal effectiveness of PEF in presence of any of the investigated additives on the population of *Salmonella* Enteritidis inoculated in LWE shown in Figs. 1 and 2, PEF technology as a sole preservation method is not sufficient to achieve a performance criterion of 5 log_{10} cycles reduction of *Salmonella* Enteritidis in LWE, which, considering all possible temperature/time situations in practice, would not be sufficient to guarantee *Salmonella*-free LWE.

However, the occurrence of cell damage justifies the application of PEF in the presence of additives with other preservation technologies.

In this investigation, the application of PEF followed by heat showed a higher lethality of *Salmonella* Enteritidis in LWE than the sum of the lethalities of the individual treatments in the presence or absence of additives (Table 1). In the absence of citrate or EDTA, a slightly synergistic lethal effect occurs (Fig. 3). However, the addition of 10 mM EDTA and especially 2% triethyl citrate markedly increased this synergistic effect (Figs. 3 and Table 1). In the literature, the application of PEF at temperatures up to 60 °C, PEF followed by heat, or heat followed by PEF treatments was investigated in LWE or egg products in order to inactivate Gram negative bacteria (Amiali et al., 2007; Bazhal et al., 2006; Hermawan et al., 2004; Huang et al., 2006; Jin et al., 2009). In these studies, inactivations of 4 $\log_{10}$ cycles in LWE and 5 $\log_{10}$ cycles in egg yolks were obtained for *Salmonella* Enteritidis or *E. coli* 0157:H7, increasing the lethality of individual treatments from 1 to 2 $\log_{10}$ cycles. The authors proposed that the reason behind the synergistic lethal effect of PEF followed by heat would be that microbial cells underwent sublethal injury during the PEF treatments, thereby becoming more sensitive to heat treatments (Jin et al., 2009). In the present investigation, this hypothesis was confirmed. In absence of additives, 0.5 $\log_{10}$ cycle of damaged cells due to PEF were observed (Fig. 1B). This small amount of cell damage would explain the slightly synergistic lethal effect of the PEF-heat treatment (Fig. 3 and Table 1). However, when 10 mM EDTA was added, a larger number of cells were damaged, which resulted in a higher synergistic lethal effect of the PEF-heat treatment (Fig. 3 and Table 1). In the case of 2% triethyl citrate, the reduction of *Salmonella* Enteritidis viability after the PEF-heat treatment could be due to not only the inactivation of PEF damaged cells but also the increased sensitivity of *Salmonella* Enteritidis to heat in the presence of triethyl citrate.

Based on the obtained results, the application of PEF followed by heat treatments in the presence of 2% triethyl citrate would make obtaining *Salmonella*-free LWE possible. In the presence of 2% triethyl citrate, the application of 25 kV/cm and 200 kJ/kg followed by a heat treatment of 55 °C/2 min caused a reduction of more than 8 $\log_{10}$ cycles of *Salmonella* Enteritidis (Table 1), which is in the range of the *Salmonella* reduction achieved with current heat pasteurization at 60 °C/3.5 min (Álvarez et al., 2006). Therefore, the heat sensitizing effect of PEF in the presence of 2% triethyl citrate has permitted a reduction in temperature and heating time from 60 °C/3.5 min to 55 °C/2 min. From a technological standpoint, the reduction in temperature and heating time would be beneficial to maintain the functional properties of treated LWE similarly to non-treated LWE. This has been confirmed by the results obtained for soluble protein content, indicating a reduction of only 1% of the citrate-PEF-heat LWE with respect to non-treated LWE. Compared to current heat pasteurization treatments, 6 and 25% reduction of the soluble protein content of LWE was observed when LWE was treated at 60 °C/3.5 min or 64 °C/2.5 min, respectively.

A synergistic lethal effect has been observed on *Salmonella* Enteritidis in LWE when applying PEF followed by heat in the presence of additives such as EDTA and triethyl citrate. Based on the heat sensitizing effect of PEF in the presence of additives, the application of PEF followed by heat to LWE with 2% triethyl citrate added has been proposed as a possible alternative to heat pasteurization to obtain *Salmonella*-free LWE. Specifically, the application of a PEF treatment of 25 kV/cm and 200 kJ/kg followed by a heat treatment of 55 °C/2 min in the presence of 2% triethyl citrate permitted to reduce more than 8 $\log_{10}$ of *Salmonella* Enteritidis in LWE with a minimal impact on the protein soluble content. Although the results are very promising, more research is necessary in order to optimize the combined process based on the mechanisms involved in the synergistic lethal effect of PEF, heat, and additives to determine the lethal efficiency of the process on other *Salmonella* serotypes and to evaluate the impact of the designed process on the functional properties of LWE.

Acknowledgments

The authors thank CICYT (project AGL 2007-62738) for the support. S.M. and G.S. gratefully acknowledge the financial support for their doctoral studies from the Department of Science, Technology and University of the Aragon Government and the Spanish Ministry for Education and Science.

References

Álvarez, I., Mañas, P., Sala, F.J., Condón, S., 2003. Inactivation of *Salmonella enteritidis* by ultrasonic waves under pressure at different water activities. Applied and Environmental Microbiology 69 (1), 668–672.

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., Smith, J.P., Raghavan, G.S.V., 2007. Synergistic effect of temperature and pulsed electric field on inactivation of *Escherichia coli* O157:H7 and *Salmonella enteritidis* in liquid egg yolk. Journal of Food Engineering 79, 689–694.

Amiali, M., Ngadi, M.O., Smith, J.P., Raghavan, G.S.V., 2006. Inactivation of *Escherichia coli* O157: H7 and *Salmonella enteritidis* in liquid egg white using pulsed electric field. Journal of Food Science 71, M88–M94.

Barbosa-Cánovas, G.V., Pierson, M.D., Zhang, Q.H., Schaffner, D.W., 2001. Pulsed electric fields. Journal of Food Science 65–79 Supplement.

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, 419–425.

Brul, S., Coote, P., 1999. Preservative agents in foods—mode of action and microbial resistance mechanisms. International Journal of Food Microbiology 50, 1–17.

Bufano, N.S., 2000. Keeping eggs safe from farm to table. Food Technology 54 (8), 192.

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

Charles-Rodriguez, A.V., Nevarez-Moorillon, G.V., Zhang, Q.H., Ortega-Rivas, E., 2007. Comparison of thermal processing and pulsed electric fields treatment in pasteurization of apple juice. Food and Bioproducts Processing 85, 93–97.

Cho, S., Jang, J.W., Jung, S.H., Lee, B.R., Oh, E., Lee, K.H., 2009. Precursor effects of citric acid and citrates on ZnO Crystal formation. Langmuir 25 (6), 3825–3831.

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.

Codex STAN, N. Norma general del Codex para los aditivos alimentarios. Adoptado en 1995. Revisión 1997, 1999, 2001, 2003, 2004, 2005, 2006, 2007, 2008, 2009 Available from: http://www.codexalimentarius.net/gsfaonline/CXS_192s.pdf Website last accessed: June 1, 2010.

Council Directive (EEC) No 89/107 of 21 December 1988 on the approximation of the laws of the Member States concerning food additives authorized for use in foodstuffs intended for human consumption (OJ L 40, 11.2.1989, p. 27)

Craven, H.M., Swiergon, P., Ng, S., Midgely, J., Versteeg, C., Coventry, M.J., Wan, J., 2008. Evaluation of pulsed electric field and minimal heat treatments for inactivation of *Pseudomonas* and enhancement of milk shelf-life. Innovative Food Science & Emerging Technologies 9, 211–216.

D'Aoust, J.Y., Emmons, D.B., Timbers, G.E., Todd, E.C.D., Sewell, A.M., Warburton, D.W., 1987. Thermal inactivation of *Salmonella* species in fluid milk. Journal of Food Protection 50 (6), 494–501.

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., GongoraNieto, M.M., BarbosaCanovas, G.V., Swanson, B.G., 2000. Effects of combined exposure of *Micrococcus luteus* to nisin and pulsed electric fields. International Journal of Food Microbiology 60, 147–152.

European Food Safety Authority, & European Centre for Diseases and Control, 2009. The Community Summary Report on Trends and Sources of Zoonoses and Zoonotic Agents in the European Union in 2007. The EFSA Journal 223 Available from: http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902269834.htm Website last accessed: June 10, 2010.

Gallo, L.I., Pilosof, A.M.R., Jagus, R.J., 2007. Effect of the sequence of nisin and pulsed electric fields treatments and mechanisms involved in the inactivation of *Listeria innocua* in whey. Journal of Food Engineering 79, 188–193.

García, D., Gómez, N., Raso, J., Pagán, R., 2005. Bacterial resistance after pulsed electric fields depending on the treatment medium pH. Innovative Food Science & Emerging Technologies 6, 388–395.

Geveke, D.J., 2008. UV inactivation of *E. coli* in liquid egg white. Food and Bioprocess Technology 1, 201–206.

Góngora-Nieto, M.M., Pedrow, P.D., Swanson, B.G., Barbosa-Cánovas, G.V., 2003. Energy analysis of liquid whole egg pasteurized by pulsed electric fields. Journal of Food Engineering 57, 209–216.

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.

Helander, I.M., von Wright, A., Mattila-Sandholm, T., 1997. Potential of lactic acid bacteria and novel antimicrobials against Gram-negative bacteria. Trends in Food Science and Technology 8, 146–150.

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.

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.

Humphrey, T.J., 1990. Growth of *Salmonellas* in intact shell eggs: influence of storage temperature. The Veterinary Record 24, 292.

Jin, T., Zhang, H., Hermawan, N., Dantzer, W., 2009. Effects of pH and temperature on inactivation of *Salmonella typhimurium* DT104 in liquid whole egg by pulsed electric fields. International Journal of Food Science & Technology 44, 367–372.

Leistner, L., 2000. Use of combined preservative factors in foods of developing countries. In: Lund, M.L., Bair-Parker, T.C., Gould, G.W. (Eds.), The Microbiological Safety and Quality of Foods. Aspen, Gaithersburg, MD, pp. 214–314.

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

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*. The Journal of Applied Bacteriology 53, 243–251.

Mañas, P., Pagan, R., 2005. Microbial inactivation by new technologies of food preservation. Journal of Applied Microbiology 98, 1387–1399.

Mañas, P., Pagán, R., Raso, J., Sala, F.J., Condón, S., 2000. Inactivation of *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Senftenberg by Ultrasonic Waves under Pressure. Journal of Food Protection 63 (4), 451–456.

Mañas, P., Pagan, R., Raso, J., Condon, S., 2003. Predicting thermal inactivation in media of different pH of *Salmonella* grown at different temperatures. International Journal of Food Microbiology 87, 45–53.

Mañas, P., Vercet, A., 2006. Effect of PEF on enzymes and food constituents. In: Raso, J., Heinz, V. (Eds.), Pulsed Electric Fields Technology for the Food Industry. Springer, New York, pp. 131–151.

McNamee, C., Noci, F., Cronin, D.A., Lyng, J.G., Morgan, D.J., Scannell, A.G.M., 2010. PEF based hurdle strategy to control *Pichia fermentans*, *Listeria innocua* and *Escherichia coli* k12 in orange juice. International Journal of Food Microbiology 138, 13–18.

Monfort, S., Gayán, E., Raso, J., Condón, S., Álvarez, I., 2010a. Evaluation of pulsed electric fields technology for liquid whole egg pasteurization. Food Microbiology 27 (7), 845–852.

Monfort, S., Gayán, E., Saldaña, G., Puértolas, E., Condón, S., Raso, J., Álvarez, I., 2010b. Inactivation of *Salmonella* Typhimurium and *Staphylococcus aureus* by pulsed electric fields in liquid whole egg. Innovative Food Science & Emerging Technologies 11, 306–313.

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.

Nohynek, L.J., Alakomi, H.L., Kahkonen, M.P., Heinonen, M., Helander, K.M., Oksman-Caldentey, K.M., Puupponen-Pimia, R.H., 2006. Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. Nutrition and Cancer 54, 18–32.

Pina-Perez, M.C., Silva-Angulo, A.B., Rodrigo, D., Martinez-Lopez, 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 130, 196–204.

Ponce, E., Pla, R., Sendra, E., Guamis, B., Mor-Mur, M., 1999. Destruction of *Salmonella enteritidis* inoculated in liquid whole egg by high hydrostatic pressure: comparative study in selective and non-selective media. Food Microbiology 16, 357–365.

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., Pagán, R., Condón, S., 2005. Nonthermal technologies in combination with other preservation factors. In: Barbosa-Cánovas, G.V., Tapia, M.S., Cano, M.P. (Eds.), Novel Food Processing Technologies. : Novel Food Processing Technologies. Marcel Dekker/CRC Press, Boca Ratón, Florida, pp. 453–476.

Real Decreto 142/2002, de 1 de febrero, por el que se aprueba la lista positiva de aditivos distintos de colorantes y edulcorantes para su uso en la elaboración de productos alimenticios, así como sus condiciones de utilización.

Rodrigez, E., Seguer, X., Rocabayera, X., Manresa, A., 2004. Cellular effects of monohydrochloride of L-arginine, N^{α}-lauroyl ethylester (LAE) on exposure to *Salmonella typhimurium* and *Staphylococcus aureus*. Journal of Applied Microbiology 96, 903–912.

Saldaña, G., Puertolas, E., Condon, S., Alvarez, I., Raso, J., 2010. Modeling inactivation kinetics and occurrence of sublethal injury of a pulsed electric field-resistant strain of *Escherichia coli* and *Salmonella* Typhimurium in media of different pH. Innovative Food Science & Emerging Technologies 11, 290–298.

USDA, 1969. Egg Pasteurization Manual. ARS 74–48. Poultry Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Albany, CA.

Walkling-Ribeiro, M., Noci, F., Cronin, D.A., Lyng, J.G., Morgan, D.J., 2009. Antimicrobial effect and shelf-life extension by combined thermal and pulsed electric field treatment of milk. Journal of Applied Microbiology 106, 241–248.