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The influence of process parameters for the inactivation of *Listeria monocytogenes* by pulsed electric fields

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Abstract

The influence of the electric field strength, the treatment time, the total specific energy and the conductivity of the treatment medium on the *Listeria monocytogenes* inactivation by pulsed electric fields (PEF) has been investigated. *L. monocytogenes* inactivation increased with the field strength, treatment time and specific energy. A maximum inactivation of 4.77 log₁₀ cycles was observed after a treatment of 28 kV/cm, 2000 μs and 3490 kJ/kg. The lethal effect of PEF treatments on *L. monocytogenes* was not influenced by the conductivity of the treatment medium in a range of 2, 3 and 4 mS/cm when the total specific energy was used as a PEF control parameter. A mathematical model based on the Weibull distribution was fitted to the experimental data when the field strength (15–28 kV/cm), treatment time (0–2000 μs) and specific energy (0–3490 kJ/kg) were used as PEF control parameters. A linear relationship was obtained between the log₁₀ of the scale factor (*b*) and the electric field strength when the treatment time and the total specific energy were used to control the process. The total specific energy, in addition to the electric field strength and the treatment time, should be reported in order to evaluate the microbial inactivation by PEF.

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Keywords: PEF; *Listeria monocytogenes*; Field strength; Specific energy; Conductivity; Weibull

1. Introduction

Pulsed electric fields (PEF) are an innovative technology that is being explored to supply safe, nutritious and high quality foods. The inactivation of vegetative forms of bacteria, yeast, molds and some enzymes related to food quality by PEF treatments has been demonstrated by different authors

(Wouters and Smelt, 1997; Barsotti and Cheftel, 1999). Although bacterial and fungal spores are not inactivated by PEF treatments (Grahl and Märkl, 1996), this technology is on intense study because of its potential as a nonthermal pasteurization process. The main objective of PEF pasteurization should include the inactivation of several log₁₀ cycles of pathogenic microorganisms present on foods to prevent their transmission to humans. The capacity to achieve these desired levels of inactivation greatly depends on the knowledge of the kinetics of microbial destruction by PEF. Heat resistance and kinetics of inactivation of the most

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important pathogens for the public health concern have been widely investigated (Doyle and Mazzotta, 2000; Stringer et al., 2000; Doyle et al., 2001). However, there is hardly any data related to the kinetics of inactivation of these pathogens by PEF.

In studies of microbial survivability to different treatments, the most general approach is to determine the influence of the treatment time at a given intensity on the microbial inactivation. Therefore, time and treatment intensity are the main control parameters of the process. Most of the studies on inactivation kinetics of microorganisms by PEF have been based on the relationship between the survival fraction and the treatment time (pulse width \times number of pulses) at different electric field strengths. However, the use of the electric field strength and the treatment time could have some limitations as suitable PEF control parameters. When a pulse is applied, a Joule heating effect of the product happens that increases its temperature, and as a consequence, the electrical conductivity. This increment could modify the pulse width and the electric field strength. Due to this fact and the less accurate measurement of the pulse width in exponential decay pulses, the total specific energy has been proposed as a new control parameter for PEF processing (Heinz et al., 1999; Heinz et al., 2001). Total specific energy is defined as an integrated parameter that involves the influence of the electric field strength, the treatment time and the electrical resistance of the treatment chamber, which depends on its dimensions and on the conductivity of the treatment medium (Heinz et al., 2001).

The objective of this investigation was to establish if the total specific energy is a more suitable PEF control parameter than the treatment time. For this purpose, the influence of the electric field strength, treatment time, specific energy and conductivity on the inactivation of *Listeria monocytogenes* by PEF treatments was studied.

L. monocytogenes is a psychrotrophic microorganism that has emerged as one of the major human foodborne pathogens (Chasseignaux et al., 2001; http://www.fsis.usda.gov/OA/topics/lm_action.htm). It has been found to contaminate different foods (Donnelly, 1994; Huss et al., 2000; Rudolf and Scherer, 2001) and, even, it has been isolated from

heat-pasteurized products (Fleming et al., 1985; Farber and Peterkin, 1991; Donnelly, 1994).

2. Materials and methods

2.1. Microorganism and growth conditions

The strain of *L. monocytogenes* (ATCC 15313) used in this investigation was supplied by the Spanish Type Culture Collection. It was maintained on slants of Tryptic Soy Agar (Biolife, Milan, Italy) with 0.6% Yeast Extract added (Biolife) (TSAYE).

A broth subculture was prepared by inoculating a test tube containing 5 ml of Tryptic Soy Broth with 0.6% Yeast Extract (TSBYE) with a single colony, followed by incubation at 35 °C for 18 h. With this subculture, flasks containing 50 ml of sterile TSBYE were inoculated to a final concentration of 10^6 cells/ml. The culture was then incubated under agitation at 35 °C during 24 h. After this incubation time microbial cells were at the stationary phase of growth. Under these growth conditions, PEF resistance of *L. monocytogenes* was maximum when it was pulsed in citrate-phosphate McIlvaine buffer of pH 7.0 (Alvarez et al., 2002).

2.2. PEF equipment

PEF equipment used in this investigation was previously described by Heinz et al. (1999). Microorganisms were treated in a parallel-electrode treatment chamber with a distance between electrodes of 0.25 cm and an area of 2.01 cm². The circuit configuration generated square waveform pulses at different frequencies, pulse widths and electric field strengths. Pulse frequency of 1 Hz, pulse width of 2 μ s and electric field strengths from 15 to 28 kV/cm were used. In all experiments, the temperature was kept under 32 °C. The temperature of the treatment medium was measured as previously described Raso et al. (2000). Actual electric field strength applied was 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 specific energy

per pulse (W') was calculated by the following equation:

$$W' = \frac{1}{\rho} \int_0^{\infty} \kappa E_{(t)}^2 dt \quad (1)$$

where ρ (kg/m^3) is the density of the sample; κ (S/m) is the electrical conductivity of the product; E (V/m) is the electric field strength; and t (s) is the duration of the pulse. The total specific energy applied (W) was calculated by multiplying the energy per pulse (W') by the number of pulses.

2.3. Microbial inactivation experiments

Before treatment, microorganisms were centrifuged at $6000 \times g$ for 5 min at 4°C and resuspended in citrate-phosphate McIlvaine buffer of pH 7.0 (Dawson et al., 1974), which concentration was adjusted to an electrical conductivity of 2 mS/cm. To study the influence of the conductivity on the microbial inactivation, cells were suspended in McIlvaine citrate-phosphate buffer of pH 7.0 diluted at different concentrations to get conductivities of 2, 3 and 4 mS/cm.

The microbial suspension at a concentration of 10^9 CFU/ml was placed into the treatment chamber with a sterile syringe. After filling, the hole of the treatment chamber was sealed with tape. After treatment, appropriate serial dilutions were prepared in sterile 0.1% peptone water and plated into TSAYE. Plates were incubated at 37°C for 48 h and, after incubation, colonies were counted with an improved image analyser automatic counter (Protos, Analytical Measuring Systems, Cambridge, UK) as previously described elsewhere (Condón et al., 1996).

2.4. Curve fitting

A mathematical model based on the Weibull distribution was used to fit the survival curves (\log_{10} of the survival fraction vs. treatment time, and \log_{10} of the survival fraction vs. total specific energy) of *L. monocytogenes*. If the microbial PEF resistance follows a Weibull distribution, the survival function will be:

$$\log_{10}S(x) = -\left(\frac{1}{2.303}\right)\left(\frac{x}{b}\right)^n \quad (2)$$

where x is the treatment time or the specific energy, and b and n are the scale and shape parameters, respec-

tively. The b value represents the time necessary to inactivate 0.434 \log_{10} cycles of the population. The n parameter accounts for upward concavity of a survival curve ($n < 1$), a linear survival curve ($n = 1$), and downward concavity ($n > 1$) (van Boekel, 2002).

To determine the b and n values, the least-squares criterion by the Solver function of the Excel 5.0 package (Microsoft, Seattle, WA) and the GraphPad PRISM® (GraphPad Software, San Diego, CA) was used. ANOVA ($p=0.05$) analyses were calculated with SPSS® (SPSS, Chicago, IL).

3. Results

3.1. Influence of the electric field strength, the treatment time and the total specific energy on *L. monocytogenes* PEF inactivation

Fig. 1 shows the influence of the treatment time on the inactivation of *L. monocytogenes* by PEF at different electric field strengths (from 15 to 28 kV/cm). Electric field strengths below 15 kV/cm did not affect the viability of this microorganism (data not shown). Over this field strength, the microbial inactivation increased with the electric field strength and the treatment time.

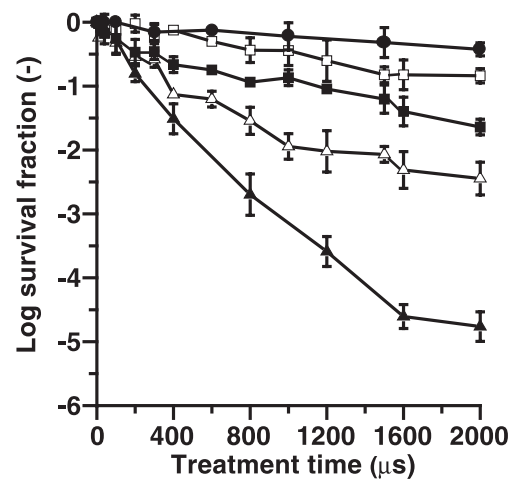


Fig. 1. Influence of the treatment time and the electric field strength on the inactivation of *L. monocytogenes* by PEF treatments in McIlvaine citrate-phosphate buffer of pH 7.0 and a conductivity of 2 mS/cm. (●) 15 kV/cm; (□) 19 kV/cm; (■) 22 kV/cm; (△) 25 kV/cm; (▲) 28 kV/cm.

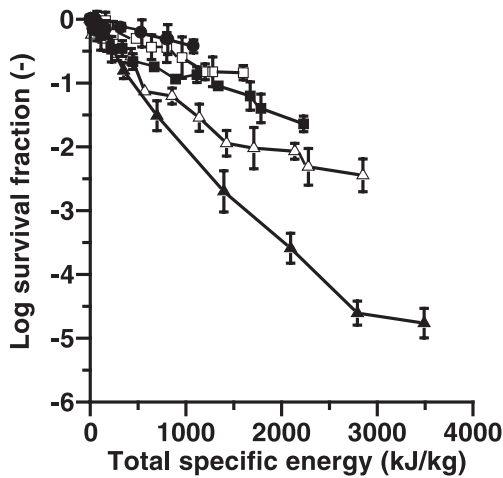


Fig. 2. Influence of the total specific energy and the electric field strength on the inactivation of *L. monocytogenes* by PEF treatments in McIlvaine citrate-phosphate buffer of pH 7.0 and a conductivity of 2 mS/cm. (●) 15 kV/cm; (□) 19 kV/cm; (■) 22 kV/cm; (△) 25 kV/cm; (▲) 28 kV/cm.

Fig. 2 shows the influence of the total specific energy on the inactivation of *L. monocytogenes* by PEF. Microbial inactivation increased with the specific energy. At the same specific energy level, treatments at higher electric field strength were more effective. For example, a treatment of 2000 kJ/kg at 22 kV/cm that inactivated 1.5 log₁₀ cycles of the population of *L. monocytogenes* at 28 kV/cm inactivated around 3.5 log₁₀ cycles.

3.2. Influence of the conductivity of treatment medium on inactivation of *L. monocytogenes* by PEF

The inactivation of *L. monocytogenes* in media of different conductivities is shown in Fig. 3. Pulses of the same electric field strength (22 kV/cm) and pulse width (2 μs) were applied in media of 2, 3 and 4 mS/cm. When a constant input voltage is delivered between two electrodes, the electric field strength achieved is influenced by the conductivity. To obtain an electric field of 22 kV/cm, input voltages applied to media of 2, 3 and 4 mS/cm were 6.9, 7.5 and 8.4 kV, respectively. According to Eq. (1), the specific energy of pulses of the same electric field strength is higher since the conductivity of the treatment medium is greater. The specific energy of pulses of 2 μs and 22 kV/cm applied to media of 2, 3 and 4 mS/cm was 2.23,

2.96 and 4.72 kJ/kg/pulse, respectively. Fig. 3A shows the influence of the conductivity on the inactivation of *L. monocytogenes* at different treatment times, and Fig. 3B at different specific energies. When the log₁₀ of the survival fraction was plotted against the treatment time, a higher inactivation was observed at higher conductivities. However, *L. monocytogenes* PEF resistance did not depend on the conductivity when

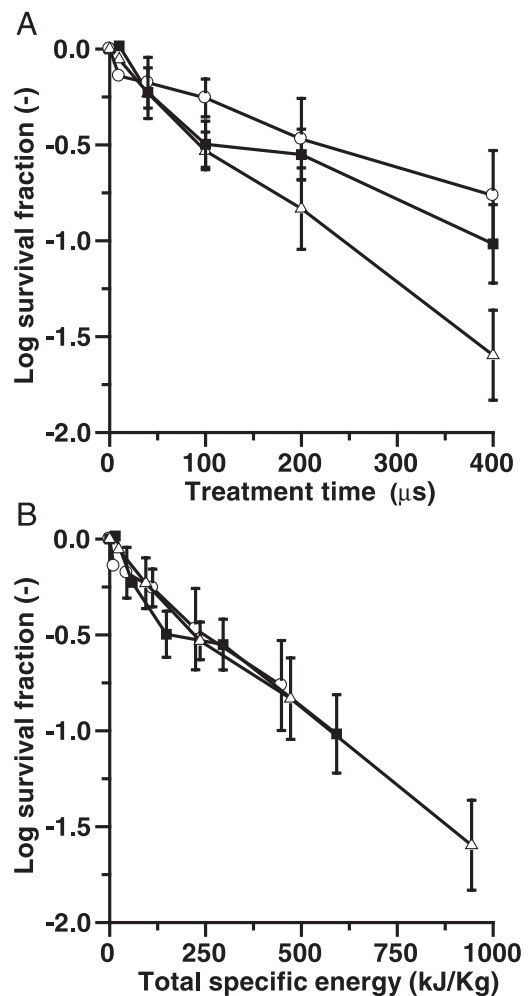


Fig. 3. Influence of the conductivity of the treatment medium on the inactivation of *L. monocytogenes* by PEF treatments at a constant field strength of 22 kV/cm at different treatment times (A) and total specific energies (B). Treatment conditions: McIlvaine citrate-phosphate buffer of pH 7.0; 2 mS/cm, 6.9 kV, 2.23 kJ/kg/pulse (○); 3 mS/cm, 7.5 kV, 2.96 kJ/kg/pulse (■); 4 mS/cm, 8.4 kV, 4.72 kJ/kg/pulse (△).

the \log_{10} of the survival fraction was plotted against the total specific energy.

3.3. Kinetics of inactivation of *L. monocytogenes* by PEF

Survival curves obtained by plotting the \log_{10} of the survival fraction against the treatment time or the total specific energy were fitted to Eq. (2). The estimated parameters b and n obtained at the different electric field strengths investigated are shown in Table 1. The determination coefficients (R^2) of each fitting are also included. The b values decreased when the electric field strength increased from 15 to 28 kV/cm. The n values obtained when treatment time and total specific energy were used as control parameters were similar. According to the n value and its confident limits, at 25 and 28 kV/cm survival curves were concave upwards. However, at 15, 19 and 22 kV/cm the curvature of the survival curves was not significant (the confident limits of the n value included the value of 1). The n values were not influenced by the electric field strength. ANOVA analysis confirmed that there were nonsignificant differences among the n values obtained at different electric field strengths when the \log_{10} of the survival fraction was plotted against the treatment time or the specific energy. As nonsignificant differences among n values were observed, survival curves were refitted with n set at its mean value (0.798). Fig. 4 shows the relationship between the \log_{10} of b values and the electric field strength when the treatment time and the total specific

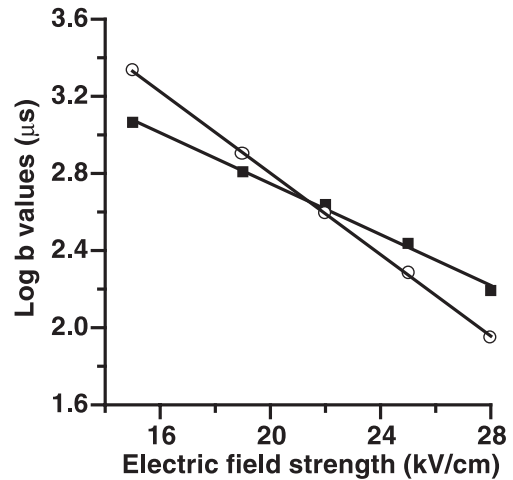


Fig. 4. Relationship between the electric field strength and the \log_{10} of the b values for the treatment time (○) and the total specific energy (■).

energy were used as control parameters in PEF treatments. In the range of the electric field strengths investigated, linear relationships ($R^2 > 0.99$) were observed for both, treatment time (Eq. (3)) and specific energy (Eq. (4)).

$$\log_{10} b = 4.914 - 0.106E \quad (3)$$

$$\log_{10} b = 4.067 - 0.066E \quad (4)$$

where E (kV/cm) is the electric field strength. This linear variation of the $\log_{10} b$ with the electric field strength allowed calculating a parameter similar to the

Table 1

b and n values estimated from the first fitting of the mathematical model based on the Weibull distribution to experimental data at different electric field strengths when the treatment time and the specific energy were used as PEF control parameters

PEF control parameter	kV/cm	b (μ s) (CL 95%) ^a	n (CL 95%) ^a	R^{2b}
Treatment time	15	2101.0 (1697.0–2505.0)	0.847 (0.561–1.132)	0.982
	19	843.8 (712.5–975.2)	0.881 (0.670–1.092)	0.975
	22	439.7 (357.9–521.5)	0.883 (0.748–1.018)	0.990
	25	111.4 (67.5–155.3)	0.621 (0.521–0.721)	0.988
	28	76.4 (39.9–112.8)	0.755 (0.633–0.877)	0.995
Specific energy	15	1135.0 (916.1–1353.0)	0.847 (0.5618–1.132)	0.982
	19	675.0 (570.0–780.1)	0.881 (0.670–1.092)	0.975
	22	490.7 (399.9–581.6)	0.883 (0.750–1.1019)	0.990
	25	158.2 (95.9–220.6)	0.621 (0.521–0.720)	0.988
	28	133.3 (70.1–196.5)	0.755 (0.634–0.876)	0.995

^a CL 95%: confidence limit.

^b R^2 : determination coefficient.

z value used in the traditional Bigelow model. These values were 9.47 and 15.16 kV/cm when the treatment time and the total specific energy, respectively, were used as PEF control parameters.

In order to determine the accuracy of the model, measured data were correlated with the estimated ones. The estimated data were calculated when the \log_{10} of the survival fraction was plotted against the treatment time, with Eq. (5), and when \log_{10} of the survival fraction was plotted against the specific energy, with Eq. (6), re-defined from Eq. (2) by

substituting the n and b values after the second fitting of the model.

$$\log_{10}S(t) = -\frac{1}{2.303} \left(\frac{t}{10^{(4.914-0.106E)}} \right)^{0.798} \quad (5)$$

$$\log_{10}S(W) = -\frac{1}{2.303} \left(\frac{W}{10^{(4.067-0.066E)}} \right)^{0.798} \quad (6)$$

The correlation coefficients between the measured and the theoretical values were 0.99 for both parameters.

Eqs. (5) and (6) were also used to generate three-dimensional relationships among the \log_{10} of the survival fraction, the electric field strength, and the treatment time or the total specific energy. These relationships are plotted in Fig. 5A and B. These figures show that the treatment time (Fig. 5A) or the total specific energy (Fig. 5B) necessary to obtain a given level of microbial inactivation decreased drastically when the electric field strength increased. For example, to obtain an inactivation of 2 \log_{10} cycles, the treatment time would decrease from 2000 to 600 μs , and the total specific energy from 2500 to 1100 kJ/kg when the electric field strength increased from 23 to 28 kV/cm.

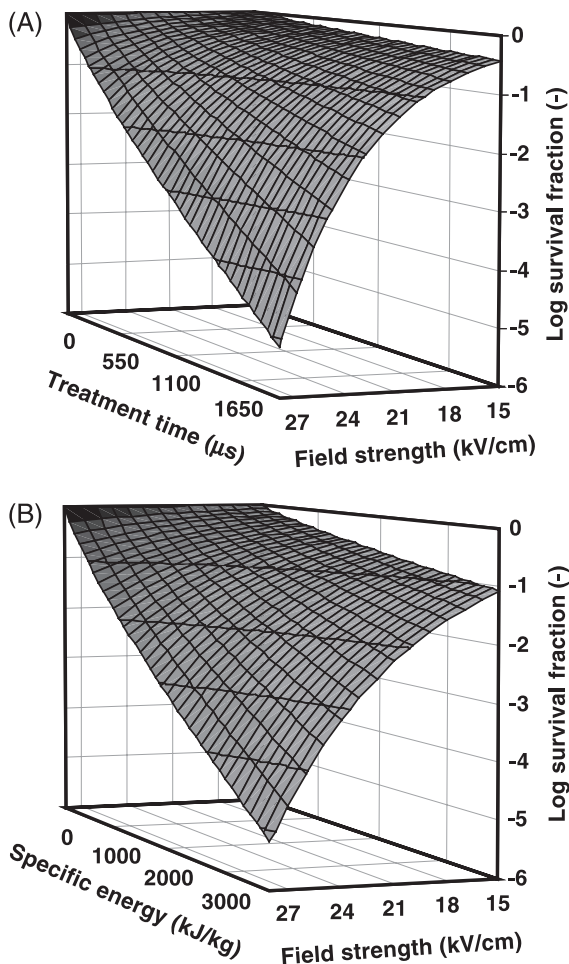


Fig. 5. Three-dimensional views of the influence of the treatment time and the electric field strength (A), or the total specific energy and the field strength (B) on the inactivation of *L. monocytogenes* by PEF.

4. Discussion

Different authors have studied the resistance of *Listeria* by PEF (Reina et al., 1998; Calderón-Miranda et al., 1999a,b; Simpson et al., 1999; Wouters et al., 1999; Dutreux et al., 2000; McDonald et al., 2000; Aronsson et al., 2001; Unal et al., 2001). Most data published correspond to the inactivation of *Listeria innocua* (Calderón-Miranda et al., 1999a,b; Wouters et al., 1999; Dutreux et al., 2000; McDonald et al., 2000; Aronsson et al., 2001), and only a few to *L. monocytogenes* (Reina et al., 1998; Simpson et al., 1999; Unal et al., 2001). Although *L. innocua* has been traditionally used as a nonpathogenic surrogate for *L. monocytogenes* (Kamat and Nair, 1996), however, it has not been demonstrated if *L. innocua* has the same PEF resistance and follows the same PEF inactivation kinetics as *L. monocytogenes*. The comparison of our data to that published is quite complicated due to different experimental conditions used in

all these studies (continuous vs. static chambers, exponential vs. square waves pulses, foods vs. buffers, etc.). Our results indicated that *Listeria* is one of the most PEF-resistant microorganisms investigated. Treatments under 15 kV/cm hardly affected the viability of our strain. A treatment of 1600 μ s at 28 kV/cm that inactivated 4.6 \log_{10} cycles of the population of *L. monocytogenes* inactivated 6.5 \log_{10} cycles of *S. senftenberg* (Raso et al., 2000). Simpson et al. (1999) obtained after 24,000 μ s at 15 kV/cm only 1 \log_{10} cycle of inactivation of the population of *L. monocytogenes*.

The total specific energy has been suggested as a control parameter of PEF treatments due to its integrated characteristic (Heinz et al., 1999). Our results were analysed by plotting the \log_{10} of the survival fraction against the treatment time or the total specific energy. However, the specific energy cannot be used as an only parameter that involves the influence of the electric field strength and treatment time because, at a constant specific energy, microbial killing depended on the electric field strength applied (Fig. 2). Thus, both total specific energy and field strength must be used to control PEF processes.

The influence of the conductivity of the treatment medium on the microbial inactivation by PEF has been investigated by different authors (Jayaram et al., 1993; Sensoy et al., 1997; Wouters et al., 1999; Álvarez et al., 2000; Dutreux et al., 2000; Wouters et al., 2001). Most studies concluded that conductivity affects microbial inactivation by PEF. However, it is unclear if the effect of the conductivity is due to the influence of this parameter in the electric field strength and pulse width, or if the conductivity influences the effect of the electric field strength on the cell membranes. Our results show that when \log_{10} of the survival fraction was plotted against the treatment time, microbial inactivation was higher at lower conductivities, but no influence of the conductivity was observed when it was plotted against the total specific energy. The higher inactivation observed at a constant treatment time and field strength when the conductivity increased could be attributed to the greater specific energy applied at higher conductivities (Fig. 3A). On the other hand, inactivation of *L. monocytogenes* was not influenced by the conductivity when the total specific energy and the electric field strength of the treatment were constant (Fig. 3B).

Therefore, the total specific energy is a parameter that must be considered when the influence of the conductivity on the microbial inactivation is studied.

Different mathematical relationships have been developed to describe the kinetics of microbial PEF inactivation (Hülshager et al., 1981; Peleg, 1995; Martín-Belloso et al., 1997; Raso et al., 2000). In these studies, the electric field strength and the treatment time have been considered the primary critical factors in reducing microbial population by PEF treatments. In this paper, survival curves were described by a mathematical model based on the Weibull distribution and specific energy was used as control parameter.

The n values obtained indicated that at the lower electric field investigated, where survival curves covered less of 1.5 \log_{10} cycles, a first-order kinetics could describe survival curves. However, at 25 and 28 kV/cm, the shape parameter was lower than 1 and therefore this kinetics did not fit the data. A first-order kinetics has already been used to describe kinetics of inactivation by PEF when survival curves cover few \log_{10} cycles (Martín-Belloso et al., 1997; Reina et al., 1998; Heinz et al., 1999). The concave upward shape of curves at the highest electric field strengths could be due to an innate variation on the PEF resistance of the individuals or a consequence of the peculiarities of the PEF treatment. Differences in morphology (size, shape) or in cell membranes (composition, structure) within the microbial population could explain a variation in resistance. However, distribution of resistances could be also due to a local variation of the electric field strength in the treatment chamber, to cell orientation of the microorganism in the treatment chamber when external electric field strength is applied, or to an adaptive response of the cells during the treatment (Heinz et al., 2001; Wouters et al., 2001).

For both control parameters, the shape parameter (n) obtained at all electric field strengths investigated was the same. This simplifies the model by using only one parameter (b value) to compare the influence of the electric field strength on PEF resistance. As the \log_{10} of the b values decreased linearly with the electric field strength, the negative value of the inverse of the slope of the regression line would represent a parameter similar to the z value (Z_{PEF}) used in the thermal processing calculations. Therefore, the PEF resistance of *L. monocytogenes* could be characterised by b , n and Z_{PEF} . Thus, if the b value can be considered as a

measurement of the PEF resistance of *L. monocytogenes*, the Z_{PEF} value would indicate the dependence of the *L. monocytogenes* PEF resistance as function of the electric field strength.

The kinetics of inactivation of *L. monocytogenes* was similar when treatment time and total specific energy were used as PEF control parameters. This fact is probably because our equipment delivers square wave pulses and experiments were carried out at low frequency to avoid an overheating of the treatment medium that could modify the conductivity of the treatment medium and pulse width.

The influence of the treatment time and the total specific energy on the inactivation of *L. monocytogenes* depended on the electric field strength (Fig. 5A and B). Treatment time and specific energy necessary to achieve a given level of inactivation decreased at higher electric field strengths. In general, reduced treatment times are important since the volume of the treatment chamber of continuous PEF apparatus is small, so long residence times are not possible. In addition, the lower specific energy needed reduces the economic cost of the process. Therefore, from an industrial application point of view of this technology, higher electric field strengths would be more suitable due to lower residence times and energy consumptions. However, the greater cost of equipments that deliver pulses of higher voltages should be considered.

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