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Control of *Enterobacter aerogenes* by high-intensity, pulsed electric fields in horchata, a Spanish low-acid vegetable beverage

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Abstract

High-intensity pulsed electric fields (HIPEF) is a non-thermal technology used in the processing of liquid foods. Inactivation of foodborne micro-organisms by this novel technology is an important alternative to traditional thermal methods with a great potential for new liquid products. However, the existence of a resistant proportion of microbial population presents a problem to establish the parameters that can guarantee the safety during the shelf-life of each food. The aim of this study was to investigate the influence of field strength, substrate inoculum size (initial cell concentration) and storage conditions after HIPEF treatment to evaluate the potential growth and subsequent spoilage by *Enterobacter aerogenes* in horchata, a Spanish low-acid vegetable beverage. Although no more than 1.1-log reductions were obtained by any of the HIPEF conditions applied, *E. aerogenes* growth in both substrates (horchata and a standard broth) at 10°C, 12°C and 16°C was affected by HIPEF treatments compared to untreated samples. The specific growth rate of the bacteria after HIPEF treatment was not modified, but the lag period was increased. A synergistic effect of HIPEF treatment, low temperature (10°C) and low inoculum size on the delay in lag phase was observed.

Keywords: *Enterobacter aerogenes*; Pulsed electric field; Non-thermal technologies; Hurdle technologies

1. Introduction

Non-thermal processing is being developed for the preservation of foods as an alternative to traditional thermal methods. Minimally processed food demand has been increased in recent years due to retention of flavour, colour and nutritive value (Martin et al., 1997). However, food-poisoning outbreaks and even death have been associated to unpasteurized foods (Beuchat, 1996). Horchata is a Spanish dairy beverage, which is extracted from tiger-nut tubers (*Cyperus sculentus*), with added sugar. Raw horchata has a pH range of 6.3–6.8 and is rich in starch; therefore, it cannot be heated above 72°C. *Enterobacter aerogenes*, a Gram-negative micro-organism is the main responsible for its rapid degradation due to its ability for gas production (Lafuente et al., 1985). This product has a high nutritional quality and therefore, a relevant potential in the present food market, which is limited by a very short shelf-life. To overcome this limitation, thermal methods of preservation (UHT) have been applied after significant composition changes, consisting in removal of starch. This implies a significant loss of flavour, compared to its traditional process, and flavourings such as vanilla have to be added. The bacterial density of the raw horchata is in the range 10^6 cfu ml^{-1} (aerobic plate count), whereas for pasteurized horchata would be around 5×10^4 cfu ml^{-1} (Selma et al., 2002).

High-intensity pulsed electric fields (HIPEF) are non-thermal methods, which can be applied to foods. HIPEF can inactivate micro-organisms by causing structural changes in the membrane, resulting in pore formation and loss of cellular constituents. Although cell membrane permeability increases, the inactivation only occurs when a certain electric field is exceeded (Barbosa et al., 1997). When pores are reversible, perforated membrane reparation can occur within seconds or minutes (Chang and Reese, 1990). There are several critical factors that influence the inactivation kinetics by pulsed electric field food processing, including process

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parameters, and microbial characteristics (Wouters et al., 2001). Due to the existence of a resistant proportion of population, this technique may be more useful as one stage in a combinated treatment and it may be capitalized on reversible pore formation using other treatments (Simpson et al., 1999; Calderon-Miranda et al., 1999a). Therefore, more research is necessary to establish the parameters that can guarantee the safety during the shelf-life of each product.

Although research is increasing in the study of survivor micro-organism fraction to other non-thermal methods such as pulsed-microwave irradiation (Hagenmaier and Baker, 1998) and irradiation (Shin and Pyun, 1997), more research is necessary to analyse the effect of HIPEF on subsequent growth of resistant population (Periago et al., 2002). Some researchers have investigated lethal and sub-lethal effects of HIPEF on micro-organisms with transmission electron microscopy (Calderon-Miranda et al., 1999b; Dutreux et al., 2000) but this damage has not been quantified. Microbial physiological factors such as micro-organisms, strains or inoculum size, as well as product parameters such as nutrients, pH and temperature could influence the results of sub-lethal effect of HIPEF on survival of bacteria. This could mean that cell injury was not immediately lethal but impair the organism's ability to survive over time (McDonald et al., 2000). The lag time of *Listeria monocytogenes* grown under suboptimal conditions (severe stress induced by starvation) was extended when the inoculum size was very small (Augustin et al., 2000). This indicates that the incidence of bacterial stress and the inoculum size may have a relevant role on the ability of bacteria to recover under exposure to a technological treatment, although there is limited information about this point. In the present work, the influence of field strength, substrate inoculum size (initial cell concentration) and storage conditions after HIPEF treatment (assessment of possible consequences) were evaluated to establish the potential growth and subsequent spoilage by *E. aerogenes* in horchata.

2. Materials and methods

2.1. Culture preparations

Culture of *E. aerogenes* CECT 684 was obtained from the Spanish Type Culture Collection, it had been isolated from humans and previously used in food studies. This culture was rehydrated in 3 ml tryptone soya broth (Oxoid CM129) and then inoculated onto separate tryptone soya agar (Oxoid CM131) plates and incubated for 24 h at 30°C. After incubation, stock bacterial culture was kept in agar slants at 4°C.

One single colony of culture was inoculated from the stock in a flask containing 100 ml of tryptone soya broth (TSB). It was then incubated at 30°C in a well-shaken water-bath for 18–20 h to achieve the stationary phase. The final cell concentration achieved was approximately 10^9 cfu ml^{-1}. Before HIPEF treatment, cells were diluted to a level of 10^5–10^6 cfu ml^{-1}, in 500 ml of 20% buffered peptone-water solution, with the same conductivity as horchata (0.2 S m^{-1} at 20°C), since HIPEF effect is influenced by the ionic strength of the conductive media (Dutreux et al., 2000; Wouters et al., 2001).

2.2. HIPEF treatment

The product was shaken at low speed and subjected to different HIPEF treatments. A bench-scale, continuous PEF system (Sensoy et al., 1997) based at the Institute of Agrochemical and Food Technology (C.S.I.C., Valencia) was used to treat the inoculated buffered peptone-water. A continuous, coaxial treatment with four chambers connected in series was used. A 2×10^{-6} s of square-wave pulse duration was selected for the treatment. This pulse wave form has been found to be more lethal than exponential decay pulses (Zhang et al., 1994). Flow rate was adjusted to 1.5×10^{-3} l s^{-1} while peak electric field strengths (E) of 3×10^6 and 2.5×10^6 V m^{-1} were applied (Table 1). The calculated resident time in each chamber was 8.11 ms. The total treatment time was calculated as 19×10^{-5} and 5×10^{-5} s for 3×10^6 V m^{-1} pulse duration and 3×10^{-5} s for 2.5×10^6 V m^{-1}. A cooling coil immersed in ice water was used to cool the sample at the entrance of the first and third treatment chambers. The treatment temperature was kept below 30°C, since it has been previously shown that it influences the membrane breakdown during HIPEF treatment (Reina et al., 1998; Evrendilek et al., 1999).

2.3. Uninoculated control

A non-inoculated buffered peptone-water was run through the system before inoculated samples were treated. The total plate count method using tryptone soya agar (TSA) was performed to assess the absence of

Table 1
Data relative to conditions of the different HIPEF treatment applied

Electric field/total treatment time	30/190	30/50	25/300
(V m^{-1} × 10^5) (s × 10^{-6})			
Effective electric field strength (V m^{-1} × 10^{-5})	30.87	30.72	26.09
Total number of pulses	95	25	150
T_I (°C)	16.0	11.0	15.0
T_F (°C)	30.0	11.0	30.6
ΔT (°C)	15.0	0.0	15.6

T_I: average temperature at the entrance of the first treatment chamber.
T_F: average temperature at the exit of the fourth treatment chamber.
ΔT: total temperature increase.

microbial contamination in the HIPEF equipment prior to treating the bacteria.

2.4. Recovery conditions

TSB and sterile horchata (100 ml) in 250 ml flasks were inoculated with 1 ml of diluted inoculum, before and after each HIPEF treatment, to give an initial concentration of about 10^3 cfu ml^{-1}. Sterile horchata was used (pH 6.73, total solids 15.3%, soluble solids 12%, minimum fat content 2%), since it has a similar composition to the pasteurized product, with less starch (pH 6.4, total solids 22.2%, soluble solids 17.2%). Alternatively, 100 ml of both media were inoculated with 1 ml of diluted inoculum to give an initial concentration of about 10 cfu ml^{-1}. Inoculated flasks with *E. aerogenes* CECT 684 were stored at 5°C, 8°C, 10°C, 12°C and 16°C. Samples were taken at appropriate time intervals, dilutions were made if necessary in peptone-water and counts in TSA were performed. Triplicate growth curves were obtained and kept for at least 60 days if growth was not detected.

2.5. Treatments applied and data modelling

The experimental design used was: two media conditions, four treatment conditions (PEF), five different temperatures of incubation and two inoculum levels. Growth curves were fitted using the function of Baranyi et al. (1993) to estimate the main growth parameters (maximum specific growth rate, lag time of micro-organisms before the onset of growth and estimated correlation coefficient, that indicates the goodness of fit of the parameters derived from experimental data). Only growth curves with at least 10 data points were used for modelling, as suggested by the authors. The correlation coefficient (R^2) for each growth curve (indicating the goodness of fit of experimental data to the equation fitted by the model) was also calculated. An ANOVA analysis was performed to establish significant differences among the treatment and recovery conditions tested using MATLAB® software.

3. Results and discussion

3.1. Effect of field strength

The effect of three HIPEF treatments on the ability of *E. aerogenes* to grow at different temperatures was evaluated. The uninoculated control with buffered peptone-water proved the absence of microbial contamination in the HIPEF equipment, since no bacterial

Table 2
Growth characteristics of *E. aerogenes* on TSB medium after different HIPEF treatments. Inoculum level of viable micro-organisms after each treatment was 10^3 cfu ml^{-1}

E (V m^{-1} × 10^5)	Treatment time (s × 10^{-6})	Incubation temperature (°C)	Lag time (time ± s.d.[a]) (h)	Maximum specific growth rate (± s.d.) (h^{-1})	Correlation coefficient (R^2)
Untreated		5	No growth	—	—
Untreated		8	No growth	—	—
Untreated		10	23.72 ± 1.01 A	0.034 ± 0.002 G	0.998
Untreated		12	10.35 ± 1.17 C	0.067 ± 0.012 H	0.998
Untreated		16	1.90 ± 0.41 E	0.161 ± 0.002 I	0.999
30	190	5	No growth	—	—
30	190	8	No growth	—	—
30	190	10	92.17 ± 10.42 B	0.036 ± 0.002 G	0.993
30	190	12	18.69 ± 1.30 D	0.051 ± 0.003 H	0.997
30	190	16	17.52 ± 5.72 F	0.149 ± 0.022 I	0.996
30	50	5	No growth	—	—
30	50	8	No growth	—	—
30	50	10	82.52 ± 5.62 B	0.041 ± 0.004 G	0.994
30	50	12	15.67 ± 1.57 D	0.059 ± 0.009 H	0.999
30	50	16	13.54 ± 2.16 F	0.172 ± 0.018 I	0.996
25	300	5	No growth	—	—
25	300	8	No growth	—	—
25	300	10	106.68 ± 16.53 B	0.035 ± 0.002 G	0.994
25	300	12	16.27 ± 1.53 D	0.051 ± 0.005 H	0.998
25	300	16	13.04 ± 2.22 F	0.150 ± 0.017 I	0.990

[a] s.d.: standard deviation.
Numbers followed by the same letter are not significantly different ($P \leqslant 0.05$) for the same incubation temperature within columns.

Table 3
Growth characteristics of *E. aerogenes* on sterile horchata at different temperatures after HIPEF treatment. Inoculum level of viable micro-organisms after each treatment was 10^3 cfu ml^{-1}

E (V.m$^{-1} \times 10^5$)	Treatment time ($s \times 10^{-6}$)	Incubation temperature (°C)	Lag time (time ± s.d.[a]) (h)	Maximum specific growth rate (± s.d.) (h^{-1})	Correlation coefficient (R^2)
Untreated		5	No growth	—	—
Untreated		8	No growth	—	—
Untreated		10	55.75 ± 11.99 A	0.039 ± 0.006 A	0.994
Untreated		12	17.56 ± 2.59 C	0.036 ± 0.001 C	0.998
Untreated		16	4.63 ± 0.79 F	0.113 ± 0.007 E	0.999
30	190	5	No growth	—	—
30	190	8	No growth	—	—
30	190	10	103.27 ± 5.35 B	0.039 ± 0.009 A	0.990
30	190	12	52.96 ± 5.60 D	0.042 ± 0.001 C	0.999
30	190	16	16.66 ± 5.14 G	0.146 ± 0.012 F	0.996
30	50	5	No growth	—	—
30	50	8	No growth	—	—
30	50	10	104.09 ± 10.95 B	0.019 ± 0.009 B	0.999
30	50	12	33.30 ± 6.36 E	0.022 ± 0.002 D	0.995
30	50	16	12.16 ± 5.71 G	0.126 ± 0.013 F	0.999
25	300	5	No growth	—	—
25	300	8	No growth	—	—
25	300	10	115.04 ± 11.43 B	0.019 ± 0.008 B	0.999
25	300	12	60.93 ± 7.21 D	0.031 ± 0.002 C	0.999
25	300	16	14.62 ± 5.83 G	0.149 ± 0.022 F	0.999

[a] s.d.: standard deviation.
Numbers followed by the same letter are not significantly different ($P \leqslant 0.05$) for the same incubation temperature within columns.

counts were obtained. Both treated and untreated cells were not able to grow at 5°C or 8°C (Tables 2 and 3). The level of inactivation achieved by HIPEF treatments ranged from 0.6 ± 0.1 (3×10^6 V m^{-1}, 5×10^{-5} s) to 1.1 ± 0.1-log reductions (3×10^6 V m^{-1}, 19×10^{-5} s and 2.5×10^6 V m^{-1}, 30×10^{-5} s). Although no more than 1.1-log reductions were obtained by any of the HIPEF conditions applied, *E. aerogenes* growth in both substrates (horchata and TSB) at 10°C, 12°C and 16°C was affected by HIPEF treatments. No significant differences were observed for HIPEF-treated bacteria at 12°C or 16°C, due to the wider variability in lag times observed after the treatment. The specific growth rate of the bacteria was not modified, but the lag period was prolonged (Tables 2 and 3). No significant differences were found between the three HIPEF treatments tested at incubation temperatures of 10°C, 12°C or 16°C (Tables 2 and 3) either recovering *E. aerogenes* in TSB or horchata. It has been previously indicated that inactivation by HIPEF may be an "all or nothing" event in which little or no sub-lethal injury is sustained before cells become completely inactivated (Russell et al., 2000). This is partly supported by the present study in which, when sub-lethal injury could be repaired after treatments, the subsequent growth rate was not affected. Therefore, only the lag phase, which often indicates the repair and adaptation period (Shin and Pyun, 1997), was enlarged for injured cells recovered in unfavourable conditions.

When *E. aerogenes* was incubated at 10°C in both substrates, the lag phase in HIPEF-treated cells was found to be significantly longer than in untreated cells (Tables 2 and 3), and this extension of the lag was more evident than that at 12°C or 16°C. This indicated that repair and adaptation of injured cells was affected by the incubation temperature. Therefore, the synergistic effects of HIPEF and low temperature after treatment contributed to slow down the recovery of sub-lethal damage in cells. This increased sensitivity of treated cells to low temperature and other stresses such as pH (Wouters et al., 1999; Valero et al., 2000) could be useful to establish HIPEF treatments, in order to produce high-quality safe foods with a longer shelf-life.

3.2. Effect of initial bacterial inoculum size

Growth curves obtained from treated *E. aerogenes* at 3×10^6 V m^{-1}, 19×10^{-5} s inoculated at either high or low initial bacterial concentration, in TSB (Fig. 1) or in horchata (Fig. 2), are shown. No significant differences in growth parameters were observed for untreated *E. aerogenes* for low or high inoculum levels. When HIPEF-treated *E. aerogenes* was incubated in TSB at 12°C, significant differences were observed between high

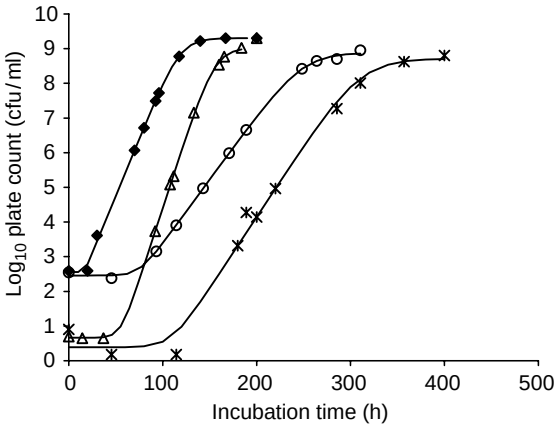

Fig. 1. Growth of *E. aerogenes* in TSB after exposure to HIPEF (3×10^6 V m^{-1}, 1.9×10^{-6} s) and high and low inoculum concentration. High inoculum level (10^3 cfu ml^{-1}) incubated at 10°C (○); low inoculum level (10^1 cfu ml^{-1}) incubated at 10°C (*); high inoculum level (10^3 cfu ml^{-1}) incubated at 12°C (◆); low inoculum level (10^1 cfu ml^{-1}) incubated at 12°C (Δ).

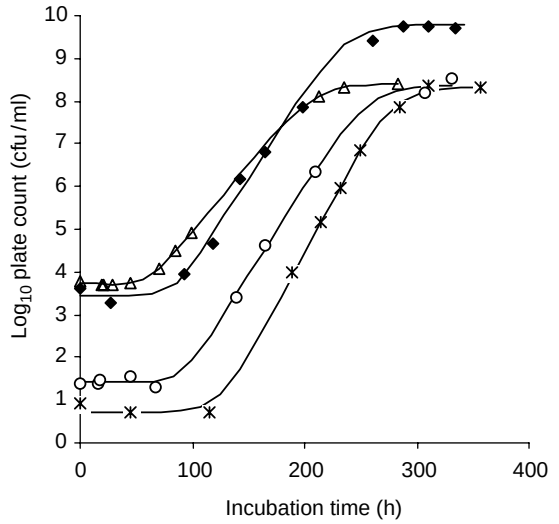

Fig. 2. Growth of *E. aerogenes* in horchata after exposure to HIPEF (3×10^6 V m^{-1}, 1.9×10^{-6} s) and high and low inoculum concentration. High inoculum level (10^3 cfu ml^{-1}) incubated at 10°C (◆); low inoculum level (10^1 cfu ml^{-1}) incubated at 10°C (*); high inoculum level (10^3 cfu ml^{-1}) incubated at 12°C (Δ); low inoculum level (10^1 cfu ml^{-1}) incubated at 12°C (○).

inoculum levels around 10^3 cfu ml^{-1} (with a lag phase of 18.7 ± 1.3 h) and low levels of 10 cfu ml^{-1} (lag phase of 53.4 ± 2.5 h) (Fig. 1). This indicated that the inoculum size determined the time needed for the damaged population to repair the injury and start growth. This delay was also observed at 10°C, with lag times for high and low inoculum levels of 92 and 131 h, respectively.

However, the growth rate was not affected, once the lag time was overcome for any of the temperatures tested (Fig. 1). This showed that the metabolic capability of the cells was fully restored, independent of the inoculum size. The lag time of untreated *E. aerogenes* cells was identical at high and low inoculum levels, which indicates that the delay in small cell populations was due to the need to repair damage and not to population variability.

When HIPEF-treated cells were inoculated in horchata substrate, the resulting growth curves showed a similar effect with low inoculum levels. At 12°C, the average lag phases obtained were significantly different (52.0 ± 5.6 and 88.6 ± 1.2 h for the high and low inoculum levels, respectively). At 10°C, they were 103.3 ± 5.3 and 125.2 ± 3.5 h. Again, the maximum specific growth rate showed no significant differences at any of the temperatures tested between high and low cell concentrations.

However, at 16°C, no significant differences were found between growth curves at high or low initial concentrations after HIPEF treatments in both substrates (TSB and horchata). These results indicate that horchata is an excellent substrate to support growth of *E. aerogenes*, since no significant differences were observed between horchata or TSB in lag times or growth rates, either at high or low inoculum levels. The behaviour of low inoculum size could partly be explained with the results reported by Gay et al. (1996), who studied the influence of preincubation temperature on growth of *L. monocytogenes*. These authors concluded that the increase in lag duration could result from the smaller starting population and the absence of synergy between cells.

Therefore, to delay proliferation of *E. aerogenes* after HIPEF treatment, a combination of unfavourable conditions is needed, this is, a low bacterial contamination and refrigeration temperatures. This is in agreement with Augustin et al. (2000), who found that the effect of small inoculum size could only be evidenced in severe stress conditions. To prevent growth of HIPEF-treated horchata, it would be necessary to control the contamination of the product with *E. aerogenes* through the line of production and to guarantee refrigeration during the distribution and storage, since recontaminations have been detected in beverage extraction processing, leading to a subsequent proliferation of bacteria (Selma et al., 2002). These two factors are critical (low contamination and refrigeration) to implement a new product, HIPEF-treated horchata, that could increase the market for this product with great potential, since it falls within the present consumer demands: natural, healthy, aditive-free, vegetable beverage.

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