Innovative Food Science and Emerging Technologies 37 (2016) 161–169

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

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

# Influence of growth and treatment temperature on *Staphylococcus aureus* resistance to pulsed electric fields: Relationship with membrane fluidity

G. Cebrián *, S. Condón, P. Mañas

*Tecnología de los Alimentos, Facultad de Veterinaria de Zaragoza, Instituto Agroalimentario de Aragón -IA2- (Universidad de Zaragoza-CITA), Zaragoza, Spain*

ARTICLE INFO

*Article history:*
Received 20 June 2016
Received in revised form 20 July 2016
Accepted 12 August 2016
Available online 13 August 2016

*Keywords:*
Pulsed electric fields
*S. aureus*
Membrane fluidity
Growth temperature
Benzyl alcohol
Combined process

ABSTRACT

The influence of growth and treatment temperature (10–42 °C) on *Staphylococcus aureus* resistance to Pulsed Electric Fields (PEF) was investigated. No statistical differences in resistance to PEF (26 kV/cm) ($p > 0.05$) were detected between *S. aureus* cells grown at 10, 20, 37, or 42 °C. In contrast, suspensions grown at 30 °C displayed significantly ($p < 0.05$) higher resistance than suspensions grown at the other 4 temperatures tested. On the other hand, an increase of treatment temperature from 10 to 42 °C resulted in a progressive decrease in PEF resistance for all suspensions. It is noteworthy that this decrease was of similar magnitude regardless of the growth temperature of *S. aureus* cells. The increase in treatment temperature also led to a fluidization of *S. aureus* cell membranes, measured by fluorescence anisotropy. However, this increase in fluidity was greater for cells grown at lower temperatures (10–20 °C) than for cells grown at higher ones (30–42 °C). Cells grown at 30 °C, which had shown a higher PEF tolerance, did not have a more rigid membrane than cells grown at 37 or 42 °C. The addition of benzyl alcohol (20 mM), which led to a fluidization of bacterial membranes, did not render *S. aureus* cells more PEF sensitive. Altogether, the results obtained cast doubt on the existence of a direct causal relationship between average membrane fluidity and the PEF resistance of bacterial cells.

*Industrial relevance:* Understanding the mechanisms of bacterial resistance to PEF is essential for in Pulsed Electric Fields process optimization and PEF-based combined processes design. Form a practical point of view, results here reported will contribute to understand the mechanisms of treatment temperature dependent bacterial sensitization to PEF. This is of particular interest regarding combined processes involving heat and PEF.



## 1. Introduction

Pulsed Electric Fields (PEF) is one of the most promising non-thermal technologies that have been proposed in order to satisfy consumer demands for foods that are fresher, less reliant on preservatives and less heavily processed, but still microbiologically safe (Mañas & Pagán, 2005; Raso & Barbosa-Cánovas, 2003). This technology mainly consists in the application of short-duration (1–100 μs), high electric field pulses (10–50 kV/cm) to food placed between two electrodes (Heinz, Álvarez, Angersbach, & Knorr, 2001). PEF's major advantage is its ability to inactivate microorganisms while causing very few changes in food sensory and nutritional quality (Hodgins, Mittal, & Griffiths, 2002; Jia, Zhang, & Min, 1999). However, and despite the increasing number of publications dealing with bacterial inactivation using this technology, many aspects are still not fully understood.

One of the questions that has generated the most controversy is the influence of growth temperature on the resistance of bacterial to PEF treatments. In most studies it has been observed that cells grown at different temperatures display different resistances to PEF (Álvarez, Condón, & Raso, 2006), but whereas some authors have observed that cells grown at higher temperatures are more PEF-resistant (Álvarez, Raso, Sala, & Condón, 2003; Russell, 2002) others have observed the opposite effect (Cebrián, Sagarzazu, Pagán, Condón, & Mañas, 2008). Also, depending on microbial species, non-dependence of PEF resistance on growth temperature (Álvarez, Pagán, Raso, & Condón, 2002) and higher PEF resistance when cells were grown at optimal temperatures (Ohshima, Akuyama, & Sato, 2002) has been reported. It is well known that a series of physiological changes occur in bacterial cells depending on growth temperature (Beuchat, 1978; Chastanet, Fert, & Msadek, 2003; Schumann, 2007) but, since bacterial membranes are considered to be the structure targeted by PEF technology (Mañas & Pagán, 2005), most authors have speculated that the changes that affect membrane stability and structure might be responsible for the differences in resistance observed.

On the other hand, although PEF technology is non-thermal, various authors have proposed the combination of PEF treatments with sublethal, or even lethal temperatures in order to increase the lethality of the process (Heinz, Toepfl, & Knorr, 2003). Those combinations have

* Corresponding author at: Tecnología de los Alimentos, Facultad de Veterinaria, C/ Miguel Servet, 177, 50013, Zaragoza, Spain.
*E-mail address:* guiceb@unizar.es (G. Cebrián).

http://dx.doi.org/10.1016/j.ifset.2016.08.011
1466-8564/

indeed been proven to enhance the lethal effect of PEF (Aronsson & Ronner, 2001; Álvarez et al., 2006; Heinz et al., 2003; Hodgins et al., 2002; Jayaram, Castle, & Margaritis, 1991; Pothakamury, Vega, Zhang, Barbosa-Cánovas, & Swanson, 1996; Reina, Jin, Zhang, & Youself, 1998; Sepúlveda, Góngora-Nieto, San-Martin, & Barbosa-Cánovas, 2005; Saldaña, Monfort, Condón, Raso, & Álvarez, 2012; Saldaña et al., 2010; Zhang, Monsalve-González, Barbosa-Cánovas, & Swanson, 1994). However, the exact mechanisms leading to the increase in the efficacy of PEF for microbial inactivation have not been yet determined. Among the theories proposed to explain this improvement in lethality, it has been suggested that the increased sensitivity of bacterial cells at high temperatures might be due to the changes in the phase state of the membrane occurring as a consequence of the increase in temperature: in other words, fluidization of bacterial membranes would make cells more sensitive to PEF.

Given the central role of the cell membrane on bacterial resistance to PEF, this investigation's objective was to determine whether the changes in membrane fluidity occurring as a consequence of changes in growth and treatment temperature might be accountable for differences in resistance to PEF. For this investigation *Staphylococcus aureus* was chosen as a Gram-positive pathogenic model microorganism (Cebrián, Sagarzazu, Pagán, Condón, & Mañas, 2007).

## 2. Materials and methods

### 2.1. Bacterial culture and media

*Staphylococcus aureus* CECT 4459 was used in this study. The bacterial culture was maintained frozen in cryovials. Stationary-phase cultures were prepared by inoculating 10 ml of tryptone soya broth (Biolife, Milan, Italy) supplemented with 0.6% yeast extract (Biolife) (TSB-YE) with a loopful of growth from tryptone soy agar supplemented with 0.6% yeast extract (TSA-YE) (Biolife), and by incubating the resulting culture overnight at 37 °C in a shaking incubator. 50 μl of this culture were inoculated into 50 ml of fresh TSB-YE at 42, 37, 30, 20 or 10 °C. For each culture temperature, samples were taken after different incubation times to construct growth curves and to determine the time required to reach stationary phase of growth. For resistance determinations, cultures were incubated for 18, 24, 24, 48 and 172 h (7 days) at 42, 37, 30, 20 and 10 °C, respectively, time needed to reach the stationary phase of growth (approximately 6–8 $\times 10^8$ cells/ml; data not shown).

### 2.2. PEF treatments

PEF treatments were carried out in exponential waveform pulse equipment (Cebrián, Mañas, & Condón, 2015; Heinz et al., 2003). High electric field pulses (pulse width ≈ 4μs) were produced by discharging a set of 10 capacitors via a thyristor switch (Behlke, Kronberg, Germany) in a batch treatment chamber. The capacitors were charged using a high voltage DC power supply (FUG, Rosenhein, Germany), and a function generator (Tektronix, Wilsonville, OR, USA) delivered the on-time signal to the switch. The treatment chamber was made of a cylindrical plastic tube closed with two polished stainless steel electrodes. The gap between electrodes was 0.25 cm and the electrode area was 2.01 $cm^2$. The actual electric field strength and electrical intensity applied were measured in the treatment chamber with a high voltage probe and a current probe respectively, connected to an oscilloscope (Tektronix). The energy associated with pulses at electric field strengths of 18, 22, 26 and 30 kV/cm was 2.24, 3.47, 4.25 and 5.83 $kJ/kg^{-1}$, respectively.

In order to adjust the temperature of the sample during PEF treatments, an ethylene-glycol solution was pumped through the ground electrode as previously described in Saldaña et al. (2010). In addition, in order to minimize sample heating, pulse frequency was fixed at 0.5 Hz. Under these conditions the increase in sample temperature -measured as described in Raso, Álvarez, Condón, and Sala (2000)- as a result of PEF treatments never exceeded 1 °C.

Prior to PEF treatments, bacterial cultures were centrifuged at 6000 × *g* for 5 min, washed once and diluted 1/10 in citrate-phosphate buffer of pH 7.0 (Dawson, Elliot, Elliot, & Jones, 1974) with conductivity adjusted to 2 mS/cm at the desired treatment temperature. The diluted microbial suspension was placed in the treatment chamber with a sterile syringe and PEF-treated. After treatments, appropriate serial dilutions were prepared in sterile TSB-YE and pour-plated.

### 2.3. Incubation of treated samples, survival count, and calculation of resistance parameters

The recovery medium was TSA-YE. Plates were incubated for 24 h at 37 °C; after incubation, colony-forming units (CFU) were counted.

Survival curves were obtained by plotting the logarithm of the fraction of survivors vs. treatment time, expressed as number of pulses. As concave upwards survival curves were observed, a mathematical model based on the Weibull distribution was used to fit experimental data (Álvarez et al., 2003). This model is described by the equation (Mafart, Couvert, Gaillard, & Leguerinel, 2002):

$$Log_{10}S(t) = -(t/\delta)^{\rho} \quad (1)$$

where $S(t)$ is the survival fraction; $t$, the treatment time (number of pulses); and $\delta$ and $\rho$ are the scale and the shape parameter, respectively. The $\delta$ value represents the number of pulses needed to reduce the first log cycle of the population, while the $\rho$ parameter indicates the survival curve's shape. $\rho$ values equal to 1 correspond to straight survival curves, $\rho$ values >1 to convex profiles and $\rho$ values <1 to concave profiles. To fit the model to the experimental data and to calculate the $\delta$ and $\rho$ parameters, GraphPad PRISM® (GraphPad Software, Inc., San Diego, Calif., U.S.A.) software was used, applying the least-squares criterion. The same software was used to calculate determination coefficients ($r^2$ values) and standard deviations, and to perform statistical analyses (analysis of variance and $t$-test) ($p = 0.05$).

All resistance determinations were performed at least three times on separate workdays. Error bars in figures correspond to the standard deviation from the mean.

### 2.4. Measurement of fluorescence anisotropy

Fluorescence anisotropy of the probe DPH (1, 6-diphenyl 1,3,5-hexatriene) (Sigma, St. Louis, USA) was used to monitor changes in membrane fluidity (Aricha et al., 2004; Zaritsky, Parola, Ibdah, & Masalha, 1985). Anisotropy values ($r$) are inversely related to membrane fluidity (Shinitzky, 1984). Briefly, samples of bacterial cultures were washed twice with PBS containing 0.25% formaldehyde (pH 7.4) for fixation, and then incubated for 45 min at 37 °C with $5 \times 10^{-7}$ M DPH (added as a $10^{-4}$ M solution in tetrahydrofuran) for probe insertion in the membrane. Steady-state fluorescence anisotropy was measured at different temperatures (between 10 and 42 °C) with a Cary-Eclipse spectrofluorometer provided with a manual polarizer accessory (Varian Inc., Mulgrave, Victoria, Australia), with excitation at 355 nm and emission at 425 nm, 5- and 5-nm slits, respectively, and a 3-s integration time.

Anisotropy values ($r$) were calculated according to Shinitzky (1984):

$$r = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2.G.I_{VH}} \text{ with } G = \frac{I_{HV}}{I_{HH}} \quad (2)$$

$_V$ and $_H$ stand for polarisation direction (vertical and horizontal directions) I: corrected fluorescence intensity obtained by:

$$I_{HH} = I_{(L)HH} - I_{(BUFFER+CELL)HH} - I_{(BUFFER+PROBE)HH} + I_{(BUFFER)HH} \quad (3)$$