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Behavior of the surviving population of *Lactobacillus plantarum* 564 upon the application of pulsed electric fields

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ABSTRACT

The behavior of the surviving population of *Lactobacillus plantarum* 564 growing in MRS broth after pulsed electric field (PEF) treatments of different intensities was monitored by isothermal calorimetry, optical density and plate counts. Bacterial cells were treated with monopolar square pulses at varying nominal electric field strengths and number of pulses, corresponding to applied energies of 34.6, 65.8 and 658.1 J/cm^3. After the PEF treatment, samples were inoculated into the MRS broth and incubated at 37 °C. The presented results show that surviving bacterial cells resume growth after a treatment-dependent delay. Both the untreated and treated cultures had similar growth rates, but the latter showed a higher growth rate during the late-growth phase, and the growth rate increased with the intensity of applied electric field. After the PEF treatment, the surviving population of bacteria was less susceptible to killing by further PEF application, showing that this subpopulation was less sensitive to the PEF treatment and could grow again.

Industrial relevance: The application of pulsed electric field (PEF) technology as a non-thermal alternative to traditional pasteurization of liquid foods has received considerable attention during the last years. Effective inactivation for most of the spoilage and pathogenic microorganisms has been shown in fruit and vegetable juices and milk with little or no impact on nutritional and sensorial properties of the food. However, very little is known about the growth abilities of the surviving population. Ensuring food safety requires a better understanding of the behavior of the surviving populations of microorganisms which may be recovering from sub lethal injury, such as PEF-induced stress. This paper reveals that the surviving population of the bacteria subjected to the PEF treatment could grow again, showing higher growth rates as the intensity of the PEF treatment increased. Also, the new bacterial population showed higher resistance to further PEF treatment. Therefore, for industrial application of the PEF technology, an in-depth characterization of surviving microorganisms in the treated product is required. Moreover, the evidence of bacterial persistence indicates that the PEF technology, as a non-thermal alternative to traditional pasteurization, could not completely replace thermal treatment, but can be applied as a supplement treatment.

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1. Introduction

Exposure of biological cells to a sufficiently strong external electric field results in transiently or permanently increased permeability of cell membranes, referred to as electroporation. High intensity pulsed electric fields (PEF) applied under conditions causing electroporation-induced death of microorganisms have been applied to a range of liquid food products such as fruit juices and milk with a reported increase of shelf life compared to untreated products (Heinz, Alvarez, Angersbach, & Knorr, 2002; Pagan, Condón, & Raso, 2005; Wan, Coventry, Swiergon, Sanguansri, & Versteeg, 2009).

During the last decade, a large amount of research and development activities have been carried out regarding the efficiency of the PEF treatment for microbial inactivation. Levels of inactivation will depend on microbial strain, growth medium and PEF conditions (Cortese et al., 2011). As examples, inactivation of 2 Log_{10} units of *Escherichia coli* ATCC 35218 in orange juice was achieved with 22 kV/cm and 45 °C, while 5 Log_{10} units inactivation of *E. coli* K12 was achieved with a slightly lower amplitude of 20 kV/cm but a higher temperature of 50 °C (Gurtler, Rivera, Zhang, & Geveke, 2010). A higher field strength of 34.8 kV/cm induced a 4-Log_{10} unit reduction of *Lactobacillus plantarum* in model beer (Ulmer, Heinz, Gänzle, Knorr, & Vogel, 2002). However, very little is known about the growth abilities of the surviving

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population. When a population of bacterial cells is exposed to a sufficiently strong treatment, the majority of the population is inactivated. By the time the treatment finishes, a small fraction of cells still survives which may resume growth and form a new population. Therefore, ensuring food safety requires a better understanding of the behavior of the surviving populations of microorganisms (Aronsson, Borch, Stenlöf, & Rönner, 2004) which may be recovering from sub lethal injury, such as PEF-induced stress.

There is considerable variation in the response of bacterial population to stress even within a genetically identical population residing in a homogeneous environment (Booth, 2002). The heterogeneity of the stress response is of high interest in treatments aimed at the death of the bacteria and it is an important criterion for judging optimal effective treatments (Ingham, Beerthuyzen, & Vlieg van Hylckama, 2008). Therefore, the identification of subpopulations such as "persisters" without a genetically acquired resistance to the treatment (Balaban, Merrin, Chait, Kowalik, & Leibler, 2004; Ingham et al., 2008) and their sensitivity to stress is essential for assessing the effect of the PEF treatment.

In this paper, the behavior of a surviving population of the strain *L. plantarum* 564 after the PEF treatment of different intensities was monitored by isothermal calorimetry. The calorimetrically measured rate of heat production is a criterion of the metabolic rate and provides a direct indication of integrated metabolic responses (Criddle, Breindenbach, & Hansen, 1991). Isothermal calorimetry has been used to measure the heat of microbial growth during fermentation and to evaluate the effect of thermal treatments and antimicrobial agents (Selma, MacNaughtan, Mitchell, & Waites, 2007; Wadsö & Gómez Galindo, 2009).

2. Materials and methods

2.1. Bacterial strain and inoculums preparation

Lactic acid bacteria strain *L. plantarum* 564 was isolated from Sjenica cheese, an autochthonous Serbian white cheese in brine (Radulović et al., 2011). The strain belongs to the collection of the Division of Industrial Microbiology, Faculty of Agriculture, University of Belgrade, Serbia. The starting culture was stored in vials with 20% (v/v) glycerol at −80 °C, from which the stocks were subsequently prepared. The stocks were prepared by inoculating the starter culture into De Man–Rogosa–Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, England) with 0.5% (v/v) inoculum, before propagation for 3 days at 37 °C. One ml of the activated culture was inoculated into 9 ml of MRS broth with 15% (v/v) glycerol, transfered to sterile vials and stored at −20 °C. This culture was again activated by inoculation into the MRS broth with 0.5% (v/v) inoculum to a final concentration of 10^7 cfu/ml. The culture was incubated at 37 °C overnight and propagated within the period of 3 days. Next day, the culture was reinoculated and treated by PEF after 4 h of incubation. Preliminary experiments showed that the culture growth reached mid-exponential phase after 4 h of incubation.

2.2. Electric treatments

The cell suspension was diluted with sterilized distilled water at room temperature in a 1:10 ratio and placed into sterile electroporation cuvettes (1 mm gap, EP-104, Cell Projects, Harrietsham Kent, UK), which were filled to totally cover the electrodes. The cell concentration in the sample was determined to be 8.6 E6 cfu/ml and its conductivity was 1.3 mS/cm.

Electric pulses were delivered by using a CEPT pulse generator (Arc Aroma Pure, Lund, Sweden). A digital oscilloscope (Fluke 123, Washington) was connected to the system to monitor the delivery of the pulses to the sample. The cell suspensions were treated with monopolar squared pulses at varying nominal electric field strengths and number of pulses as follows: 22.9 kV/cm, 10 pulses; 31.6 kV/cm, 10 pulses and 31.6 kV/cm, 100 pulses. The pulse width was 5 μs and the period between pulses was 10 ms. The energy input given by the applied PEF treatments was calculated as (Dejmek & Trägårdh, 1994):

$$\omega = E^2 \lambda \, t \quad (1)$$

where E is the electric field strength, λ is the conductivity and t is the treatment time. The calculated energy input corresponded to 34.6, 65.8 and 658.1 J/cm^3. Each treatment was done in duplicates. The control bacterial samples were placed into the sterile cuvettes connected to the pulse generator but were kept untreated.

After the PEF treatment, bacterial samples were inoculated into the MRS broth to a final concentration of 10^7 cfu/ml and incubated at 37 °C for analysis within 24 h.

2.3. Isothermal calorimetry analysis

The rate of heat production after the application of PEF was continuously measured in a TAM Air isothermal calorimeter (Thermometric AB, Järfälla, Sweden), as described by Rocculi et al. (2007). This calorimeter has a sensitivity (precision) of ±10 μW (Wadsö, 2005) and contains eight twin calorimeters. In each calorimeter, heat is allowed to flow between the reaction vessel containing the sample and a heat sink, the temperature of which is kept essentially constant. The heat transfer takes place through a heat flow sensor that is located between the vessel and the heat sink.

Each bacterial sample was transferred from the PEF cuvette to one ampoule with 10 ml MRS broth to bring to a final concentration of 0.5% (v/v), thermostated at 37 °C for 30 min, sealed and placed in the calorimeter at 37 °C. Each calorimeter had its own reference that consisted of a sealed 20 ml glass ampoule containing 10 ml of water. The eight calorimeters permitted simultaneous measurements of the rate of heat production of the ampoules containing the bacterial cells. In this way, controls and treated samples were distributed in the calorimeters. Two complete replicates were recorded for each PEF condition.

The isothermal measurements were performed for the period of 24 h. Baselines (BL) were recorded before or after each measurement. The primary output from the heat flow sensors in the calorimeters (a voltage) was recorded by computer from the digitized output of the calorimeters. The corresponding thermal powers (heat production rates) were calculated by the following equation:

$$P = \varepsilon \, (V_s - V_{BL})/M \quad (2)$$

where P (mW g^{-1}) is the specific thermal power of the sample, ε (mW mV^{-1}) the calibration coefficient of the calorimeter, V_S (mV) the voltage signal from the calorimeter, V_{BL} (mV) the corresponding voltage recorded for the baseline and M (g) is the mass of the sample. The calibration coefficients were calculated from electrical calibrations made at 37 °C with heaters (100.0 Ω precision resistors) placed in the same type of ampoule as used in the experiments.

2.4. Plate counts

After the PEF treatment, replicate bacterial samples were incubated for 24 h at 37 °C. Plate counts were done to evaluate the effect of the different PEF treatments and to evaluate the population of the bacteria at selected time points of the calorimetry measurements. After each time point, samples were serially diluted in sterile 0.1% (w/v) peptone physiological salt solution (Oxoid Ltd., Basingstoke, UK). One ml of the appropriate dilutions was spread out on plastic petri dishes and 10 ml of MRS agar was poured out on the plates. After incubation at 37 °C for 48 h under anaerobic conditions in a gas-pack system (Oxoid Ltd., Cambridge, UK) the colonies were counted.

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