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Use of a fluorescent viability stain to assess lethal and sublethal injury in food-borne bacteria exposed to high-intensity pulsed electric fields

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ABSTRACT

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Aims: To apply scanning electron microscopy, image analysis and a fluorescent viability stain to assess lethal and sublethal in food-borne bacteria exposed to high-intensity pulsed electric fields (PEF).

Methods and Results: A rapid cellular staining method using the fluorescent redox probes 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 4′,6-diamidino-2-phylindole was used for enumerating actively respiring cells of *Listeria mononcytogenes*, *Bacillus cereus* and *Escherichia coli*. This respiratory staining (RS) approach provided good agreement with the conventional plate count agar method for enumerating untreated and high-intensity PEF-treated bacteria suspended in 0.1% (w/v) peptone water. However, test organisms subjected to similar levels of lethality by heating at 56°C resulted in *ca* 3-log-unit difference in surviving cell numbers ml^{-1} when enumerated by these different viability indicators. PEF-treated bacteria were markedly altered at the cellular level when examined by scanning electron microscopy.

Conclusions: While PEF-treatment did not produce sublethally injured cells ($P < 0.05$), substantial subpopulations of test bacteria rendered incapable of forming colonies by heating may remain metabolically active.

Significance and Impact of the Study: The fluorescent staining method offers interesting perspectives on assessing established and novel microbial inactivation methods. Use of this approach may also provide a better understanding of the mechanisms involved in microbial inactivation induced by PEF.

Keywords: fluorescent metabolic probes, microbial inactivation, pulsed electric fields, scanning electron microscopy, sublethal injury.

INTRODUCTION

The potential to utilize high intensity, pulsed electric field (PEF) technology for food processing applications is currently receiving considerable attention as this electro-technology may significantly improve the quality of certain liquid food products (Heinz *et al.* 2001; Wouters *et al.* 2001). As an emerging nonthermal food preservation method, PEF pasteurization is of interest to the food industry as this treatment technology has the potential to retain the organoleptic and nutritive properties that are characteristic of fresh food products. PEF treatment involves the application of PEF with a magnitude usually greater than 20 kV cm^{-1}, for very short durations (500 ns to 4 μs), to liquid foods (MacGregor *et al.* 2000; Heinz *et al.* 2001). Numerous research groups have demonstrated the biocidal potential of PEF, having shown that a wide range of food spoilage and pathogenic micro-organisms can be inactivated in test liquids and in various food products (MacGregor *et al.* 2000; Heinz *et al.* 2001; Wouters *et al.* 2001). Whilst the mechanism(s) underlying the inactivation

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of micro-organisms by PEF remains to be fully elucidated, the most commonly suggested theory is that of severe electroporation (i.e. the formation of pores in cell membranes by the action of high voltage electric-fields) (Wouters *et al.* 2001). Despite intensive scientific and developmental interests in PEF-technology, very little research has been directed towards gaining a better understanding of the recovery processes associated with sublethally injured micro-organisms after PEF treatment: previous studies have relied heavily on enumerating microbial survivors using conventional plate count agar techniques.

In this study, image analysis and the novel fluorescent redox probe 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) were used to investigate respiratory activity in *Listeria monocytogenes*, *Bacillus cereus* and *Escherichia coli* cells that were exposed to separate PEF and heat treatments. In particular, enumeration of PEF and heat-injured cells by respiratory staining was compared with similarly treated samples that were enumerated by the conventional plate count agar method. Here, we report that the former epifluorescence approach offers a rapid and quantitative assay for determining the extent of sublethal cellular injury in heat and PEF-treated food-borne bacteria.

MATERIALS AND METHODS

Bacterial strains used

Listeria monocytogenes (NCTC 11994), the diarrhoeagenic strain of *B. cereus* (NCTC 11145), and *E. coli* (NCTC 9001) used in this study were obtained from the National Collection of Type Cultures (Public Health Laboratory Service, Colindale, UK). Cells were initially grown in 50-ml typtone soya broth supplemented with 0·6% yeast extract (TSYEB) for 24 h at 37°C with agitation (125 rev min^{-1}). Following this, a 1-ml sample of the 10^{-5} dilution was transferred to fresh 50-ml TSYEB (giving a starting cell population of *ca* 10^3 cells ml^{-1}), and was incubated at 125 rev min^{-1} for 6 h at 37°C to obtain vegetative cells in the mid-exponential growing phase. Cells were pelleted by centrifugation (3000 *g* for 10 min at 4°C), and were washed three times and resuspended in 0·1% (w/v) peptone water to yield a suspension of 10^7 CFU of test organisms ml^{-1} (determined spectrophotometrically at 540 nm, model UV-120-02 instrument; Shimadzu Corp., Kyoto, Japan) before transfer to the PEF treatment chamber. The absence of bacterial endospores was confirmed by spore-staining of triplicate samples of *B. cereus* and by heating at 80°C for 15 min prior to enumeration on TSYEA plates. Stored bacteria were kept at −70°C in phosphate-buffered saline (PBS) with 20% glycerol (v/v) until used.

PEF treatment of test bacteria

Test bacteria were suspended in 30 ml of 0·1% (w/v) peptone water and were subjected to high voltage, PEF (*ca* 30 kV cm^{-1}) in a circulated, closed-loop system. This comprised a holding reservoir, treatment chamber, interconnections, and peristaltic pump as described previously, with modifications (MacGregor *et al.* 2000). The flow rate of the system was 200 ml min^{-1}, the volume of the system was *ca* 100 ml, and the test or treatment chamber volume was 30 ml. The test chamber consisted of a disk of 10 mm Perspex (SamTech Ltd, Glasgow, UK), with a central hole cut through it to hold the 30 ml volume of sample. Two separate channels were drilled from the outer edge of the Perspex to the central hole, thus allowing for syringe injection and removal of samples. Flat brass plates were fitted on both sides of the central hole, which formed the electrodes of the test chamber. Predetermined cell populations were treated with 7000–8000 pulses at 25°C (in order to achieve *ca* 5 log reduction in CFU of test bacteria ml^{-1} as determined by the conventional plate count method), at a pulse repetition frequency that was limited to 5 pulses per second in order to ensure that there were no thermal inactivation effects associated with the energy dissipation in the test chamber. It should be noted, the PEF system was not optimized for inactivation of test bacteria. The test chamber was immersed in a circulating constant temperature water bath (model HE30; Grant Instruments Ltd, Cambridge, UK) equipped with a thermoregulator capable of maintaining temperature to within ±0·05°C (model TE-8A; Techne Ltd, Cambridge, UK) to maintain the desired treatment temperatures at 25°C. A thermocouple was also employed throughout the studies in order to verify the temperature of the treated liquid. A 100-kV high voltage DC generator (Glassman EH50R02; Glassmann Europe Ltd, Hampshire, UK) was used to charge a co-axial cable Blumlein pulse generator (Type TLG B-01, Samtech Ltd, Glasgow, UK) through a charging resistance of 10 MΩ. The co-axial cable generator was constructed from 100 m of URM67, 40 kV cable (Samtech).

The high voltage output pulse from the generator was 500 ns in duration, and the generator had an output impedance of 100 Ω, a switching impedance of 50 Ω, and an open circuit gain of two. The generator was wound inductively on a 30-cm diameter former in order to minimize secondary transmission line losses. The electrical and charging circuits were arranged to ensure that the generator charging currents did not flow through the test cells. The pulse generator was charged from one end of the cable, and was fired by switching the inner conductor to ground at the opposite end. Although both sides of the test chamber were earthed, the output from the cable generator, which was connected to one side of the test chamber, was

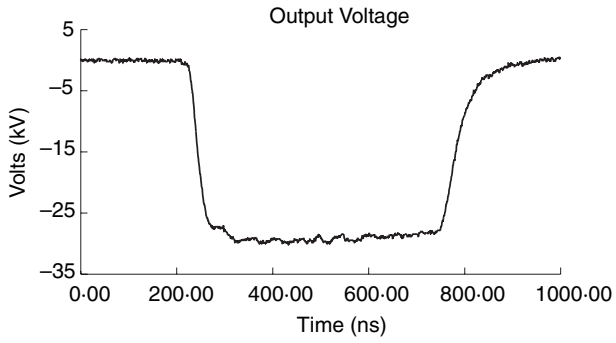

Fig. 1 Measured output pulse waveform applied to the test chamber for PEF treatment

transiently de-coupled from earth during application of the voltage pulse. The Blumlein generator was fired using a triggered corona stabilized switch. The output pulse from the generator was monitored throughout the experiments using a 1000 : 1, Tektronix high voltage probe (Tektronix P6015A; Imex Ltd, Coatbridge, UK). The voltage pulse profile used for PEF treatment, measured across the test cell, is shown in Fig. 1. Recovery of surviving populations (log CFU of test bacteria ml^{-1}), was determined for experimental and control suspensions and dilutions thereof by spread and spiral plating samples (model B; Sprial Systems Inc., Shipley, UK) onto TSYEA agar (Oxoid) plates; these were incubated for 48 h at 37°C before enumeration.

The test bacteria, *L. monocytogenes*, *B. cereus* and *E. coli* were also subjected to heating at 56°C in order to achieve a similar level of inactivation (*ca* 5-log units in CFU ml^{-1}) to that obtained by PEF treatment, according to methods described previously (Rowan and Anderson 1998). Test bacteria were suspended in 1·9 ml of preheated 0·1% (w/v) peptone water to a density of *ca* 10^7 CFU ml^{-1} in 3-ml shrimp cap glass vials (Phase Separations Ltd, Watford, Hertfordshire, UK). The vials were sealed and kept below the level of a circulating constant temperature water bath (model HE30; Grant Instruments Ltd) equipped with a thermoregulator capable of maintaining temperature to within ±0·05°C (model TE-8A; Techne Ltd). The vials were sealed and kept 4 cm below the level in the water bath for the treatment period. Recovery and enumeration of surviving populations of the test bacteria were performed as mentioned above.

Use of image analysis, fluorescent redox probes and conventional plating counting method to enumerate cell numbers after heating and PEF treatment

Image analysis and the fluorescent redox probes CTC and 4′,6-diamidino-2-phylindole (DAPI) were used to investigate cellular activity in the test bacteria according to previously described procedures, with modifications (Rodriguez *et al.* 1992; Gavin *et al.* 2000). One-millilitre cell suspensions were harvested by centrifugation (4°C for 10 min at 3000 *g*) and washed three times with 0·1 M phosphate-buffered saline (PBS). Experimental (i.e. PEF and heat-treated samples) and control preparations, and dilutions thereof, were resuspended in 300 μl of 5 mM CTC (Polysciences, Inc, St Louis, MO, USA) and were incubated for 1 h in the dark at 20°C with agitation (200 rev min^{-1}). CTC is readily reduced to insoluble, highly fluorescent and intracellularly accumulated CTC-formazon, through bacterial respiration. After incubation, experimental and control preparations, and dilutions thereof, were counterstained for 8 min at 20°C with 5 μg of DAPI ml^{-1} (Sigma, St Louis, MO, USA) and were enumerated using methods described previously with modifications (Besnard *et al.* 2000; Gavin *et al.* 2000). Counterstaining with the DNA-binding DAPI allowed concurrent determinations of total (i.e. viable plus nonviable) bacteria and viable (i.e. only cells exhibiting red CTC-formazan fluorescence) bacteria. Epifluorescence observations of CTC-treated preparations were microscopically examined (Nikon Optiphot microscope, Tokyo, Japan) using a blue 420–480-nm excitation filter (combined with a 580-nm dichroic mirror and a 590-nm barrier filter), where eight to 10 fields were counted at a magnification of ×1,000 (expressed in terms of $\log_{10}$ number of corresponding bacteria ml^{-1} of sample). CTC- and DAPI-strained bacteria in the same preparation were also viewed and counted simultaneously with a 365-nm-excitation filter, and emission filter and a 400-nm cutoff filter. Stained cells were distinguished from nonspecific reactions by overlaying the fluorescence and phase-contrast images. The image analysis system comprised a Sony charge-coupled device camera and a Seescan Solitaire image analyser (both Seescan Ltd, Cambridge, UK) with archiving to hard disk. Direct microscopic counts of bacterial suspensions were also carried out by using bright-field microscopy and Thoma counting chamber preparations (Gunasekera *et al.* 2002).

Assessment of PEF-treated bacteria for cellular damage by using scanning electron microscopy

PEF-treated samples were examined visually for cellular damage by using scanning electron microscopy where test bacteria were resuspended for 1 h in 0·1 M phosphate buffer containing 2·5% gluteraldehyde. Fixed cells were subjected to three 5-min washes in 0·1 M phosphate buffer containing 2% (w/v) glucose and were stained for 1 h using 1% osmium tetroxide. Samples were rinsed three times in sterile distilled water and were then treated in the dark for 1 h with 0·5% aqueous uranyl acetate. Samples were then placed on 0·2 μm polycarbonate filters and dehydrated using 10-min

immersions in an increasing series of acetone (30, 50, 70 and 90%, and absolute). Samples were left submerged in dried absolute acetone overnight before transfer to critical point dryer for 1·5 h where the liquid CO_2 was changed every 15 min. Samples were then mounted on carbon double side tape and gold coated using a scanning electron microscopy coating system (Polaran SC515; Quorum Technologies, Newhaven, UK). Samples were viewed using a Philips 500 scanning electron microscope (Eindhoven, the Netherlands).

Statistical analysis

All of the experiments in this study were performed in triplicate, and results are reported as averages. Significant differences in the experimental (heating and PEF treatment) and untreated control results were calculated at the 95% confidence interval using analysis of variance (oneway) with Minitab software Release 11 (Minitab Inc., State College, PA, USA).

RESULTS AND DISCUSSION

Thermal treatment or holding times required to achieve 5 log reductions in cell populations for *L. monocytogenes*, *B. cereus* and *E. coli* at 56°C were 44, 38 and 42 min, respectively. This corresponded to $D_{56°C}$ values (decimal reduction time: the time required to kill a 1-log unit concentration of bacteria) of 8·8, 7·6 and 8·4 min, respectively (data not shown). Good agreement was obtained between the CTC-fluorescence or respiratory staining (RS) method and the conventional plate count agar (PC) method for enumerating untreated cell populations of test bacteria ($P < 0·05$) (Table 1). The PC method demonstrated that heat-treated samples of *L. monocytogenes*, *B. cereus* and *E. coli* were reduced by 5·2, 5·3 and 5·3 log CFU ml^{-1}, respectively: this markedly contrasted with reductions of 2·6, 3·0 and 2·2 log cell numbers of actively respiring bacteria ml^{-1} as determined by the rapid RS ($P < 0·05$) (Table 1). However, no significant difference was observed between conventional PC and rapid RS methods after enumerating PEF-treated *L. monocytogenes*, *B. cereus* and *E. coli* cell populations ($P < 0·05$) (Table 1). Results showed that the 4·2, 5·1, and 3·7 log unit reductions recorded by the PC method for the respective PEF-treated bacteria were similar to that of the respective 3·9, 4·6, and 3·2 log unit reductions determined by the rapid RS approach ($P < 0·05$) (Table 1). While *L. monocytogenes* and *E. coli* appeared equally heat and PEF tolerant, the larger *B. cereus* cells were more susceptible to the lethal action of both PEF treatment. The latter finding is in agreement with other researchers, where larger microbial cells such as yeast are more susceptible to irreversible electroporation (Heinz *et al.* 2001; Wouters *et al.* 2001). Use of image analysis and respiratory staining showed that the PC method did not significantly underestimate the numbers of cell survivors after PEF treatment, which markedly contrasted with the *ca* 3-log unit difference in surviving cell populations that had been heated. The latter results agree with the recent findings of Gunasekera *et al.* (2002), where these researchers used *de novo* expression of a *gfp* reporter gene and membrane integrity based on propidium iodide exclusion as viability indicators to show that a substantial portion of *E. coli* and *Pseudomonas putida* cells in heat-treated milk are metabolically active but are incapable of forming colonies.

Use of fluorescent redox probes for direct visualization of actively respiring bacteria is gaining in popularity amongst research groups investigating the viable but not culturable (VBNC) phenomenon (i.e. stress-injured bacteria which are not capable of the cellular division required for growth in the usual culture media, but, yet remain physically active for several metabolic functions) in food and water-borne bacteria (Besnard *et al.* 2000; Caruso *et al.* 2003). There is increasing evidence for the existence of a VBNC state in microbes, particularly in the stressed environment presented by modern foods with their varied pretreatment and packaging strategies (Rowan 1999). In particular, use has been made of CTC as a metabolic staining technique for reporting the possible existence of VBNC states in *Micrococcus luteus* (Kaprelyants and Kell 1993), *L. monocytogenes* (Besnard *et al.* 2000) and in *E. coli* (Caruso *et al.* 2003): CTC reduction reflects the presence of a functional electron transport (i.e. respiratory) system in the microbial cell

Table 1 Enumeration of *Listeria monocytogenes*, *Bacillus cereus* and *Escherichia coli* cell numbers by respiratory staining (RS) and CFU by conventional plate counting (PC) after heating or PEF treatment

	Log CFU or cell numbers ml^{-1}					
Test bacteria	Untreated (PC)	Untreated (RS)	Heat (PC)	Heat (RS)	PEF (PC)	PEF (RS)
L. monocytogenes	7·3 (±0·2)^{A}	7·5 (±0·3)^{A}	2·1 (±0·3)^{D}	4·9 (±0·3)^{B}	3·1 (±0·5)^{C}	3·6 (±0·4)^{C}
B. cereus	7·1 (±0·2)^{A}	7·0 (±0·1)^{A}	1·9 (±0·4)^{D}	4·8 (±0·4)^{B}	2·0 (±0·5)^{D}	2·4 (±0·3)^{D}
E. coli	6·8 (±0·3)^{A}	7·3 (±0·3)^{A}	1·4 (±0·6)^{D}	3·5 (±0·3)^{C}	1·7 (±0·3)^{D}	2·1 (±0·5)^{D}

Values followed by the same upper case letter in separate columns do not significantly differ at the 95% confidence intervals ($P < 0·05$).

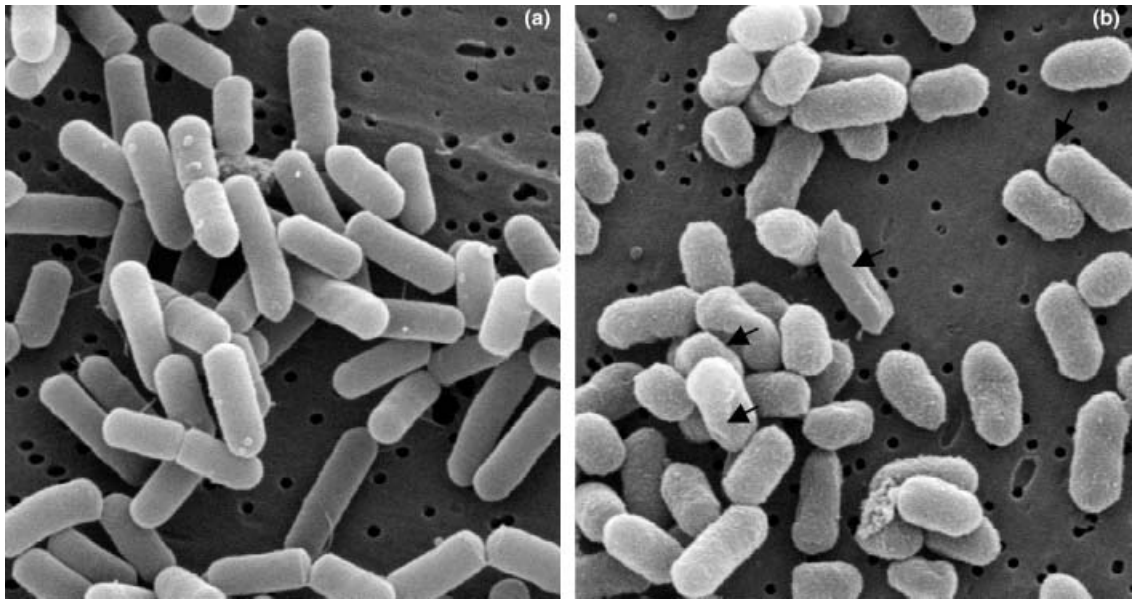

Fig. 2 Scanning electron micrographs of untreated (a) and PEF-treated (b) *Escherichia coli* cells, where arrows indicate points at which significant cellular damage may have occurred. Magnification ×12 000

membrane where insoluble fluorescent CTC-formazan crystals accumulate in metabolically active bacteria in a time-dependent manner. This present study has provided evidence that a significant portion of *L. monocytogenes*, *B. cereus* and *E. coli* cells that are rendered incapable of forming colonies by heat or PEF treatments are metabolically active. These results demonstrate the possible existence of a viable but nonculturable (VBNC) state in vegetative cells of *B. cereus* that has not been reported previously. There is compelling evidence that bacteria in a VBNC state may, in suitable conditions, regain the ability to grow and/or produce toxins that can be pathological to those consuming the food (Booth 1998; Rowan 1999). Results presented previously suggest that this seemingly anomalous situation may result from damage caused by exposure of stress-damaged bacteria to the nutrient-rich conditions in conventional media where this artificial nutritious environment may lead to a decoupling of microbial catabolic and anabolic processes (i.e. some stressed-injured bacterial cells are unable to cope metabolically with nutritious growth media and die through oxidative suicide) (Booth 1998; Rowan 1999). Alternatively, it has been reported that the VBNC state may be a survival mechanism adopted by bacteria when exposed to adverse environmental conditions (Jones *et al.* 1991).

Although there has been considerable research on the design and operation of PEF systems, much less is known about the different types of sublethal damage that PEF exerts on microbial cells (Heinz *et al.* 2001). Whilst the mechanisms underlying the inactivation of micro-organisms by PEF has yet to be fully elucidated, it is generally considered that the formation of irreversible pores in the cell membrane by strong electric fields (i.e. electropermeabilization) contributes significantly to cell death (Rowan *et al.* 2000; Heinz *et al.* 2001). Findings from scanning electron microscopy studies revealed that a significant proportion of PEF-treated bacteria were altered at the cellular level (Fig. 2), where arrows indicate areas of significant cellular damage. While the scanning electron micrographs revealed cellular change as a consequence of exposure to a high intensity electric field (Fig. 2), these images alone do not provide conclusively that irreversible damage occurred. However, when the latter findings are considered in conjunction with the fluorescent CTC-staining data, it is the likely that a single lethal injury (such as electropermeabilization), rather than multiple injuries (associated with thermal inactivation), was responsible for cell death in PEF-treated bacteria. Future use of additional vitality indicator stains such as propidium iodide (Caruso *et al.* 2003), which assesses microbial membrane integrity, may further enhance the accuracy and reliability of metabolic stains in determining the viability status of problematic bacteria in different environments.

In conclusion, results from the present study demonstrate the usefulness of CTC for rapid detection and quantification of actively respiring bacteria that have been subjected to biocidal treatments. The fluorescent staining method offers interesting perspectives on assessing established and novel microbial inactivation methods, particularly when the differentiation between dead and living cells is required for a more precise assessment of treatment or processing efficiency and bacteriological quality. Use of this approach may

also provide a better understanding of the mechanisms involved in microbial inactivation induced by PEF, as well as determining the critical factors influencing inactivation, developing better PEF equipment, and defining the conditions to inactivate micro-organisms in food without over-processing the product.

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