

ORIGINAL ARTICLE

Evidence of lethal and sublethal injury in food-borne bacterial pathogens exposed to high-intensity pulsed-plasma gas discharges

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Abstract

Aims: To apply scanning electron microscopy, image analysis and a fluorescent viability stain to assess lethal and sublethal injury in food-borne bacteria exposed to pulsed-plasma gas discharges (PPGD).

Methods and Results: The fluorescent redox probe 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was used for enumerating actively respiring cells of *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium that were suspended in sterile water at 4°C and exposed to separate PPGD and heat treatments. While there was good agreement between use of respiratory staining (RS) and direct-selective agar plate counting (PC) for enumerating untreated bacteria, there were *c.* 1 and 3 log-unit differences in surviving cell numbers per millilitre for test organisms subjected to PPGD and heat treatments respectively, when enumerated by these different viability indicators. PPGD-treated bacteria were markedly altered at the cellular level when examined by scanning electron microscopy.

Conclusions: Use of this RS method revealed that substantial subpopulations of test bacteria rendered incapable of forming colonies by separate PPGD and heat treatments may remain metabolically active.

Significance and Impact of the Study: Use of this RS method offers interesting perspectives on assessing established and novel microbial inactivation methods, and may also provide a better understanding of mechanisms involved in microbial inactivation induced by high-intensity PPGD treatments.

Introduction

Pulsed power technologies have been investigated as major alternative approaches for the destruction of microbial pathogens on contaminated surfaces (Rowan *et al.* 1999), in food and drink (Rowan *et al.* 2001; Sentandreu *et al.* 2006), in domestic and industrial waste water (Aniplov *et al.* 2002, 2004) and for the degradation of pollutants (Hao *et al.* 2007). Albeit limited, some recent studies have reported on the application of high-voltage pulses to gas-injected test liquids that results in microbial inactivation through the formation of a plasma that causes the generation of free radicals, free electrons,

ultraviolet light, acoustic and shock waves and electric fields at levels between 10 and 40 kV cm⁻¹ (Espie *et al.* 2001; Aniplov *et al.* 2002). The application of high-voltage pulses to gas-sparged test liquids, such as laboratory-based media (Espie *et al.* 2001) and domestic and industrial waste water (Aniplov *et al.* 2002, 2004), results in partial discharge activity and ionization of the gas that leads to complete breakdown of the gas in these liquid media. Previous studies have shown that in addition to the generation of the aforementioned plasmachemical components in test liquids that have recognized antimicrobial properties, substantial levels of ozone also accompany this electrical discharge process when oxygen is used

as the sparged gas (Espie *et al.* 2001). Despite scientific and developmental interests in pulsed-plasma gas discharge (PPGD) technology, very little research has been directed towards gaining a better understanding of the recovery processes associated with sublethally injured micro-organisms after PPGD treatment: previous studies have relied heavily on enumerating microbial survivors using conventional plate count (PC) agar techniques.

In this study, image analysis and fluorescent redox probe 5-cyano-2,3-ditoyl tetrazolium chloride (CTC) were used to investigate respiratory activity in *Listeria monocytogenes*, *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* and *Campylobacter jejuni* that were exposed to separate PPGD and heat treatments. Here, we report on the use of respiratory staining (RS) for rapidly quantifying the extent of sublethal cellular injury in PPGD and heat-treated food-borne bacteria.

Materials and methods

Bacterial strains used and preparation of inocula

Single strains of *C. jejuni* ATCC 33560, enterotoxigenic *E. coli* NCTC 11601, nonpathogenic *E. coli* K-, *L. monocytogenes* NCTC 9863, *S. enterica* serovar Typhimurium ATCC 14028, *Staph. aureus* NCTC 4444 and *B. cereus* NCTC 11145 were used in this study. All test strains were maintained in Microbank storage vials (Cruinn Diagnostic, Ireland) at -70°C . *Campylobacter jejuni* was grown to single colonies on modified charcoal-cefoperazone-deoxycholate agar (CCDA) plates (Unipath, Bedford, UK) at 42°C for 48 h in a microaerophilic environment generated by CampyPak gas generators (Unipath) generating 5% O_2 , 10% CO_2 and 85% N_2 . Strains of *E. coli*, *L. monocytogenes*, *Staph. aureus* and *Salmonella* spp. were grown separately to single colonies on MacConkey agar (MCA), *Listeria* selective agar (LSA), Baird Parker agar (BPA; Oxoid) and xylose lysine desoxycholate (XLD; Oxoid, Basingstoke, UK) agar, respectively at 37°C for 48 h aerobically. While *B. cereus* was grown at 37°C for 5 days on *B. cereus* selective agar (BCSA; Oxoid) supplemented with 3 mg l^{-1} of $\text{MnSO}_4\cdot\text{H}_2\text{O}$, the latter component stimulates endospore formation.

Bacteria were harvested from respective agar plates, washed thrice in 0.1 mol l^{-1} phosphate-buffered saline (PBS) pH 7.2 and sedimented by centrifugation at 4000 g for 20 min at 4°C . *Campylobacter jejuni* was resuspended in 10 ml of PBS and transferred to a 1.5-l fermentation vessel containing 500 ml of Brucella broth (Difco Laboratories, Detroit, Michigan, USA), and grown in a Bioflow 3000 bioreactor (New Brunswick Scientific, St Albans, UK) for 24 h at 42°C under the following batch culture settings: agitation 125 rev min^{-1} ; sparged gas composition

5% O_2 , 10% CO_2 and 85% N_2 ; and pH was maintained at 6.8 using 0.1 mol l^{-1} NaOH and 0.1 mol l^{-1} H_2SO_4 . After 24 h growth (early-stationary phase) bacteria were resuspended in 10 ml of sterile distilled water (dH_2O) at 4°C , and the optical density (OD) was adjusted at 540 nm to 2.0 ($c. 10^9\text{ CFU ml}^{-1}$) by spectrophotometric (model UV-120-02 instrument; Shimadzu Corp., Kyoto, Japan) determination. Inocula for the other test bacteria were prepared similarly with the following modifications; growth at 37°C in trypticase soy broth supplemented with 3% (w/v) yeast extract (Difco) with agitation (250 rev min^{-1}) using sparged atmospheric air. The presence and degree of endospore formation was confirmed by heat treating the PBS suspension of *B. cereus* for 15 min at 85°C in a circulating constant temperature waterbath (model HE30; Grant Instruments Ltd, Shepreth, UK) equipped with a thermoregulator capable of maintaining temperature to within $\pm 0.05^{\circ}\text{C}$ (model TE-8A; Techne Ltd, Cambridge, UK), and by subsequent enumeration of treated samples on BCSA plates after 48 h at 37°C .

PPGD treatment of test bacteria

Ten-millilitre aliquots of OD_{540} adjusted test bacterial suspensions ($c. \geq 10^9\text{ CFU ml}^{-1}$) were added to 247 ml of sterile dH_2O at 4°C before transfer to the coaxial treatment chamber (total volume 257 ml). Predetermined starting cell populations were prepared similarly for each test bacterium. The treatment chamber was constructed from 2-cm diameter stainless steel pipe forming the outer earthed electrode, with a 1-mm copper wire forming the coaxial high-voltage electrode. The test chamber was also immersed in chilled water bath in order to maintain the temperature at $\leq 4^{\circ}\text{C}$, which was monitored with a thermocouple. Once the pulse power system had been activated, oxygen was injected to the treatment chamber using a venturi gas injector. Plasma discharge activity was achieved in the test liquids using high-voltage pulses that were applied to the coaxial treatment chamber using a pulse-forming line (PFL) circuit, consisting of eight lengths of 12.62 m coaxial cable as described previously, with modifications (Espie *et al.* 2001). The PFL was charged using a high-voltage 40 kV DC capacitor charging power supply, connected via a resistance/diode protection circuit (RLIM). Using an SF6/air-pressurized, triggered spark gap switch [Samtech CSS-01, Samtech TG-01(B)], the PFL output was connected to the treatment chamber via a further 2 m, 50Ω , transmission line. The electrical operating parameters used were pulse energy of 3.7 J, PFL charging voltage of 23.5 kV, pulse rate of 124 pps and gas flow rate of 10 l min^{-1} . The gas flow rate was controlled by use of a Brooks mass flow controller (model 5851S), allowing continual adjustment to compensate for pressure

and temperature variances. When the spark gap switch was triggered, a voltage pulse was launched along the transmission line feed cable to the treatment chamber. Upon reaching the treatment chamber, the pulse was applied to suitably contoured electrodes, resulting in ionization of the surrounding gas bubbles in the liquid leading to ozone formation. Samples were taken in triplicate at designated intervals and after 30 s treatment time had elapsed, the pulsed power system was shut off and the gas supply was disconnected. Measurement of the dissolved ozone level was carried out using a BMT 963AQ ozone-in-water sensor ultraviolet (UV) photometer as per methods described previously (Espie *et al.* 2001). Conductivity, pH and temperature were measured using a Hanna Instruments WT-50 Water Test meter (RS Components, Northants, UK), which had the following ranges and accuracy: temperature, 0 to 60.0°C ± 1°C; pH, -0 to 14.0 ± 0.2; conductivity -0 to 1999 µS cm⁻¹, ±2% full scale. Samples were removed after predetermined exposure times and enumerated as described earlier.

The test bacteria were also subjected to heating at 56°C in order to achieve a similar level of inactivation (*c.* 4 log units in CFU ml⁻¹) to that obtained by PPGD treatment, according to methods described previously, with modifications (Yaqub *et al.* 2004). Test bacteria were suspended in sterile dH₂O to a density of *c.* 10⁸ CFU ml⁻¹ in 3-ml shrimp cap glass vials (Phase Separations Ltd, Watford, Hertfordshire, UK). The vials were sealed and kept 4 cm below the level of a circulating constant water bath for the treatment period. After separate treatments, heat and PPGD samples were diluted as appropriate in PBS and were spiral-plated onto appropriate agar as described earlier using a spiral system (model B; Spiral Systems Inc., 6740 Clough Cincinnati, USA). Undiluted samples were enumerated using the pour-plate technique. Typical colonies of each test strain were randomly selected from respective selective agar plates after 24 h and 48 h at 37°C (and 42°C for *Campylobacter*) with the highest dilution, and were confirmed by use of appropriate physiological and biochemical tests as described earlier. Survivor cell populations and untreated controls were expressed in terms of colony-forming units per millilitre (CFU ml⁻¹) and the corresponding death rate kinetic curves were generated.

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

PPGD-treated samples were examined visually for cellular damage by using scanning electron microscopy (SEM). Briefly, test samples were centrifuged (10 min, 10 000 g, 4°C), and the supernatants were discarded. The pellets were washed with PBS twice and fixed with 2.5% glutaraldehyde (Sigma-Aldrich, Gillingham, Dorset, UK). Cells were then

filtered onto 0.2-µm-pore-size Isopore GTTP membrane filters (Millipore, Watford, UK). The cells were dehydrated once in 50%, 70%, 80% and 90% ethanol and twice in 100% ethanol, treated with 100% isoamyl acetate, and critical point dried. Finally, cells were sputter-coated with 150-nm gold particles and with the model JSM-T200 SEM (JEOL).

Use of epifluorescence microscopy, image analysis and fluorescence redox probes to enumerate respiring cell numbers after heating and PPGD treatments

Epifluorescence microscopy, image analysis and the fluorescent redox probes CTC and 4',6-diamidino-2-phenylindole (DAPI) were used to investigate respiratory activity in test strains according to previously described procedures, with modifications (Yaqub *et al.* 2004). One-millilitre cell suspensions were harvested by centrifugation (4°C for 10 min at 3000 g) and washed thrice with PBS. Experimental and control preparations were resuspended in 300 µl of 5 mmol l⁻¹ CTC (Polysciences, Inc., St. Louis, MO, USA) and incubated in microaerophilic (for *Campylobacter* only) or aerobic environment for 1.5 h in the dark at 20°C with agitation (200 rev min⁻¹). After incubation, experimental and control preparations, and dilutions thereof, were counterstained for 8 min at 20°C with 5 µg of DAPI (Sigma, St. Louis, MO, USA) per millilitre and the samples were transferred to a Petroff-Hausser counting chamber for enumeration. 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 viewed using a blue 420–480-nm excitation filter (combined with a 580-nm dichromic mirror and a 590-nm barrier filter) in a Nikon Optiphot microscope. CTC- and DAPI-stained bacteria in the same preparation were viewed simultaneously with a 365-nm excitation filter, and emission filter and a 400-nm cut-off 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 (Seescan Ltd., Cambridge, UK). Counts were determined from five randomly selected squares on the chamber etched-grid in triplicate experiments and results were expressed as the log number of the corresponding bacteria per millilitre of the sample.

Statistical analysis

Analysis of variance – balanced model (Minitab software Release 14; Minitab Inc., State College, PA, USA) was used to compare the effects of PPGD treatments on

microbial inactivation. Experiments were replicated thrice with duplicate treatments in each replication, and results are reported as means \pm standard deviations. Significant differences were reported at the 95% ($P < 0.05$) and confidence interval.

Results

Enumeration of heat-treated test bacteria using RS and direct-selective PC

Thermal treatment or holding times required to achieve 4 log reductions in cell populations for *L. monocytogenes* NCTC 11994, nonpathogenic *E. coli* K12, enterotoxigenic *E. coli* NCTC 11601, *C. jejuni*, *Staph. aureus* and *S. enterica* serovar Typhimurium at 56°C were 36.4, 32, 28.8, 31.2, 35.4 and 33.6 min, respectively. This corresponded to $D_{56^\circ\text{C}}$ values (decimal reduction time: the time required to kill 1-log unit concentration of bacteria) of 9.1, 8, 7.2, 7.8, 8.8 and 8.4, respectively (data not shown). Good agreement was obtained between the CTC fluorescence or RS method and the conventional direct PC method for enumerating untreated cell populations of test bacteria ($P < 0.05$) (Table 1). The PC method demonstrated that heat-treated samples of *C. jejuni*, nonpathogenic *E. coli*, enterotoxigenic *E. coli*, *S. enterica* serovar Typhimurium, *L. monocytogenes* and *Staph. aureus* were reduced by 4.3, 3.7, 3.1, 4.1, 3.9 and 3.6 log CFU ml⁻¹, respectively; this markedly contrasted with reductions of 1.9, 2.7, 1.5, 1.6, 2.7 and 2 log cell numbers of actively respiring bacteria per millilitre as determined by the rapid RS method ($P < 0.05$) (Table 1).

Enumeration of PPGD-treated test bacteria using RS and direct-selective PC

PPGD treatments rapidly reduced predetermined populations (≤ 8 log CFU ml⁻¹) of all vegetative test bacteria sus-

ended separately in sterile dH₂O at 4°C to nondetectable levels within 24 s when enumerated by the PC approach (Fig. 1). Inactivation kinetic data obtained from Fig. 1 was used to determine PPGD exposure times (s) that produced similar levels of lethality (c. 4-log units in CFU ml⁻¹) to that obtained by heating: this corresponded to exposure times of 3, 4, 6, 7, 8 and 30 s for *C. jejuni*, nonpathogenic *E. coli* K-12, enterotoxigenic *E. coli*, *S. enterica* serovar Typhimurium, *L. monocytogenes* and *B. cereus* spores, respectively. Findings showed that a similar trend emerged when enumerating PPGD-treated bacteria using the different viability counting methods compared with that of treating similar samples with heating (Table 1). The PC method demonstrated that PPGD-treated samples of *L. monocytogenes*, nonpathogenic *E. coli*, enterotoxigenic *E. coli*, *C. jejuni*, *S. enterica* serovar Typhimurium and *Staph. aureus* were reduced by 3.5, 3.5, 3.2, 3.2, 3.6 and 3.1 log CFU ml⁻¹, respectively, which markedly contrasted with reductions of 2.5, 1.5, 2.0, 2.2, 2.8 and 2.3 log cell numbers of actively respiring bacteria per millilitre as determined by the rapid RS method ($P < 0.05$) (Table 1). Mean differences in cell numbers for pooled vegetative test bacteria enumerated by PC and RS methods after heating at 56°C was 3.86 ± 0.49 and 1.98 ± 0.46 log CFU ml⁻¹, respectively. A similar trend emerged for PPGD-treated samples ($P < 0.05$), where the mean difference for pooled test bacteria enumerated by PC and RS approaches was 3.5 ± 0.21 and 2.2 ± 0.51 log CFU ml⁻¹, respectively. Whilst heating did not inactivate *B. cereus* spores (data not shown), application of PPGD reduced spore populations by c. 3.4 log CFU ml⁻¹ after 30 s at 4°C (Fig. 1).

Discussion

In general, it would appear that subpopulations of PPGD- and heat-treated test bacteria (c. 1 and 3 log CFU ml⁻¹, respectively) are capable of respiration (which suggests

Table 1 Enumeration of test bacterial cell numbers by respiratory staining (RS) and colony-forming units (CFU) by direct plate counting (PC) after heating and pulsed plasma gas-discharge (PPGD) treatment

| Test bacteria | Log CFU or cell members ml ⁻¹ | | | | | |
|---------------------------------|--|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| | Untreated (PC) | Untreated (RS) | Heat (PC) | Heat (RS) | PPGD (PC) | PPGD (RS) |
| <i>Campylobacter jejuni</i> | 7.8 (± 0.2) ^A | 7.6 (± 0.3) ^A | 3.5 (± 0.3) ^D | 5.7 (± 0.3) ^C | 4.3 (± 0.5) ^D | 5.1 (± 0.6) ^C |
| <i>Escherichia coli</i> K-12 | 7.6 (± 0.3) ^A | 7.3 (± 0.2) ^A | 3.9 (± 0.7) ^D | 4.6 (± 0.3) ^D | 4.1 (± 0.3) ^D | 5.8 (± 0.4) ^C |
| <i>E. coli</i> 11601 | 7.4 (± 0.2) ^A | 7.3 (± 0.2) ^A | 4.3 (± 0.5) ^D | 5.8 (± 0.4) ^C | 4.2 (± 0.4) ^D | 5.3 (± 0.5) ^C |
| <i>Salmonella</i> Typhimurium | 7.5 (± 0.3) ^A | 7.4 (± 0.1) ^A | 3.4 (± 0.4) ^D | 5.8 (± 0.3) ^C | 4.3 (± 0.6) ^D | 5.2 (± 0.4) ^C |
| <i>Staphylococcus aureus</i> | 7.6 (± 0.3) ^A | 7.3 (± 0.3) ^A | 4.0 (± 0.4) ^D | 5.3 (± 0.2) ^D | 4.5 (± 0.4) ^D | 5.0 (± 0.5) ^D |
| <i>Listeria monocytogenes</i> | 7.7 (± 0.3) ^A | 7.5 (± 0.3) ^A | 3.8 (± 0.6) ^D | 5.3 (± 0.3) ^C | 4.1 (± 0.5) ^D | 4.7 (± 0.4) ^D |
| <i>Bacillus cereus</i> (spores) | 6.9 (± 0.1) ^B | NA [§] | 6.8 (± 0.1) ^B | NA | 4.5 (± 0.4) ^D | NA |

Values followed by the same upper case letter in separate columns do not significantly differ at the 95% confidence interval ($P < 0.05$).

§Not applicable, as 5-cyano-2,3-ditoyl tetrazolium chloride (CTC)-4',6-diamidino-2-phenylindole (DAPI) RS does not stain endospores.

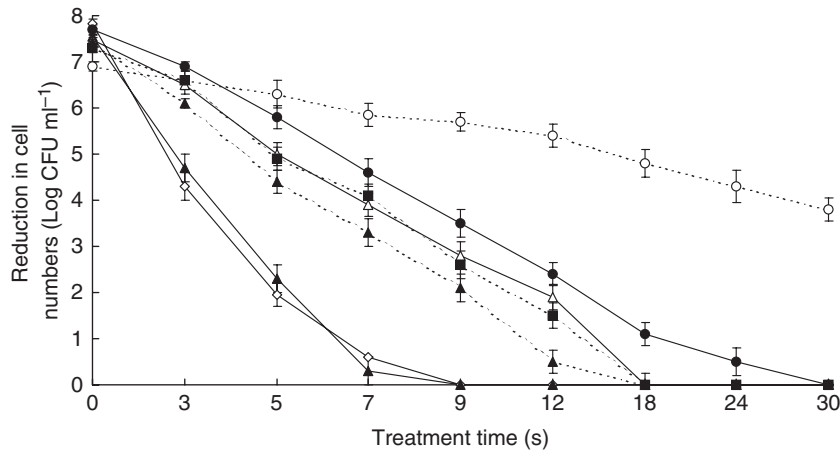


Figure 1 Influence of pulsed plasma gas-discharge treatment on the inactivation of test bacteria suspended in distilled water at 4°C. —◇— *C. jejuni*; —▲— *E. coli* K-12; —△— *S. typhimurium*; —●— *L. monocytogenes*; ...▲... *E. coli* 11601; ...○... *B. cereus* (spores); ...■... *S. aureus*.

cell viability), but are unable to grow on selective laboratory-based culture media because of sublethal injury. Results from the latter thermal inactivation study agree with the 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 proportion of *E. coli* and *Pseudomonas putida* cells in heat-treated milk are metabolically active but are incapable of forming colonies. These findings are significant as food technologists routinely use agar PC to detect spoilage and pathogenic bacteria (Rowan 2004).

Reductions in *Campylobacter* numbers ($\leq 8 \log \text{CFU ml}^{-1}$) were achieved in just 9 s at 4°C (Fig. 1), which may be attributable in part to the particular sensitivity of this notable enteropathogen to highly oxygenated environments (Purdy *et al.* 1999). In general, both gram-positive and -negative test bacteria appeared similarly susceptible to the effects of plasma treatment, despite there being significant differences in cellular wall structure (Fig. 1). *Listeria monocytogenes* was shown to be more resistant to the lethal action of PPGD compared with other vegetative test bacteria ($P < 0.05$), which corroborates previous studies that demonstrated the resilience and adaptability of this problematic bacteria to a wide range of applied lethal stresses (Lou and Yousef 1997). It is interesting to note that the nonpathogenic strain *E. coli* K-12 was more susceptible to the lethal action of PPGD treatment compared with that of the enterotoxigenic *E. coli* NCTC 11601 strain, which is in line with previous studies that investigated the relationship between virulence factor expression and stress tolerance (Rowan 2004).

Use of fluorescent redox probes (such as CTC) for direct visualization of actively respiring bacteria is gaining popularity among research groups investigating the viable but nonculturable (VBNC) phenomenon (i.e. stress-

injured bacteria which are not capable of cellular division required for growth in usual culture media, but, yet remain physically active for several metabolic functions) in food- and water-borne bacteria (Chaveerach *et al.* 2003; Gupte *et al.* 2003; Yaqub *et al.* 2004; Cappelier *et al.* 2007). CTC reduction reflects the presence of a functional electron transport (i.e. respiratory) system in the microbial cell membrane where insoluble fluorescent CTC-formazon crystals accumulate in metabolically active bacteria in a time-dependent manner. However, as nonselective agar plates were not used for the recovery of heat- or PPGD-treated test micro-organisms, the CTC redox probe used in this present study reports on microbial viability in the context of demonstrating respiration that may not in itself be sufficient to prove growth of living cells post-treatments. This present study has provided evidence that a significant proportion of *C. jejuni*, *L. monocytogenes*, *S. enterica* serovar Typhimurium and *E. coli* cells that rendered incapable of forming colonies by heat or PPGD treatments are metabolically active. However, previous studies (Yaqub *et al.* 2004) that reported on the use of image analysis and RS showed that the PC method did not significantly underestimate the numbers of cell survivors after pulse electric field (PEF) treatment, which markedly contrasts from the earlier findings where *c.* 2-log unit difference in surviving cell populations was evident after heating or PPGD treatment. While the mechanisms underlying the inactivation of micro-organisms by PPGD have yet to be fully elucidated, it is generally considered that microbial inactivation may be attributed to the combined influence of numerous antimicrobial components formed during this plasma discharge process, such as the generation of ozone and other free radicals, free electrons, ultraviolet light, acoustic and shock waves (Espie *et al.* 2001; Aniplov *et al.* 2004). In this study, the residual ozone level produced during PPGD treatments of test micro-organisms in distilled water after 30 s was

c. 250 ppm. Findings from SEM studies revealed that a significant proportion of PPGD-treated bacteria were altered at the cellular level (data not shown). It should be noted, however, that Aronsson *et al.* (2005) reported that the finally observed irreversible membrane permeabilization may not be the initial mechanistic basis for the inactivation of yeast cells. Interestingly García *et al.* (2005) reported on the occurrence of sublethal injury after extended PEF treatments, which depended on the type of the test micro-organism, the treatment medium, pH and the intensity of the treatment investigated. From these and other data it may be inferred that the damage inflicted upon microbial cells such as stresses is multifactorial and that therefore the stress response of the cells will be of a complex nature. Therefore, fundamental insights into the type of damage that inflicts single or multiple injuries in PEF or PPGD-treated micro-organisms merits further experimentation. While a broad spectrum of useful approaches can be pursued to investigate the mode of action, of particular relevance would be the use of genome-wide transcription analysis to assess sublethal conditions in a bacterial model strain such as that described recently by Iwahashi *et al.* (2005).

Albeit limited in scope, previous studies carried out by this (Espie *et al.* 2001) and other research groups (Aniplov *et al.* 2002) demonstrated that quality of plasma-treated water fulfilled the necessary standards of the European Union and was deemed fit for human consumption. However, these studies have not focused on the possibility that application of electric discharge to agricultural, municipal or industrial effluents containing organic material may generate unwanted by-products such as bromate which is a recognized carcinogen derived from bromide (Kim *et al.* 2003). The pH of test bacterial suspensions decreased during plasma treatment at 4°C, where the pH values measured after 30 s was 3.9 compared with 6.03 for untreated sterile distilled water. Reasons as to why sparging with oxygen during the electric discharge process resulted in a decrease in pH is not presently known, but may be attributed to the possible formation of weak nitric and carbonic acids from N₂ and CO₂-based compounds present in the microbial milieu during plasma treatment. It is also likely that the rapid reduction in pH to 3.9 may have contributed to microbial inactivation, especially because *C. jejuni* has been reported previously to be sensitive to extremes in pH, especially acidic conditions (Zhao and Doyle 2006).

In conclusion, this is the first study to report on the efficacy of CTC as a rapid approach for quantifying actively respiring food-borne bacteria that were subjected to PPGD treatments. Our studies also indicate that the use of PPGD was very effective not only in rapidly reducing populations of bacterial enteropathogens suspended

in sterile distilled water at 4°C, and therefore, could possibly act as a critical control point in a hazard analysis critical control point (HACCP) by preventing cross-contamination of the processing environments.

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