Pulsed-Plasma Gas-Discharge Inactivation of Microbial Pathogens in Chilled Poultry Wash Water

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Running title: **Pulsed Plasma Decontamination of Poultry Wash Water**

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ABSTRACT

This study reports on the development of a pulsed-plasma gas-discharge (PPGD) system for the novel decontamination of chilled poultry wash water. Results showed that treatment of poultry wash water in the plasma generation chamber for up to 24s at 4^{o}C reduced (≤8 log CFU/ml) *Escherichia coli* NCTC 9001, *Campylobacter jejuni* ATCC 33560*, Campylobacter coli* ATCC 33559, *Listeria monocytogenes* NCTC 9863, S*almonella enterica* serovar Enteritidis ATCC 4931 and *S. enterica* serovar Typhimurium ATCC 14028 populations to nondetectable levels. While similar PPGD-treatments at 4^{o}C also produced significant reductions (≥3 log CFU/ml) in recalcitrant *B. cereus* NCTC 11145 endospore numbers within 30s, the level of endospore reduction was shown to be dependent on the nature of the sparged gas used in plasma treatments. Use of SEM revealed that significant damage occurred at the cellular level in PPGD-treated test organisms. Studies also showed that while there was good agreement between use of conventional direct plate counts (PC) and novel respiratory staining for enumerating PPGD-treated *Campylobacter*, the former PC technique overestimated the level of sublethally injured *Salmonella* by 1.3 log CFU/ml. This green-friendly electrotechnology delivers energy in intense ultrashort bursts generating liquids of multiple bactericidal properties (comprising ozone, ultraviolet light, acoustic and shock waves, and pulsed electric fields), which revert back to water leaving no unwanted chemical residues. This technology offers an exciting complementary or alternative approach for treating raw poultry wash and for preventing cross-contamination of processing environments.

Keywords: poultry, recycling wash water, pulsed power technologies, ozone, HACCP

Salmonella and *Campylobacter* species are leading bacterial causes of human food-borne illness (*14, 25*). The United States Department of Agriculture estimates the costs associated with food-borne illness to be approximately $22 billion per year. Outbreaks of enteritis associated with these pathogens have been associated with consumption of contaminated meat, particularly poultry products (*11*). Because of the relatively high frequency of contamination of poultry with these pathogens, raw poultry products have perceived to be responsible for a significant amount of human illness. These enteric pathogens frequently contaminate chicken skin and exposed surfaces during slaughter operation, the development of an effective process for their reduction or elimination on the surface of poultry is important (*25*). Contamination rates of *Campylobacter* spp. on poultry purchased at retail establishments in the United States and in the United Kingdom range from 68 to 83% (*8*).

While various processes have been proposed as alternatives to eliminate or substantially decrease bacterial populations on poultry carcases (*12*), most of these approaches have not been completely acceptable due to the chemical residues, discolouration of chicken carcases, and high cost of limited effectiveness. Chlorine rinses are generally used during processing of poultry for pathogen reduction (*25*), however use of chlorine by the food industry is coming under increasing scrutiny by regulators due to toxicity issues and disinfection by-products (DBPs). Recent surface water rules promulgated by the United States Environmental Protection Agency relating to chlorine and chloride derived DBPs will undoubtedly stimulate operators to seek technologies which will assure discharge compliance (*9*). The poultry industry is also a large volume consumer of water and the potential for reuse or recycling of poultry processing water represents an attractive economic benefit to the industry.

Pulsed electric field technology has been investigated as major alternative approaches for the destruction of microbial pathogens on contaminated surfaces (*18*), in food and drink (*13, 20, 22*), in potable and waste water (*1, 2, 7*), and degradation of pollutants (*4*). The electrical discharge treatment of pumpable liquids can be highly effective with respect to microbial reduction and cost. The application of high-voltage pulses to gas-injected test liquids results in the formation of a plasma which 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, 2, 5*). The application of high voltage pulses to gas-sparged test liquids results in partial discharge activity and ionisation of the gas that leads to

complete breakdown of the gas in the liquid medium. It is through this ionisation process that substantial levels of ozone (up to 5400 ppm) and other physical bactericidal properties are formed in test liquids, which subsequently reverts back to water post treatment (*5*). This technology has been used reported previously by other researchers for the treatment potable water artificially-contaminated with *E. coli* (*2*). While ozone has been successfully used to treat liquids particularly water for commercial and industrial applications since the beginning of the last century (*9*), it is very difficult to achieve high ozone transfer rates and high residual dissolved ozone levels in water using conventional ozone generators due to the properties of ozone and its inherent ability to decompose into its constituent oxygen. This electrotechnology is therefore environmental friendly as it does not involve the introduction of toxic chemicals into test liquids (*2*), as in the case of chlorination, which can lead to the formation of carcinogenic and/or mutagenic disinfection by-products (cited in *9*).

Despite advances made in the development of pulsed power technologies, plasma and plasmochemical processes accompanying electric discharge in water and industrial effluents have received very limited study to date. Therefore the aim of this study was to develop an efficient method of decontaminating poultry wash water containing a range of relevant microbial pathogens using a high-voltage, pulsed-plasma, gas-discharge (PPGD) system. It is proposed that the application of high voltage pulses to gas-sparged test liquids could produce substantial inactivation of test organisms as several antimicrobial mechanisms are produced during this process.

MATERIALS AND METHODS

Bacterial strains. Single strains of *Escherichia coli* NCTC 9001, *Campylobacter jejuni* ATCC 33560, *Campylobacter coli* ATCC 33559, *Listeria monocytogenes* NCTC 9863, *Salmonella enterica* serovar Enteritidis ATCC 4931, *Salmonella enterica* serovar Tpyhimurium ATCC 14028, and *B. cereus* NCTC 11145 were used in this study. All test strains were maintained in Microbank storage vials (Cruinn Diagnostic, Ireland) at -70ºC. Each *Campylobacter* strain was grown to single colonies on modified charcoal-cefoperazone-deoxycholate agar (CCDA) plates (Unipath) at 42ºC for 48h 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*, and *Salmonella* spp were grown

separately to single colonies on MacConkey agar (MCA), *Listeria* Selective agar (LSA) and Xylose Lysine Desoxycholate (XLD, Oxoid) agar respectively at 37ºC for 48 h aerobically. While *B. cereus* was grown at 37ºC for 5 days on *Bacillus cereus* selective agar (BCSA, Oxoid) supplemented with 0.5 mg/l $MnSO_4.H_2O$, the latter component stimulates endospore formation. Confirmation of *Salmonella* strains was accomplished by using biochemical tests including triple sugar iron agar slants (Difco) and API 20 E strips (Biomerieux, Inc.), characteristic colony growth on XLD and subsequent serological reactions with somatic (O) and flagellar (H) antigens (Difco). *E. coli* was confirmed by characteristic colony growth on MCA and use of API 20 E strips. *Campylobacter* strains were confirmed by characteristic colony growth on CCDA, and by use of immunolatex assay (Dryspot *Campylobacter* Test, Oxoid). *B. cereus* was confirmed by characteristic colony appearance on BCSA, by endospore production under aerobic growth conditions, and by use of API 50 CHB and API 20 E biochemical strips. *L. monocytogenes* was confirmed by characteristic growth on LSA at 4ºC, tumbling motility at 25ºC, CAMP (Christie, Atkins, Munch-Peterson) test reaction and by API *Listeria* (Biomerieux, Inc.) profile.

Preparation of inocula. Bacteria were harvested from respective agar plates, washed three times in 0.1 M phosphate-buffered saline (PBS) pH 7.2 and sedimented by centrifugation at 4,000 X *g* for 20 min at 4ºC. *Campylobacter* were resuspended separately in 10 ml of PBS and transferred to a 1.5 L fermentation vessel containing 500 ml of Brucella broth (Difco Laboratories, Detroit, Mich.), and grown in a Bioflow 3000 bioreactor (New Brunswich Scientific, UK) for 24 h at 42ºC under the following batch culture settings: agitation 125 rpm, sparged gas composition 5% O_2, 10% CO_2 and 85% N_2, and pH was maintained at 6.8 using 0.1 M NaOH and 0.1 M H_2SO_4. After 24 h growth (early-stationary phase) bacteria were resuspended in 10 ml of sterile distilled water that had been refrigerated overnight at 4ºC, and the optical density was adjusted at 540 nm to 2.0 (ca. 10^9 CFU/ml) 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 37ºC in trypticase soy broth supplemented with 3% (w/v) yeast extract (Difco) with agitation (250 rpm) 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 85ºC in a circulating constant temperature waterbath (Model HE30, Grant Instruments Ltd, Uk) equipped with a thermoregulator

capable of maintaining temperature to within ±0.05ºC (model TE-8A, Techne Ltd, Cambridge, UK), and by subsequent enumeration of treated samples on BCSA plates after 48 h at 37^{o}C.

Determination of appropriate sparged gas for subsequent pulsed-plasma gas-discharge treatments. 10 ml aliquots of OD_{540} adjusted *B. cereus* endospore suspensions were added to 247 ml of sterile distilled water that had been refrigerated overnight at 4^{o}C before transfer to the coaxial treatment chamber (total volume 257ml). The treatment chamber was constructed from 1 inch 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 ≤4ºC, which was monitored with a thermocouple. Once the pulse power system had been activated, the treatment gas was injected to the treatment chamber using a venturi gas injector. Four different treatment gases were investigated separately, which were nitrogen, carbon dioxide, oxygen and air. In addition to the 4 gases used, a control experimental test was also conducted with no gas added to the test liquid, which results in pulsed electric field generation. 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 12.62m coaxial cable as described previously, with modifications (*5*) (Fig. 1). The PFL was charged using a high voltage 40kV DC capacitor charging power supply, connected via a resistance/diode protection circuit (RLIM). Using an SF6/Air pressurised, triggered spark gap switch (Samtech CSS–01, Samtech TG–01(B)), the PFL output was connected to the treatment chamber via a further 2m, 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 10 L/min. 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 ionisation of the surrounding gas bubbles in the liquid leading to ozone formation (depending on gas type used). Samples were taken in triplicate at designated intervals and after 30s treatment time had elapsed, the pulsed power system was shut off and the gas supply disconnected. Measurement of the dissolved ozone level was carried out using a BMT 963AQ ozone–in–water sensor UV photometer as per

methods described previously (*5*). Although this peak ozone level produced may decay slightly before measurement, much of the residual ozone remains in solution allowing the effect of the tested parameters on microbial reduction to be evaluated. 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 1999µS/cm, ±2% full scale.

Chicken wings. Fresh chicken wings were purchased from the local retail store. Representative samples (two chicken wings) selected randomly from the same bag were assayed before inoculation of the bacterial test strains by the protocols described below.

Pulsed plasma gas-discharge (PPGD) treatments of distilled water and poultry wash water containing test organisms. Three chicken wings (each ca. 8 cm long, 4.5 cm wide and ca. 45 to 50 g) were submerged in a stomacher bag containing 300 ml of distilled water seeded with ca. 10^8 CFU/ml of test organism (confirmed spectrophotometrically as described earlier). Inocula for each test strain was prepared as described earlier. The seeded chicken wing suspensions were then stomached for 15 s, and 30 ml samples was added to 270 ml of sterile distilled water that had been refrigerated overnight at 4^{o}C. The mean pH of poultry wash water containing test bacteria was 6.74 ±0.18, while the pH of untreated sterile distilled water was 6.03 ±0.21. A total aerobic mesophilic count (enumerates both microbial flora from chicken in addition to seeded test organism) was then performed on duplicate 5 ml samples as described previously (*25*), in addition to enumerating untreated levels of each test organism using appropriate selective agar as mentioned above. 257 ml sample was then transferred to plasma chamber for treatment at 4^{o}C as described earlier. Samples were removed after pre-determined exposure times and enumerated as described earlier. Each test organism was also suspended separately in sterile distilled water only at 4^{o}C to a population of ca. 10^7 CFU/ml, and then subjected to plasma treatment with commensurate enumeration as above. This was carried out as a control to determine the impact, if any, of poultry wash on microbial inactivation. Typical colonies of each test strain were randomly selected from respective selective agar plates after 24h and 48 h at 37ºC (and 42ºC for *Campylobacter*) with the highest dilution, and were confirmed by use of appropriate physiological and biochemical tests as described earlier.

Plate count. Treated and control 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). Undiluted samples were subjected to the pour-plate technique. Survivor cell populations and untreated controls were expressed in terms of colony forming units per ml (CFU/ml) and corresponding death rate kinetic curves were generated.

Scanning electron microscopy (SEM). Briefly, test samples were centrifuged (10 min, 10,000 X *g*, 4^{o}C), and the supernatants were discarded. The pellets were washed with PBS twice and fixed with 2.5% gluteraldehyde (Sigma-Aldrich). Cells were then filtered onto 0.2-µm-pore-size Isopore GTTP membrane filters (Millipore). The cells were dehydrated once in 50, 70, 80 and 90% ethanol and twice in 10% ethanol, treated with 100% isoamyl acetate, and critical point dried. Finally, cells were sputter coated with 150nm Au particles and with a model JSM-T200 scanning electron microscope (JEOL).

Use of epifluorescence microscopy, image analysis and fluorescence redox probes to investigate respiratory activity in test bacteria. Epifluorescence microscopy, image analysis and the fluorescent redox probes 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 4',6-diamidino-2-phylindole (DAPI) were used to investigate respiratory activity in *Campylobacter* and *Salmonella* test strains only according to previously described procedures, with modifications (*3, 24*). One-millilitre cell suspensions were harvested by centrifugation (4ºC for 10 min at 3,000 X *g*) and washed three times with PBS. Experimental and control preparations were resuspended in 300 µl of 5mM CTC (Polysciences, Inc, St. Louis, Mo.) and incubated in microaerophilic (for *Campylobacter*) or aerobic (for *Salmonellae*) environment for 1.5 h in the dark at 20ºC with agitation (200 rpm). After incubation, experimental and control preparations, and dilutions thereof, were counterstained for 8 min at 20ºC with 5 µg of DAPI (Sigma, St. Louis, Mo.) ml^{-1} and 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 to 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-cutoff filter. Stained cells were distinguished from non-specific 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 corresponding bacteria per millilitre of sample.

Statistical analysis. Analysis of variance - balanced model (Minitab software Release 11, Minitab Inc., State College, PA) was used to compare the effects of pulsed plasma gas-discharge treatments on microbial inactivation. Experiments were replicated three times with duplicate treatments in each replication, and results are reported as means ± standard deviations. Significant differences were reported at the 95% ($P < 0.05$) and confidence interval.

RESULTS AND DISCUSSION

Effect of sparged-gas composition on pulsed-plasma induced inactivation of *B. cereus* **endospores.** The results showed that the presence of oxygen in the sparged gas composition significantly affected ($P<0.05$) the level of reduction of *B. cereus* endospores achieved in the plasma treatment chamber (Fig. 2). Use of oxygen alone produced the greatest level of endospore reduction (3.4 log CFU/ml) after 30 s at 4ºC, which may be attributed in part to the greater amounts of residual ozone (250 ppm) produced in test liquids using oxygen alone. The significant increase in dissolved ozone using sparged oxygen is likely to result from the substantial increase in oxygen molecules present in the treatment gas mix, thus allowing much greater ozone production and hence dissolved ozone levels in the test liquid. The greater levels of oxygen molecules present in the treatment chamber the greater the production and dissolution ozone. Use of pulsed electric fields (PEF) alone had no influence of *B. cereus* spore numbers (Fig. 2), which is line with findings from previous studies (*19*).

In addition to the generation of multiple bactericidal properties such as high-intensity ultraviolet light, acoustic and shock waves, PEF, and free radicals as reported previously (*1, 2, 5*), production of plasma discharges in sparged-water containing *B. cereus* endospores had a notable effect on pH levels that was not investigated previously (*1, 2, 5*). The pH of the *B. cereus* spore suspension decreased during plasma treatment at 4ºC, where the pH values measured after 30 s were 3.6, 3.8, 3.9

and 4.4 using sparged-N_2, air, oxygen, and CO_2 respectively. The pH of untreated sterile distilled water for all gaseous studies was 6.03. Reasons as to why N_2 produced the lowest pH value as a result of plasma treatment is not presently known, but maybe attributed to the availability of oxygen molecules from the breakdown of water during plasma treatment. Under such conditions, N_2-based compounds such as nitrate could be formed during plasma treatment, which would subsequently dissolve into the treated test liquid producing an acidic solution (e.g. nitric acid) that would promote a rapid reduction in pH. A 3-log reduction in *B. cereus* spore numbers per ml was also achieved using sparged CO_2 or air (Fig. 2). This may be attributed to the formation of a weak carbonic acidic solution during plasma treatment. Commensurate increases in liquid conductivity occurred during plasma treatment with value of 65 µs/cm obtained using CO_2 after 30 s at 4ºC. Use of oxygen during plasma treatment resulted in a small but constant increase in conductivity to a value of 5.8 µs/cm. Water is a very suitable dielectric medium for plasma treatment as it has a high permittivity ($\varepsilon = 8.01$) that allows for a high electric field to be generated across the injected gas bubbles of low permittivity ($\varepsilon \approx 1$ for most gases) (*5*). Water also have a low conductivity (2 and 40 µs/cm for distilled and tap water respectively) that minimizes energy loss and temperature rise occurring during resistive heating (*2*). As oxygen alone produced the greatest level of endospore reduction in the plasma chamber, this gas was used for the subsequent treatments of other test organisms during this study.

Effect of plasma discharge treatment on the viability of test organisms suspended separately in distilled water and in poultry wash water. Findings showed that plasma discharge treatment of all vegetative test bacteria suspended separately in sterile distilled water at 4ºC resulted in rapid reductions in microbial numbers (≤8 log CFU/ml) within 30 s exposure to nondetectable levels (Fig. 3). The order of increasing sensitivity to pulsed plasma treatment was *B. cereus* endospores, *L. monocytogenes*, *S.* Typhimurium, *S.* Enteritidis, *E. coli, C. jejuni* and *C. coli*. Reductions in *Campylobacter* numbers (≤8 log CFU/ml) were achieved in just 9 s at 4ºC, which may be attributable in part to the particular sensitivity of these enteropathogens to highly oxygenated environments (*15*). Suspending untreated (control) test bacteria in sterile distilled water at 4ºC for 30 s did not significantly affect population numbers (data not shown). In general, Gram negative test bacteria were more susceptible to the lethal action of pulsed plasma gas-discharge treatment ($P < 0.05$). This maybe due to the complexity of the cell envelope associated with Gram negative bacteria, which is targeted

ozone and other oxygenated free radicals (*17*). The latter statement is supported by scanning electron micrograph (SEM) images (Fig 4b) of PPGD-treated *Campylobacter* showing significant changes to the outer cell envelope at the cellular level (arrows indicate areas of injury). *L. monocytogenes* was shown to be more resistant to the lethal action of PPGD compared to other vegetative test bacteria ($P < 0.05$), which corroborates previous studies that demonstrated the resilience and adaptability of this problematical bacteria to a wide range of applied lethal stresses (*10, 17*). Confirmation of the possibility that disinfection distilled water using electric discharges did not lead to the production of toxic by-products was achieved as per methods described previously (*2, 5*) (data not shown). However, pH in the distilled water-bacterial suspension decreased rapidly from pH 6.03 (untreated control) to pH 3.9 during plasma treatment, which may have contributed in part to microbial inactivation. *C. jejuni* has been reported previously to be sensitive to extremes in pH, especially acidic conditions (*25*). Zhao and Doyle (*25*) recently exploited this sensitivity by showing that a combination of calcium sulphate and lactic acid (pH 2.1) can be used for the decontamination of *C. jejuni* on artificially-seeded poultry surfaces.

Results showed that plasma discharge treatment of all vegetative test bacteria suspended in poultry wash water at 4^{o}C also resulted in rapid reductions in microbial numbers (≤8 log CFU/ml) within 30 s exposure to nondetectable levels (Fig. 5). While similar inactivation kinetic data was observed for vegetative test organisms treated in distilled water (Fig. 3) and in poultry wash water (Fig. 5), in general, a more rapid reduction in test bacteria cell numbers was evident during treatment in the latter poultry wash water ($P < 0.05$). This may possibly be attributed in part to conversion of oils, fatty acids and proteins released during poultry washing into associated nitric and carbonic acids during plasma treatment. Interestingly, *S. enterica* serovar Typhimurium appeared more resistant to the lethal effects of pulsed plasma treatment compared to the other poultry-related *Campylobacter* and *Salmonella* pathogens tested. The latter resilience and survivability of this particular strain may in part reflect the growing importance of its multidrug resistance status, and as a cause of intestinal disease in humans (*6*).

The average total aerobic mesophilic count for untreated poultry wash water was 4.3 (± 1.1) log CFU/ml which was reduced to a non-detectable level after 30 s plasma treatment at 4ºC. Similar levels of *B. cereus* endospore reduction (*ca*. 3 log CFU/ml) was achieved during plasma treatments in

chilled distilled water (Fig. 3) and in poultry wash water (Fig. 5). Use of fluorescent redox probes revealed that there were slight overestimations in the level of *Campylobacter* (by *ca*. 0.7 ± 0.4 log CFU/ml) and *Salmonella* (by *ca*. 1.3 ± 0.3 log CFU/ml) cells killed in pulsed plasma gas-discharged treated distilled water at 4^{o}C compared to enumerating similarly treated test bacteria using the conventional direct plate count technique (Table 1.). The former technique measures respiratory activity in plasma treated test bacteria, and was also reported previously by other researchers as a rapid and effective means of enumerating microbial survivors (*3*). In general, it would appear that a sub-population (ca. 1 log CFU/ml) of PPGD-treated *Salmonella* and *Campylobacter* are capable of respiration (which intimates cell viability), but are unable to grow on laboratory-based culture media due to sub-lethal injury. This difference in microbial enumerating techniques is far less significant than compared to using conventional decontamination methods such as heating, where ≥3 log CFU/ml difference between direct plate count and respiratory staining was observed for *L. monocytogenes* and vegetative *B. cereus* cells (*24*). Conventional plate count methods are routinely employed to detect living microorganisms, but they are time consuming (because of lengthy incubations) and cannot be used to directly observe metabolically active cells in situ (*17*). Rodriguez et al. (*16*) previously reported on the used of the redox sensitive dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) for microscopic detection of respiring bacteria in aquatic environments. An aqueous solution of CTC is nearly colourless and nonfluorescent, but it is readily reduced to the water-insoluble fluorescent CTF product via the microbial electron transport system. CTF is deposited intracellularly like other formazans in a time-dependent manner and indicates respiratory activity. While numerous research groups have since reported on the use of CTC as an effective means of assessing respiratory activity in food and water-borne microorganisms that were subjected previously to a range of biocidal conditions (3, 16, 17, this is the first study to report on the efficacy of CTC for enumerating viable cells subjected to pulsed plasma gas-discharge treatments.

Our laboratory-based studies indicate that the use of pulsed plasma gas-discharge was very effective not only in reducing populations of bacterial enteropathogens in poultry wash water at 4^{o}C within 30s, but also could act as a critical control point in a hazard analysis critical control point (HACCP) by preventing cross-contamination of processing environments due to the non survival of *Campylobacter* and *Salmonella* spp. in the treated test liquids. The potential for poultry wash water to

be recycled using this pulsed plasma approach appears strong, particularly as this technology reduces microbial organisms treated to non-detectable levels in very short exposure times and the bactericidal properties associated with the plasma treatment itself are short lived reverting back to water post treatment leaving no unwanted chemical residues. However, further studies are needed to be undertaken in poultry-processing facilities to validate the efficacy of this plasma treatment under actual use conditions.

ACKNOWLEDGMENTS

We are grateful to David Currie for technical assistance on electron microscopy.

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Fig. 1 Schematic of pulsed plasma gas-discharge system for test liquids

Fig. 2 Relationship between sparged-gas composition and the inactivation of *B. cereus* endospores in pulsed plasma gas-discharge system containing distilled water at 4ºC

Fig. 3 Influence of pulsed plasma gas-discharge treatment on the inactivation of test bacteria suspended in distilled water at 4ºC

Fig. 4 Scanning electron micrograph images of PPGD-treated (a) and untreated (b) *C. jejuni* cells (magnification X12,000; white arrows indicate cellular damage).

Fig. 5 Influence of pulsed plasma gas-discharge treatment on the inactivation of test bacteria suspended in poultry wash water at 4ºC

Fig. 1.

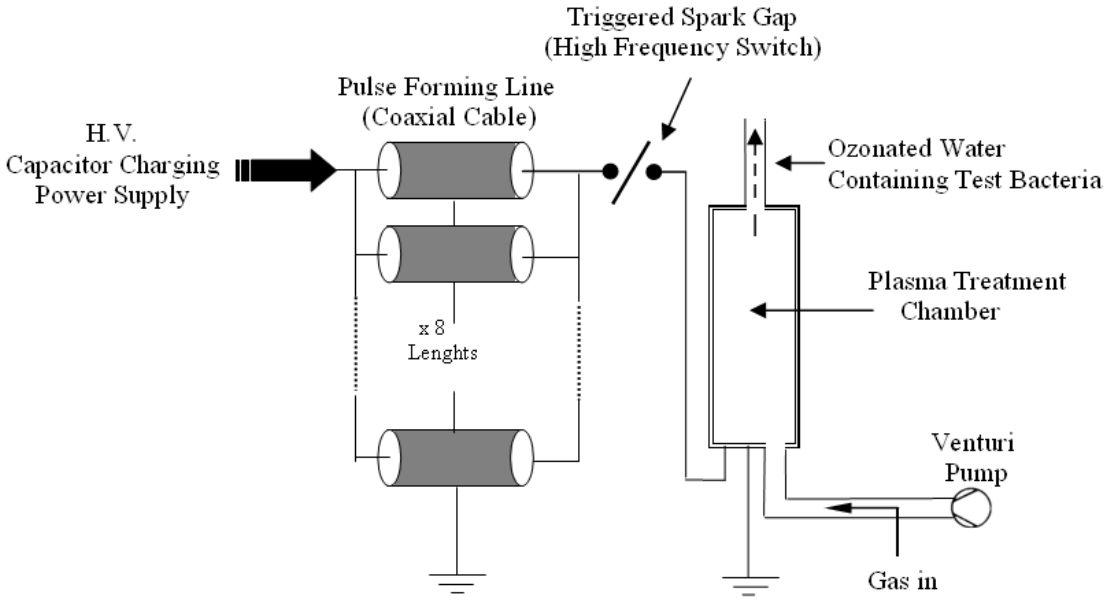
Triggered Spark Gap
(High Frequency Switch)
Pulse Forming Line
(Coaxial Cable)
H.V.
Capacitor Charging
Power Supply
Ozonated Water
Containing Test Bacteria
Plasma Treatment
Chamber
x 8
Lenghts
Venturi
Pump
Gas in

Fig. 2

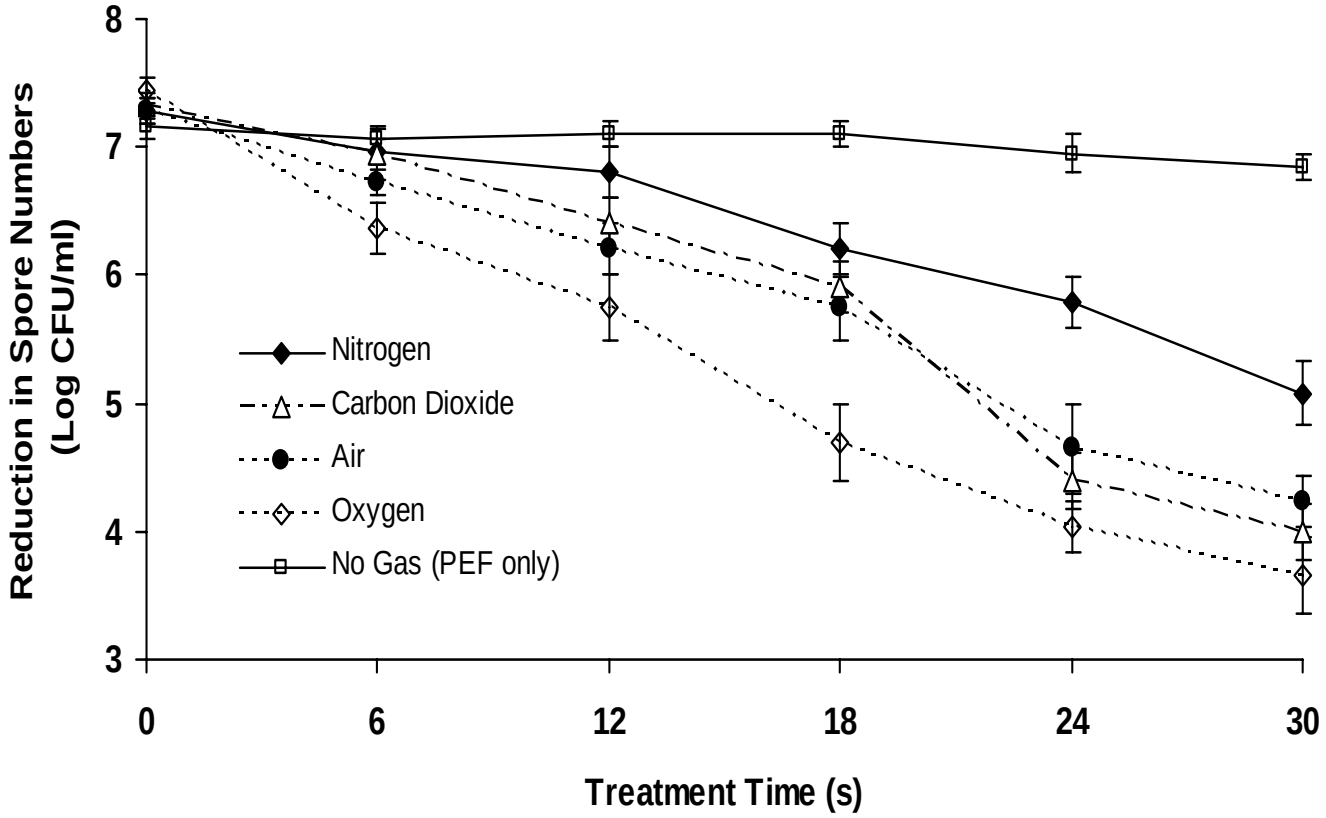
Reduction in Spore Numbers (Log CFU/ml)
8
7
6
5
4
3
0
6
12
18
24
30
Treatment Time (s)
Nitrogen
Carbon Dioxide
Air
Oxygen
No Gas (PEF only)

Fig. 3.

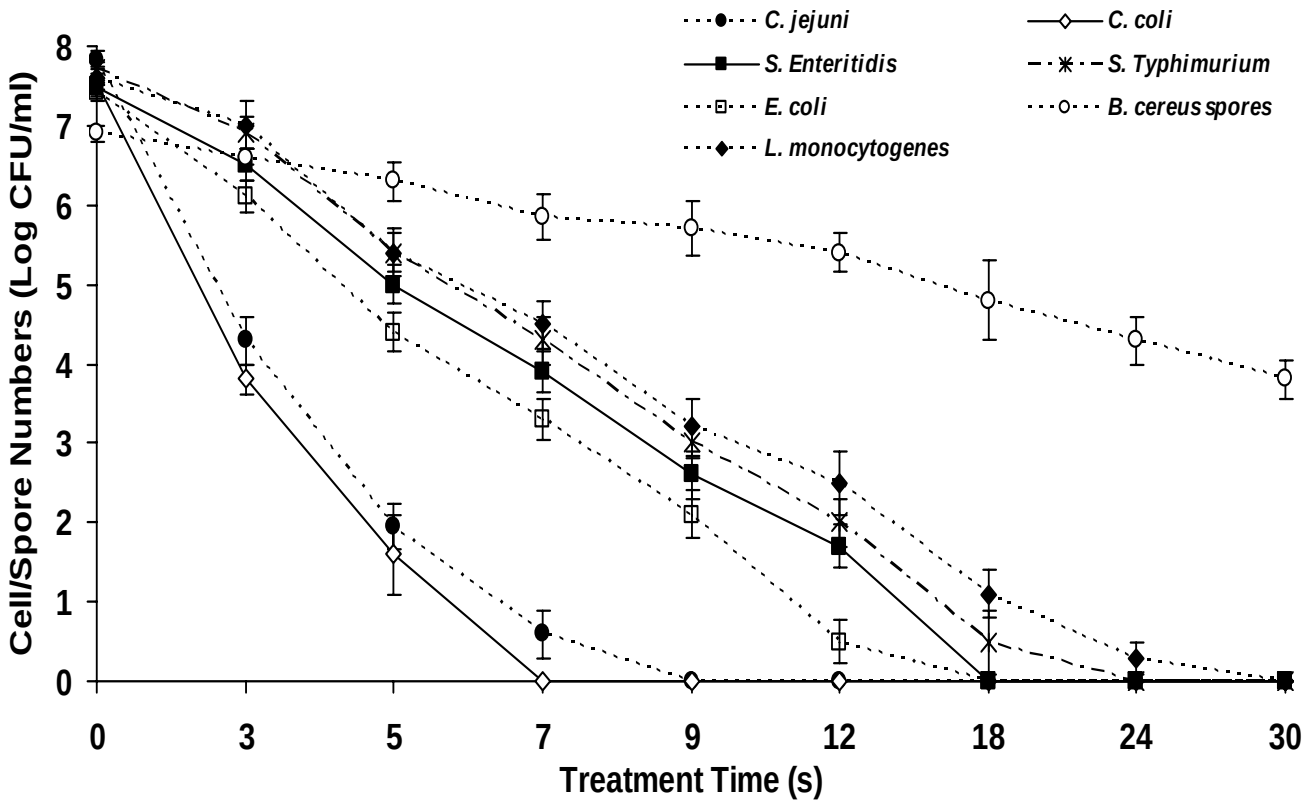
C. jejuni
C. coli
S. Enteritidis
S. Typhimurium
E. coli
B. cereus spores
L. monocytogenes
Cell/Spore Numbers (Log CFU/ml)
0
1
2
3
4
5
6
7
8
0
3
5
7
9
12
18
24
30
Treatment Time (s)

Fig. 4

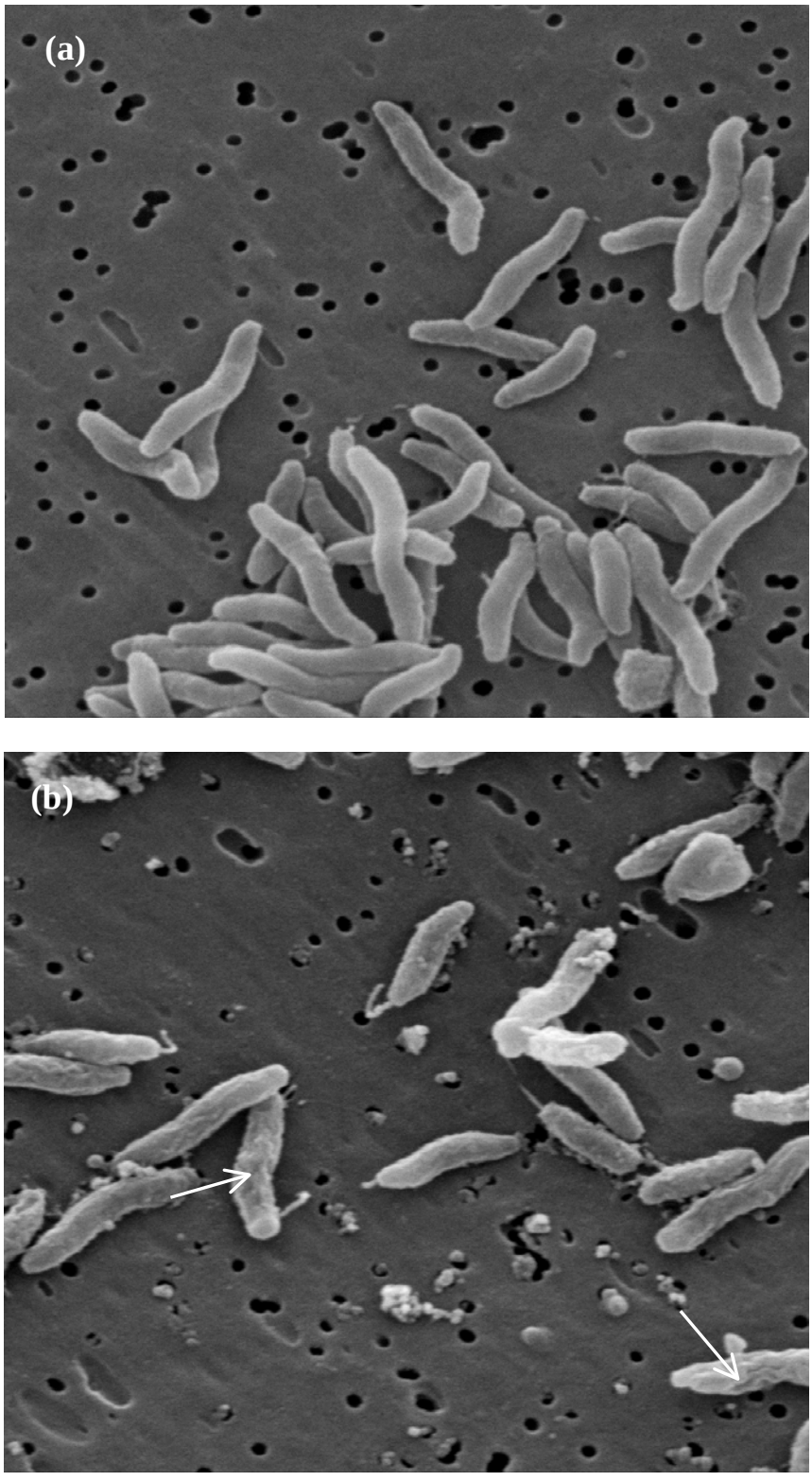

Fig. 5

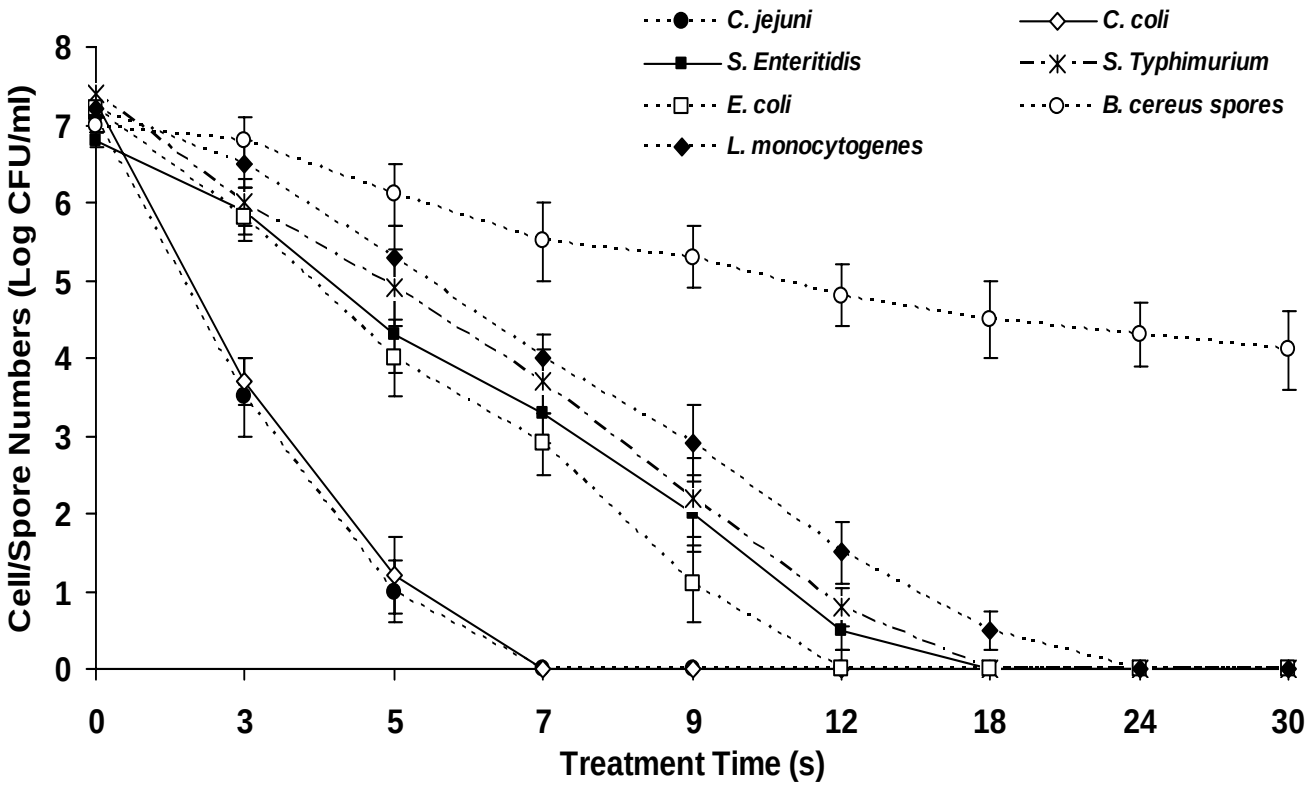
C. jejuni
C. coli
S. Enteritidis
S. Typhimurium
E. coli
B. cereus spores
L. monocytogenes
Cell/Spore Numbers (Log CFU/ml)
0
1
2
3
4
5
6
7
8
0
3
5
7
9
12
18
24
30
Treatment Time (s)

Table 1. Enumeration of *Campylobacter* and *Salmonellae* cell numbers by respiratory staining (RS) and CFU by direct plate counting (PC) after PPGD treatment

Test Bacterium	**PPGD[a]-Treatment Time (s)**	**Surviving population (Log CFU or Cells/ml)[b]**			**Significant Difference**
		PC	**RS**	**PC-RS[c]**	***P* < 0.05**
C. jejuni	0	7.8 ± 0.1	7.7 ± 0.2	0.1	No
	5	1.9 ± 0.4	2.7 ± 0.5	0.8	No
	7	0.5 ± 0.5	1.4 ± 0.4	0.9	No
	9	0	0.6 ± 0.5	0.6	Yes
	30	0	0	0	No
C. coli	0	7.6 ± 0.1	7.4 ± 0.1	0.2	No
	5	1.6 ± 0.6	2.3 ± 0.5	0.7	No
	7	0	0.4 ± 0.2	0.4	Yes
	9	0	0	0	No
	30	0	0	0	No
S. Typhimurium	0	7.7 ± 0.1	7.6 ± 0.1	0.1	No
	5	5.4 ± 0.5	6.3 ± 0.3	0.9	Yes
	9	3.0 ± 0.6	4.6 ± 0.5	1.6	Yes
	12	1.9 ± 0.3	2.9 ± 0.4	1.0	Yes
	30	0	0	0	No
S. Enteritidis	0	7.5 ± 0.2	7.5 ± 0.2	0	No
	5	5.0 ± 0.4	6.1 ± 0.3	1.1	Yes
	9	2.6 ± 0.6	3.8 ± 0.4	1.2	Yes
	12	1.6 ± 0.5	3.0 ± 0.4	1.4	Yes
	30	0	0	0	No

[a] Pulsed-plasma gas-discharge (PPGD) – treatments; 0 s refers to untreated control

[b] Values are the means of triplicate replicated measurements ± SD

[c] Log difference in enumeration techniques between cell numbers (by respiratory staining or RS) and CFU (by conventional direct plate counting or PC)