Bacterial Inactivation by High Voltage Atmospheric Cold Plasma: Influence of Process Parameters and Effects on Cell Leakage and DNA

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Bacterial inactivation by High Voltage Atmospheric Cold Plasma: Influence of process parameters and effects on cell leakage and DNA.

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Running Title: Bacterial inactivation by Atmospheric Cold Plasma
Abstract

Aims: This study investigated a range of atmospheric cold plasma (ACP) process parameters for bacterial inactivation with further investigation of selected parameters on cell membrane integrity and DNA damage. The effects of high voltage levels, mode of exposure, gas mixture and treatment time against *Escherichia coli* and *Listeria monocytogenes* were examined.

Methods and Results: $10^8$ CFU ml$^{-1}$ *E. coli* ATCC 25922, *E. coli* NCTC 12900 and *L. monocytogenes* NCTC11994 were ACP treated in 10ml phosphate buffered saline (PBS). Working gas mixtures used were; Air (gas mix 1), 90% N$_2$+10% O$_2$ (gas mix 2) and 65% O$_2$+30% CO$_2$+5% N$_2$ (gas mix 3). Greater reduction of viability was observed for all strains using higher voltage of 70 kV$_{RMS}$, and with working gas mixtures with higher oxygen content in combination with direct exposure. Indirect ACP exposure for 30 s inactivated below detection level both *E. coli* strains. *L. monocytogenes* inactivation within 30 s was irrespective of the mode of exposure. Leakage was assessed using A$_{260}$ absorbance and DNA damage was monitored using PCR and Gel electrophoresis. Membrane integrity was compromised after 5 s, with noticeable DNA damage also dependent on the target cell after 30 s.

Conclusions: Plasma treatment was effective for inactivation of challenge microorganisms, with a greater sensitivity of *L. monocytogenes* noted. Different damage patterns were observed for the different bacterial strains, attributed to the membrane structure and potential resistance mechanisms.

Significance and Impact of study: Using atmospheric air as working gas resulted in useful inactivation by comparison with high nitrogen or high oxygen mixes. The mechanism of inactivation was a function of treatment duration and cell membrane characteristics, thus offering potential for optimised process parameters specific to the microbial challenge.
Key words: DBD-ACP, Escherichia coli, Listeria monocytogenes, voltage level, cell integrity, DNA damage

Introduction

Plasma is a neutral ionised gas which is composed of particles including free electrons, radicals, positive and negative ions, quanta of electromagnetic radiation, excited and non-excited molecules (Misra et al. 2011). Plasma generated at room temperature and pressure is called atmospheric cold plasma (ACP). Due to advantages presented in terms of cost, environmental compliance and ease of processing the potential applications of ACP now encompass environmental and food treatment as well as clinical and health care areas. In developing ACP for food applications, it is important to recognise that flavour change may occur as a result of lipid peroxidation (Misra et al, 2011) and that sensory analysis should be included in process development. Research on ACP system development is rapidly progressing for complex environmental and biological applications such as cancer treatment or healing of open wounds (Müller and Zahn 2007; Eto et al. 2008; Sensenig et al. 2008; Dobrynin et al. 2011b).

ACP has been proved effective for microbial inactivation (Deng et al. 2007; Joshi et al. 2011; Ziuzina et al. 2013). Plasma discharge results in the generation of a wide range of reactive species responsible for the antimicrobial effects. Depending on the cell envelope differences, different inactivation responses were observed in plasma sterilization studies, where Gram-positive bacteria were found to be more resistant than Gram-negative bacteria (Lee et al. 2006; Ermlaeva et al. 2011). In contrast other studies indicated no significant differences in the effect of plasma treatment between Gram positive and Gram negative bacteria (Kayes et al. 2007; Venezia et al. 2008).

The inactivation efficacy of ACP is governed by system and process variables including power input, mode of exposure, duration of exposure and gas composition, as well as
features of the target such as microbial cell type (Deng et al. 2007; Fridman et al. 2007; Ghomi et al. 2009; Takamatsu et al. 2011; Liu et al. 2013). Higher system voltage and extended treatment time have been associated with greater inactivation efficacy (Deng et al. 2007; Ghomi et al. 2009; Joshi et al. 2011; Liu et al. 2013). With regard to mode of exposure, the magnitude of the field generated with direct voltage gap between electrodes can cause shear stress to cells, and energetic ions can directly affect cells subjected to direct exposure (Dobrynin et al. 2009; Dobrynin et al. 2011a). The diffusion of recombined or longer lived species through the medium and the target may lead to different inactivation patterns in association with mode of exposure (direct/indirect) and post treatment storage time (Ziuzina et al. 2013). Therefore, in this study we assessed the effect of the system parameters of high voltage levels and mode of exposure in conjunction with the gas composition.

Working gas type may influence the range and type of reactive species formed with an expected significant effect on microorganisms (Lerouge et al. 2000; Purevdorj et al. 2003; Zhang et al. 2013). Using air as a working gas, the reactive species generated could include reactive oxygen species (ROS), reactive nitrogen species (RNS), ultraviolet (UV) radiation, energetic ions and charged particles. ROS can play the most crucial role in the inactivation of microbes (Joshi et al. 2011). Hydrogen peroxide, singlet and atomic oxygen have a strong oxidative effect on microbes as well as ozone (Dobrynin et al. 2009), which can all be generated using plasma discharge in air and oxygen-containing mixtures. Dobrynin et al (2009) concluded that oxygen was required for fast and effective inactivation of bacteria, however, their study compared a range of single gas types with air. In contrast, Boxhammer et al. (2012) analysed the relative contribution of ROS and RNS produced by ACP in air and concluded that the bactericidal effect was related to a combination of oxidative and nitrosative effects. Discharging plasma in an air or nitrogen containing gas mixture can also
generate reactive nitrogen species (NO$_x$). However, it was the combined application of NO and H$_2$O$_2$ that yielded a higher inactivation effect on E. coli than a treatment with NO or H$_2$O$_2$ alone (Boxhammer et al., 2012). Another NO$_x$ species, peroxynitrite, which can be rapidly endogenously formed due to the reaction of nitric oxide and superoxide, can damage proteins, lipids and DNA as reported in Shigenaga et al. (1997). Thus, the type and range of reactive species generated can influence the mechanism of inactivation and this range of reactive species may vary with the working gas composition employed.

Therefore this study compared the inactivation effect of dielectric barrier discharge atmospheric cold plasma (DBD-ACP) using air with two different working gas mixtures commonly used in modified atmosphere packaging technology. The interactive effects with two high voltage levels, mode of exposure and treatment time were examined. Evaluating the interaction between system and process parameters with the target cell type will deepen understanding of the key extrinsic control parameters associated with membrane and intracellular processes. Therefore the relationship between inactivation patterns and the intracellular damage patterns that could be achieved were further explored. Cell membrane integrity and genomic DNA damage were selected as indicators. This study evaluated the effects described above on two target organisms E. coli and L. monocytogenes to compare the different damage mechanisms for Gram negative and positive bacteria. Furthermore, two strains of E. coli with different virulence characteristics were compared.

**MATERIALS AND METHODS**

**Bacteria types and Growth Conditions**

Three bacteria types were used in this study. E. coli ATCC 25922 and L. monocytogenes NCTC 11994, were obtained from microbiology stock culture of the School of Food Science and Environmental Health, Dublin Institute of Technology. E. coli NCTC 12900, (non-toxigenic O157:H7) was obtained from National Collection of type cultures of the Health...
Protection Agency (HPA, UK). Strains were selected to present both Gram positive and 
Gram negative foodborne challenges and to facilitate comparison with other studies. Strains 
were maintained as frozen stocks at -70 °C in the form of protective beads, which were plated 
onto tryptic soy agar (TSA, Scharlau Chemie) and incubated overnight at 37 °C to obtain 
single colonies before storage at 4 °C.

Preparation of Bacterial Cell Suspensions

Cells were grown overnight (18 h) by inoculating isolated colony of respective bacteria in 
tryptic soy broth without glucose (TSB-G, Scharlau Chemie), at 37 °C. Cells were harvested 
by centrifugation at 8,720 g for 10 min. The cell pellet was washed twice with sterile 
phosphate buffered saline (PBS, Oxoid LTD, UK). The pellet was re-suspended in PBS and 
the bacterial density was determined by measuring absorbance at 550 nm using McFarland 
standard (BioMérieux, Marcy-l'Étoile, France). Finally, cell suspensions with concentration 
of 10^8 CFU ml^{-1} were prepared in PBS.

ACP system configuration

The dielectric-barrier discharge (DBD) ACP system used in this study (Fig. 1) consists of a 
high voltage transformer (with input voltage 230 V at 50 Hz), a voltage variac (0 – 100%, 
output voltage controlled within 0–120 kV). ACP discharge was generated between two 15-
cm diameter aluminium electrodes. The system was operated at voltage levels of either 56-
kV_{RMS} or 70 kV_{RMS} at atmospheric pressure. Voltage and input current characteristics of the 
system were monitored using an InfiniVision 2000 X-Series Oscilloscope (Agilent 
Technologies Inc., USA). The two electrodes were separated by a dielectric barrier i.e. the 
polypropylene container, which acted as a sample holder. The distance between the two 
electrodes was kept identical (2.2 cm) for all experiments.

ACP treatment
For direct plasma treatment, 10 ml of bacterial cell suspensions in PBS were aseptically transferred to a sterile plastic petri dish, which was placed in the centre of the container, between the electrodes. For indirect plasma treatment, a separate container was used, where the sample petri dish was placed on the upper left corner of the container, outside the plasma discharge (Fig. 1). Each container was sealed in a high barrier polypropylene bag (B2630; Cryovac Sealed Air Ltd, Dunkan, SC, USA) using atmospheric air (gas mix 1) as a working gas for ACP generation. For the two gas mixtures, i.e. 90% N\textsubscript{2} + 10% O\textsubscript{2} (gas mix 2) and 65%O\textsubscript{2} + 30% CO\textsubscript{2} +5% N\textsubscript{2} (gas mix 3) the required working gas was filled into a sealed package using a flow regulator at a controlled flow rate of 0.5 L min\textsuperscript{-1} for 1 min. Bacterial samples were then treated with ACP at either 56 kV\textsubscript{RMS} or 70 kV\textsubscript{RMS} for 30 s, respectively. After ACP treatment, samples were subsequently stored at room temperature for 24 h (Ziuzina et al. 2013). Ozone concentrations generated were measured using GASTEC gas tube detectors (Product # 18M, Gastec Corporation, Kanagawa, Japan) immediately after treatment and also after 24 h storage. All experiments were carried out in duplicate and replicated twice.

**Microbiological Analysis**

To quantify the effects of plasma treatment, 1 ml of treated samples were serially diluted in MRD and 0.1 ml aliquots of appropriate dilutions were surface plated on TSA. In order to obtain low microbial detection limits, 1 ml of the treated sample was spread onto TSA plates as described by EN ISO 11290-2 method (ISO 11290-2, 1998). The limit of detection was 1 Log CFU ml\textsuperscript{-1}. Plates were incubated at 37 °C for 24 h and colony forming units were counted. Any plates with no growth were incubated for up to 72 h and checked for the presence of colonies every 24 h. Results are reported in Log CFU ml\textsuperscript{-1} units.

**Cell membrane integrity**
Membrane integrity was examined by determination of the release of material absorbing at 260 nm and 280 nm (Virto et al. 2005). The UV absorbance at 260 nm and 280 nm ($A_{260}$ and $A_{280}$) were used to indicate the effect on cell membrane integrity. Untreated (bacterial cells in PBS) and ACP-treated samples were centrifuged at 13,200 g for 10 min. Untreated controls determined the release of any intracellular material before ACP treatment. 200 µL supernatant of each sample was transferred into microtitre plate wells and measured by Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments Inc.) at 260 nm and 280 nm.

**Genomic DNA extraction and Polymerase chain reaction (PCR)**

Plasma treated aliquots were precipitated by ethanol with a final concentration of 70% for 5 min and centrifuged at 13,200 g for 10 min to obtain the pellet. Genomic DNA was then extracted from the pellet by Wizard Genomic DNA Purification Kit (Promega) as per manufacturer’s instructions. The amount of DNA was quantified by measuring the absorbance at 260 nm. The genomic DNA amplification of conserved bacterial regions i.e. 16S rRNA was performed using the primers listed in Table 1. All reactions were performed with GoTaq Colorless Mastermix (Promega). 25 µL PCR reaction system was used that contained, 12.5 µL Mastermix, 2 µL of each primer (0.2 nmol), 2 µL of genomic DNA as template (0.2 ng) and sterilized water to make final volume up to 25 µL. Amplification programme was conducted as: initial denaturation step at 95 ºC for 5 min, followed by 25 cycles at 95 ºC 45 s for denaturation, at 51 ºC 30 s annealing, at 72 ºC 90 s extension, and 72 ºC 10 min for final extension.

Electrophoresis was carried in 0.8% agarose gel, with Ethidium Bromide (Sigma Aldrich Ltd, Dublin, Ireland) staining at 140 V. Genomic DNA samples (20 ng) were loaded for each well, with exACTGene 1 kb plus marker (Fisher BioReagents). 16S rRNA PCR products were loaded with BenchTop pGEM DNA marker (Promega).
Statistical Analysis

Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, U.S.A). Data represent the means of experiments performed in duplicate and replicated at least twice. Means were compared using analysis of variance (ANOVA) using Fisher’s Least Significant Difference-LSD at the 0.05 level.

RESULTS

The effect of voltage level on DBD-ACP inactivation efficiency

The effect of voltage levels on ACP inactivation efficacy was investigated for *E. coli* strain ATCC 25922. ACP treatment of *E. coli* in PBS at 56 kV\textsubscript{RMS}, using air as a working gas type, decreased the cell population by 1.8 and 1.6 log cycles after direct and indirect exposure, respectively. Similar effects were noted using gas mix 2, where the reductions noted were 1.0 and 1.2 log cycles following direct and indirect ACP exposure, respectively (Table 2). Increased voltage level of 70 kV\textsubscript{RMS} achieved significantly greater inactivation effects compared to lower voltage level tested (p≤0.05). The indirect plasma exposure at 70 kV\textsubscript{RMS}, for 30s either in gas mix 1 or 2, decreased the population by 7.9 and 3.2 log cycles, respectively. Similarly, direct ACP exposure at higher voltage level using either gas mixture resulted in better inactivation effects compared to lower voltage level tested (Table 2).

The effect of gas mixtures on DBD-ACP inactivation efficiency

In order to assess the effect of gas mixtures on ACP inactivation efficacy, the higher voltage level was used against bacterial strains studied. With direct exposure of ACP generated in either in gas mix 1, 2 or 3, the population of *E. coli* ATCC 25922 was reduced by 3.4, 2.8 and 6.6 log cycles, respectively (p≤0.05). However, indirect exposure in gas mix 1 and 3 resulted in greater inactivation rates whereas only 3.2 log cycles reduction were recorded when gas mix 2 was utilised (Table 3). While in the case of non-toxigenic strain *E. coli* NCTC 12900, inactivation below detection was achieved only after indirect exposure in gas mix 3. The
other gas mixtures were less effective. *L. monocytogenes* population was below detection level after indirect exposure of ACP generated in all three gas mixtures tested. With direct ACP exposure only, gas mixes 1 and 3 were more effective than the gas mix 2 for inactivation of *L. monocytogenes* (Table 3).

**Ozone measurements**

Ozone concentrations were measured immediately after plasma exposure with GASTEC gas tubes. Ozone concentrations of 1280 and 1000 ppm was noted immediately after direct ACP treatment in gas mix 1 or 2, respectively. Indirect ACP treatment recorded slightly increased concentrations of 1440 ppm and 1367 ppm, respectively. Higher concentrations of 2000 and 4000 ppm were noted after direct or indirect ACP treatment in gas mix 3, respectively. No ozone concentrations were recorded for stored samples after 24 h of post-treatment storage.

**Effect on cell membrane integrity**

Figure 2 represents inactivation curve of bacterial strains following ACP exposure. It was observed that, all bacterial strains studied were inactivated below detection level with 60 s of ACP treatment in air irrespective to the mode of exposure (Fig. 2). *E. coli* ATCC 25922 populations were reduced below detection level after 30 s of indirect ACP treatment however, direct ACP treated population reduced by 3.4 log cycles. *E. coli* NCTC 12900 was more resistant, with reduction of around 2 log cycles recorded after both direct and indirect exposure. With 5 s exposure, both *E. coli* ATCC 25922 and *E. coli* NCTC 12900 had less than 1 log cycle reduction. However, *L. monocytogenes* NCTC 11994 was more sensitive, where 5 s of treatment achieved reductions of 3.1 and 1.8 log cycles with direct and indirect exposure, respectively. Meanwhile, 30 s of treatment decreased population by about 6 log cycles, regardless of the mode of exposure.

Figure 3 presents the release of intracellular components (nucleic acid) absorbing at 260 nm following treatment. The results obtained at 280 nm (protein) were similar (data not shown).
For both *E. coli* strains, the absorption curves showed similar trends. A sharp increase in absorbance followed by a steady stage was recorded, indicating the cell integrity was compromised within 5 s of ACP treatment. While a similar trend was observed for the absorbance of *L. monocytogenes* NCTC 11994, the leakage recorded even after 120 s treatment was significantly less than that for *E. coli* strains within 5 s treatment.

**DNA Damage**

Bacterial cells were treated with plasma and harvested cells were tested for DNA amplifications by performing PCR as described before. Figure 4 represents extracted genomic DNA and PCR amplified products of untreated and ACP treated samples run on agarose gel electrophoresis. DNA samples were quantified by absorbance at 260 nm and adjusted to same loading amount in each lane. Genomic DNA from ACP treated samples showed weaker band intensity than the control i.e. untreated cells, thus indicating ACP treatment resulted in damage of DNA. With longer treatment time of 30 s, more damage was observed than with 5 s treatment (Fig. 4 a, b, c). This pattern was noted for all strains studied, which was well correlated with microbial inactivation assessed by colony count method.

PCR results which are more sensitive for detection of small amounts of DNA showed no noticeable difference between treated and untreated samples of *E. coli* (Fig. 4 d and e). However, in the case of *L. monocytogenes*, 30 s of ACP treatment resulted in more DNA damage which showed a band with a weaker intensity than 5 s ACP treated and control untreated samples (Fig. 4 f). *L. monocytogenes* was more sensitive than the other two strains of *E. coli* studied. These observations were also correlated with the low survival rate of *L. monocytogenes* after ACP treatment.

**DISCUSSION**

In order to ensure system efficacy, ACP was tested against different types of bacteria which can demonstrate different responses against plasma stress as noted previously (Hury *et al.*
1998; Laroussi et al. 2003, Kvam et al. 2012). Applied voltage level had an impact on ACP antimicrobial efficacy, where at the higher voltage level greater microbial inactivation was achieved. The energy of ACP discharge is decided by applied voltage and frequency, thus generating different amounts of reactive species which influence inactivation (Deng et al. 2007; Liu et al. 2013). Liu et al. (2013) studied the relationship between reactive species generation using helium as working gas and atmospheric non thermal plasma jet voltage level over time and found that four kinds of active species, N$_2^+$, OH, He and O, increased gradually with increasing applied voltage, which they attributed as responsible for the increasing inactivation efficacy. In the preliminary stages of our study, higher operating voltage resulted in higher inactivation efficacy, which could also be attributed to the concentration of generated reactive species influencing the inactivation rate.

The mode of ACP exposure showed some interesting inactivation effects interacting with the type of bacteria and working gas used. Direct plasma exposure was reported to have greater bactericidal effects than indirect exposure due to role of charged particles in synergy with the generated reactive species (Fridman et al. 2007; Dobrynin et al. 2009). In our study, interestingly, overall the indirect mode of exposure was more effective than direct exposure for microbial inactivation (p < 0.05). The possible explanation for this could be recombination of reactive radicals prior to reaching the target sample, generating reactive species with strong bactericidal effects, in addition to the action of ozone that might also occur especially with gas mixes 1 and 3. In common to other studies, there was a clear link to plasma treatment time and its inactivation efficacy (Deng et al. 2007; Ghomi et al. 2009; Joshi et al. 2011).

Besides voltage level, the working gas utilised for ACP discharge had a major effect on inactivation. Overall gas mix 3 was associated with greater inactivation (p < 0.05). No significant differences were observed between gas mix 1 and 2 effects overall (p>0.05). However, there was a significant interactive effect of microorganism with gas mixture on the
inactivation efficacy. For *E. coli* ATCC 25922 with direct exposure and *E. coli* NCTC 12900 with indirect exposure, the gas mixture with higher oxygen content (gas mix 3) was the most effective for inactivation. Similar effects have been noted previously where oxygen gas plasma were very effective for microbial reduction (Hury *et al.* 1998; Laroussi and Leipold 2004; Hong *et al.* 2009). ACP discharge in a gas mixture containing oxygen generates highly reactive chemical species such as hydroxyl radicals (OH\(^-\)) and ozone (O\(_3\)). The presence of water either as humidity in gas or as liquid in a system such as in this study, during the plasma discharge, results in an abundance of OH\(^-\) radicals, H\(_2\)O\(_2\) and hydronium ions H\(_3\)O\(^+\) (Dobrynin *et al.* 2011a; Parvulescu *et al.* 2012). Thus, production of highly oxidizing species previously reported as having strong bactericidal effects yielded significant inactivation effects. ACP inactivation efficacy in air was further influenced by treatment time (Fig. 2) and type of target cell. Boxhammer *et al.* (2012) investigated the relative role of reactive species generated with plasma discharge in air and proposed that the high bactericidal effect of ACP in air was due to an interaction of both RNS and ROS, as indicators of ROS or RNS alone did not yield significant microbial reductions, but a 4 min ACP treatment using air delivered a 5 log reduction of *E. coli*. In our study, we report greater bactericidal tendency using gas mix 3 which was composed of high oxygen, high carbon dioxide but low nitrogen levels than that achieved using gas mix 2 with high nitrogen and low oxygen levels. However, a useful efficacy was achieved using gas mix 1 (air). It is likely that varying the ratio of working gases will lead to different ratios of reactive species which in turn may further elucidate the relative importance of specific species for inactivation of particular target cells. ACP discharge in air (gas mix 1) also recorded better inactivation effects than gas mix 2, although these were not always significant. The gas mixes studied here reflect those commonly employed for modified atmosphere packaging in the food industry. Therefore it was interesting to note that significant antimicrobial effects could be achieved using atmospheric air in very short treatment times, in place of a specific gas mix targeted for microbiological quality control.

The effects of ACP inactivation were also dependent on bacterial strains studied. Literature reports differing bacterial sensitivity towards plasma, based on their cell wall structures (Ma *et al.* 2008; Ermolaeva *et al.* 2011; Liang *et al.* 2012) while others suggested no clear differences in inactivation by cold atmospheric plasma treatment in relation to cell wall structure (Klämpfl *et al.* 2012). In our study, with respect to inactivation, Gram positive *L.*
monocytogenes populations were more sensitive than Gram negative E. coli cells. Bacterial inactivation by non-thermal plasma is a complex process and its mechanism of action is a subject of interest which is still not completely understood. Related to the system in use, it is warranted to evaluate specific system and process parameters in conjunction with the potential target as one way of enhancing understanding of the mechanism of action.

To gain insight to the relationship between system and process parameters and mechanism of ACP action, we investigated some biological consequences following ACP exposure. Cell leakage and DNA damage were assessed. Joshi et al. (2011) attributed cell death to oxidation of DNA, protein and lipid during ACP treatment. However, Dobrynin et al. (2009) reported the primary target as the cell membrane. These differences are not surprising given the contrasting and inconsistent inactivation effects against bacteria with different cell membranes as described above. However, both the cell wall and vital intracellular macromolecules are reported as main targets of reactive species (Dobrynin et al. 2009; Machala et al. 2009; Roth et al. 2010). In our study, cell leakage measured by absorbance 260 nm and 280 nm, following ACP exposure showed different responses for Gram negative and Gram positive bacteria. The cell leakage (reflecting release of intracellular material such as proteins, DNA, RNA) results in our studies suggested more compromised cell membrane integrity for Gram negative bacteria, for short duration ACP exposure. With regard to effects on membrane integrity, Laroussi et al. (2003) reported that the cell wall of Gram negative bacteria (outer membrane of lipopolysaccharide and thin layer of peptidoglycan) was more vulnerable than the more stable peptidoglycan structure of the cell envelope for Gram positive bacteria.

Comparing cell leakage and inactivation results with a 5 s ACP exposure, a high leakage rate was detected for all strains (Fig.3), however, there were only minor effects on the cell culturability (Fig. 2). For E. coli strains, approximately 1 log reductions were achieved after
5 s in association with a large spike in the material leaking from the cell. However, for *Listeria*, up to 3 log reductions were noted after 5 s but with a much smaller spike cell leakage recorded. The possible explanation could be that the short ACP exposure of 5 s results in reversible damage with the likelihood of activation of a cell response system for repairing the damage (Dobrynin *et al.* 2009). Nevertheless, when treatment time was increased, bacterial populations were reduced to undetectable levels for all strains. The diffusion of generated reactive species into the cell results in either irreparable damage to cell membrane and/or major cell constituents.

The inactivation efficacy was also related to anti-oxidative activities of the target bacteria. Compared to *E. coli* ATCC 25922, the non-toxigenic *E. coli* NCTC 12900 has been reported to have a stronger resistance to acid stress, multi-drug resistance and higher rate of mutations, which has cross protective effect against a wide range of environmental stresses including oxidative stress (Braoudaki and Hilton 2004; Maurer *et al.* 2005; Hosein 2010,.). These characteristics could impact the resistance of *E. coli* NCTC 12900 resulting in different responses yielding greater resistance to ACP stress by comparison with *E. coli* ATCC 25922. Comparing the results of inactivation, *L. monocytogenes* was more sensitive to ACP treatment than the two *E. coli* strains studied. In the case of *L. monocytogenes*, the diffusion of ROS and RNS across the membrane would cause a severe irreversible damage of macromolecules including DNA, making the bacterial cells susceptible to ACP treatment. Therefore, we performed further investigations to assess ACP effects on genomic DNA damage and amplified DNA products.

In our study using high voltage plasma, the extent of genomic DNA damage was dependent on type of bacteria and treatment time. Thus indicating that the concentration of ACP generated reactive species increased with time, resulting in time dependent genomic DNA damage (Figs. 4 a, b, c), which potentially increased the sensitivity towards plasma generated
oxidative stress. To further assess DNA fragmentation by high voltage plasma, amplification of DNA by PCR was performed which revealed the extent of DNA damage was dependent not only on type of bacteria but on ACP treatment time (Fig. 4 d, e, f). Extensive DNA damage has been related to bacterial type and system parameters (Cooper et al. 2010; Joshi et al. 2011). Using the low voltage of 15Kv, Joshi et al. (2011) demonstrated the fragmentation of E. coli genomic DNA depended on the length of plasma exposure (treatment time) and energy dose (J/cm²) using floating electrode DBD plasma application. Cooper et al. (2010) investigated DBD plasma treatment of Bacillus stratosphericus under a dry environment at 30 kV for 120 s and suggested direct interaction of charged particles or photons within the plasma with the bacterial cell membrane thus directly exposing internal components to extensive DNA damage. In our study, 5 s of ACP treatment showed significant effects on membrane integrity with a strong increase in leakage, but no significant impact on DNA damage was noted, thus suggesting that repair is possible when the microbiological target is subject to very short treatment times even at high voltage. Enzymes, such as Ahp, SOD and Kat, are reported to have clearance effects for ROS (Imlay, 2013), resulting in less intracellular damage, such as DNA cleavage or enzyme inactivation. The repair systems in E. coli and L. monocytogenes could mitigate the effect of ROS generated by ACP and diffused inside the cell. Alternatively, the 5s treatment time might be too short for ROS to cause detectable DNA damage. With longer treatment times, the ROS accumulation could exceed the cell clearance capability, and resulting in damage visible on agarose gel. After 30 s of treatment, population viability was significantly reduced in tandem with sustained leakage, while significant DNA damage was only evident for L. monocytogenes. Little DNA damage was noted for E. coli strains, even after 30 s treatment. The plasma reactive species may interact with the multi-layered Gram negative cell membrane and the polysaccharide chains and compromise membrane integrity, thus DNA damage may not be
the primary mechanism of action for short treatment times at high voltage. Further increasing
treatment time could cause more adverse effects on nucleic acids resulting in irreversible
DNA damage with loss of cell culturability. The PCR results reveal multi-site DNA strand
breakage. Cell viability could be maintained with the low level DNA damage observed with
the very short plasma treatment time of 5 s (Fig. 4); specifically, activities related to multi-
copy genes may be unaffected. Increasing the treatment time up to 30 s at high voltage, the
predominant effect of our system was related to the target cell; where membrane damage may
be the primary effect but for *L. monocytogenes* intracellular components were major targets.
Recent studies highlighted activation of repair systems of plasma treated bacteria in addition
to up or down regulation of specific genes under ACP stress (Roth *et al.* 2010, Sharma *et al.*
2009). To understand in detail the ACP effects on intracellular targets, investigations on
regulatory factors of ACP treated bacteria could elucidate the interaction between reactive
species and cell response.

Overall, there was a strong effect of the ACP process parameters of working gas mixture and
treatment time on inactivation of *E. coli* and *L. monocytogenes*. The working gas ratios were
associated with different bactericidal efficacies. We have found using a high voltage of 70
kV<sub>RMS</sub> that the reactive species generated in a very short treatment time of 5 s had significant
effects on cell integrity. Extending treatment time to 30 s, caused significant bacterial
reduction with mode of action dependent on bacterial type.

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References:


Geneva: ISO.


**Table 1: Designed primers for PCR**

<table>
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<tr>
<th>Organism</th>
<th>5’-3’ Sequence</th>
<th>PCR product length</th>
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<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>Forward: CAG GCC TAA CAC ATG CAA GT</td>
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<td><em>E. coli</em> NCTC 12900</td>
<td>Reverse: CGA AGG TTA AGC TAC CTA CTT</td>
<td>1410 bp</td>
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<tr>
<td><em>L. monocytogenes</em> NCTC 11994</td>
<td>Forward: TAAAGAGAGT TTGATCCTGG C</td>
<td>1418 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCT ACC GAC TTC GGG TGT T</td>
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**Table 2: Effect of voltage levels on ACP inactivation efficacy for E. coli ATCC 25922**

<table>
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<tr>
<th>Voltage (kV&lt;sub&gt;RMS&lt;/sub&gt;)</th>
<th>Gas mixtures</th>
<th>Mode of Plasma Exposure</th>
<th>[\text{Initial cell density (Log CFU ml}^{-1}\text{)}]</th>
<th>Reduction (Log CFU ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>SD*</th>
<th>[\text{Initial cell density (Log CFU ml}^{-1}\text{)}]</th>
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<td></td>
</tr>
<tr>
<td>56</td>
<td>1*</td>
<td>8.0</td>
<td>1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.6</td>
<td>8.0</td>
<td>1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2*</td>
<td>7.7</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2</td>
<td>7.7</td>
<td>1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>1*</td>
<td>7.9</td>
<td>3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4</td>
<td>7.9</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2*</td>
<td>7.7</td>
<td>2.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.2</td>
<td>7.7</td>
<td>3.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

Different letters indicate a significant difference at the 0.05 level between voltage level and between gas types.

*SD: standard deviation

ND*: Under detection limit

Experimental conditions: 30 s ACP treatment, 24 h post-treatment storage

*Gas mix 1: Air

Gas mix 2: 90% N<sub>2</sub>+10% O<sub>2</sub>
Table 3: Effect of gas mixtures on ACP inactivation efficacy

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gas mixtures</th>
<th>Mode of Plasma Exposure</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>SD*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Direct</td>
<td>Initial cell density (Log CFU ml(^{-1}))</td>
<td>Reduction (Log CFU ml(^{-1}))</td>
<td>SD*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli ATCC 25922</em></td>
<td>1</td>
<td>7.9</td>
<td>3.4(^a)</td>
<td>0.4</td>
<td>7.9</td>
<td>ND*(^d)</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.7</td>
<td>2.8(^b)</td>
<td>0.2</td>
<td>7.7</td>
<td>3.2(^{a,b})</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.0</td>
<td>6.6(^c)</td>
<td>0.1</td>
<td>8.0</td>
<td>ND*(^d)</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td><em>E. coli NCTC 12900</em></td>
<td>1</td>
<td>7.9</td>
<td>1.8(^{a})</td>
<td>0.2</td>
<td>7.9</td>
<td>1.6(^a)</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.0</td>
<td>1.4(^a)</td>
<td>0.4</td>
<td>8.0</td>
<td>1.8(^{b})</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.0</td>
<td>2.1(^b)</td>
<td>0.5</td>
<td>8.0</td>
<td>ND*(^c)</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>2</td>
<td>8.3</td>
<td>ND*(^a)</td>
<td>0.7</td>
<td>8.3</td>
<td>ND*(^a)</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>NCTC 11994</td>
<td>3</td>
<td>8.2</td>
<td>4.1(^b)</td>
<td>0.1</td>
<td>8.2</td>
<td>ND*(^a)</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SD*</td>
<td></td>
</tr>
</tbody>
</table>

Different letters indicate a significant difference at the 0.05 level between gas mixtures and mode of exposure for each strain.

*SD: Standard Deviation

ND*: Under detection limit

Experimental conditions: 70 kV\(_{\text{RMS}}\) 30 s treatment, 24 h post-treatment storage

*Gas mix 1: Air

Gas mix 2: 90% \(N_2\)+10% \(O_2\)

Gas mix 3: 65% \(O_2\) + 30% \(CO_2\)+5% \(N_2\)
Fig. 1: A schematic diagram of the experimental plasma device.
Fig. 2: ACP inactivation of bacterial strains in PBS

Experimental conditions: Voltage: 70 kV_{RMS}; Treatment time: 0–120 s; Post treatment storage time: 24 h; Gas mix: Air

● *E. coli* ATCC 25922; ■ *E. coli* NCTC 12900; ■ *L. monocytogenes* NCTC 11994

Solid line: direct exposure; Dotted line: indirect exposure
Fig. 3: Absorbance 260 after ACP treatment in PBS
Voltage: 70 kV\textsubscript{RMS}; Treatment time: 0–120 s; Post treatment storage time: 24 h; Gas mix: Air

\textbullet E. coli ATCC 25922; \textblacktriangle E. coli NCTC 12900; \textblacklozenge L. monocytogenes NCTC 11994

Solid line: direct exposure; Dotted line: indirect exposure
Fig. 4: Agarose gel electrophoresis showing genomic DNA and PCR amplified products of untreated and ACP treated samples

Voltage: 70 kV\textsubscript{RMS}; Treatment time: 0–30 s; Post treatment storage time: 24 h; Gas mix: Air

Genomic DNA damage of (a) *E. coli* ATCC 25922; (b) *E. coli* NCTC 12900; (c) *L. monocytogenes* NCTC 11994

16S rRNA PCR results of (d) *E. coli* ATCC 25922; (e) *E. coli* NCTC 12900; (f) *L. monocytogenes* NCTC 11994

Lane 1: Non plasma treatment control; 2: 5 s directly treated samples; 3: 5 s indirectly treated samples; 4: 30 s directly treated samples; 5: 30 s indirectly treated samples