2014-1

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

Lu Han
Technological University Dublin, lu.han@tudublin.ie

Sonal Patil
Technological University Dublin, sonalpatil81@gmail.com

Kevin M. Keener
Purdue University, kkeener@purdue.edu

See next page for additional authors

Recommended Citation

Han, L., Patil, S., Keener, K., Cullen, P. J., Bourke, P. (2014). Bacterial inactivation by High Voltage Atmospheric Cold Plasma: Influence of process parameters and effects on cell leakage and DNA, *Journal of Applied Microbiology*,116 (4), p.784-794. DOI: 10.1111/jam.12426

Funder: European Community

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

Lu H.[1], Patil S.[1], Keener, K.M[2]., Cullen, P.J.[1], & Bourke, P[1]*.

1. *School of Food Science and Environmental Health, Dublin Institute of Technology, Ireland*
2. *Purdue University, Nelson Hall of Food Science, Rm 3215745 Agriculture Mall DriveWest Lafayette, IN 47907-2009*

*Corresponding Author

Paula Bourke

School Food Science and Environmental Health

Dublin Institute of Technology

Dublin 1, Ireland

paula.bourke@dit.ie

00 353 1 4027594

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 sheer 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_2O_2 that yielded a higher inactivation effect on *E. coli* than a treatment with NO or H_2O_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 ºC in the form of protective beads, which were plated onto tryptic soy agar (TSA, Scharlau Chemie) and incubated overnight at 37 ºC to obtain single colonies before storage at 4 ºC.

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 ºC. 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 Vat 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

153 For direct plasma treatment, 10 ml of bacterial cell suspensions in PBS were aseptically
154 transferred to a sterile plastic petri dish, which was placed in the centre of the container,
155 between the electrodes. For indirect plasma treatment, a separate container was used, where
156 the sample petri dish was placed on the upper left corner of the container, outside the plasma
157 discharge (Fig. 1). Each container was sealed in a high barrier polypropylene bag (B2630;
158 Cryovac Sealed Air Ltd, Dunkan, SC, USA) using atmospheric air (gas mix 1) as a working
159 gas for ACP generation. For the two gas mixtures, i.e. 90% N_2+ 10% O_2 (gas mix 2) and
160 65%O_2 + 30% CO_2 +5% N_2 (gas mix 3) the required working gas was filled into a sealed
161 package using a flow regulator at a controlled flow rate of 0.5 L min^{-1} for 1 min. Bacterial
162 samples were then treated with ACP at either 56 kV_{RMS} or 70 kV_{RMS} for 30 s, respectively.
163 After ACP treatment, samples were subsequently stored at room temperature for 24 h
164 (Ziuzina *et al.* 2013). Ozone concentrations generated were measured using GASTEC gas
165 tube detectors (Product # 18M, Gastec Corporation, Kanagawa, Japan) immediately after
166 treatment and also after 24 h storage. All experiments were carried out in duplicate and
167 replicated twice.

168 ***Microbiological Analysis***

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

176 ***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_{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_{RMS} achieved significantly greater inactivation effects compared to lower voltage level tested ($p \leq 0.05$). The indirect plasma exposure at 70 kV_{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 ($p \leq 0.05$). 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 mixes1 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

303 inactivation efficacy. For *E. coli* ATCC 25922 with direct exposure and *E. coli* NCTC 12900
304 with indirect exposure, the gas mixture with higher oxygen content (gas mix 3) was the most
305 effective for inactivation. Similar effects have been noted previously where oxygen gas
306 plasma were very effective for microbial reduction (Hury *et al.* 1998; Laroussi and Leipold
307 2004; Hong *et al.* 2009). ACP discharge in a gas mixture containing oxygen generates highly
308 reactive chemical species such as hydroxyl radicals ($OH^{\cdot}$) and ozone (O_3). The presence of
309 water either as humidity in gas or as liquid in a system such as in this study, during the
310 plasma discharge, results in an abundance of $OH^{\cdot}$ radicals, H_2O_2 and hydronium ions H_3O^+
311 (Dobrynin *et al.* 2011a; Parvulescu *et al.*2012). Thus, production of highly oxidizing species
312 previously reported as having strong bactericidal effects yielded significant inactivation
313 effects. ACP inactivation efficacy in air was further influenced by treatment time (Fig. 2) and
314 type of target cell. Boxhammer *et al.* (2012) investigated the relative role of reactive species
315 generated with plasma discharge in air and proposed that the high bactericidal effect of ACP
316 in air was due to an interaction of both RNS and ROS, as indicators of ROS or RNS alone did
317 not yield significant microbial reductions, but a 4 min ACP treatment using air delivered a 5
318 log reduction of *E. coli*. In our study, we report greater bactericidal tendency using gas mix 3
319 which was composed of high oxygen, high carbon dioxide but low nitrogen levels than that
320 achieved using gas mix 2 with high nitrogen and low oxygen levels. However, a useful
321 efficacy was achieved using gas mix 1 (air). It is likely that varying the ratio of working
322 gases will lead to different ratios of reactive species which in turn may further elucidate the
323 relative importance of specific species for inactivation of particular target cells. ACP
324 discharge in air (gas mix 1) also recorded better inactivation effects than gas mix 2, although
325 these were not always significant. The gas mixes studied here reflect those commonly
326 employed for modified atmosphere packaging in the food industry. Therefore it was
327 interesting to note that significant antimicrobial effects could be achieved using atmospheric
328 air in very short treatment times, in place of a specific gas mix targeted for microbiological
329 quality control.
330 The effects of ACP inactivation were also dependent on bacterial strains studied. Literature
331 reports differing bacterial sensitivity towards plasma, based on their cell wall structures (Ma
332 *et al.* 2008; Ermolaeva *et al*. 2011; Liang *et al.* 2012) while others suggested no clear
333 differences in inactivation by cold atmospheric plasma treatment in relation to cell wall
334 structure (Klämpfl *et al.* 2012). In our study, with respect to inactivation, Gram positive *L.*

335 *monocytogenes* populations were more sensitive than Gram negative *E. coli* cells. Bacterial
336 inactivation by non-thermal plasma is a complex process and its mechanism of action is a
337 subject of interest which is still not completely understood. Related to the system in use, it is
338 warranted to evaluate specific system and process parameters in conjunction with the
339 potential target as one way of enhancing understanding of the mechanism of action.
340 To gain insight to the relationship between system and process parameters and mechanism of
341 ACP action, we investigated some biological consequences following ACP exposure. Cell
342 leakage and DNA damage were assessed. Joshi *et al.* (2011) attributed cell death to oxidation
343 of DNA, protein and lipid during ACP treatment. However, Dobrynin *et al*. (2009) reported
344 the primary target as the cell membrane. These differences are not surprising given the
345 contrasting and inconsistent inactivation effects against bacteria with different cell
346 membranes as described above. However, both the cell wall and vital intracellular
347 macromolecules are reported as main targets of reactive species (Dobrynin *et al.* 2009;
348 Machala *et al.* 2009; Roth *et al.* 2010). In our study, cell leakage measured by absorbance
349 260 nm and 280 nm, following ACP exposure showed different responses for Gram negative
350 and Gram positive bacteria. The cell leakage (reflecting release of intracellular material such
351 as proteins, DNA, RNA) results in our studies suggested more compromised cell membrane
352 integrity for Gram negative bacteria, for short duration ACP exposure. With regard to effects
353 on membrane integrity, Laroussi *et al.* (2003) reported that the cell wall of Gram negative
354 bacteria (outer membrane of lipopolysaccharide and thin layer of peptidoglycan) was more
355 vulnerable than the more stable peptidoglycan structure of the cell envelope for Gram
356 positive bacteria.
357 Comparing cell leakage and inactivation results with a 5 s ACP exposure, a high leakage rate
358 was detected for all strains (Fig.3) , however, there were only minor effects on the cell
359 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

385 oxidative stress. To further assess DNA fragmentation by high voltage plasma, amplification
386 of DNA by PCR was performed which revealed the extent of DNA damage was dependent
387 not only on type of bacteria but on ACP treatment time (Fig. 4 d, e, f). Extensive DNA
388 damage has been related to bacterial type and system parameters (Cooper *et al*. 2010; Joshi *et*
389 *al*. 2011). Using the low voltage of 15Kv, Joshi *et al.* (2011) demonstrated the fragmentation
390 of *E. coli* genomic DNA depended on the length of plasma exposure (treatment time) and
391 energy dose (J/cm^2) using floating electrode DBD plasma application. Cooper *et al.* (2010)
392 investigated DBD plasma treatment of *Bacillus stratosphericus* under a dry environment at
393 30 kV for 120 s and suggested direct interaction of charged particles or photons within the
394 plasma with the bacterial cell membrane thus directly exposing internal components to
395 extensive DNA damage. In our study, 5 s of ACP treatment showed significant effects on
396 membrane integrity with a strong increase in leakage, but no significant impact on DNA
397 damage was noted, thus suggesting that repair is possible when the microbiological target is
398 subject to very short treatment times even at high voltage. Enzymes, such as Ahp, SOD and
399 Kat, are reported to have clearance effects for ROS (Imlay, 2013), resulting in less
400 intracellular damage, such as DNA cleavage or enzyme inactivation. The repair systems in *E.*
401 *coli* and *L. monocytogenes* could mitigate the effect of ROS generated by ACP and diffused
402 inside the cell. Alternatively, the 5s treatment time might be too short for ROS to cause
403 detectable DNA damage. With longer treatment times, the ROS accumulation could exceed
404 the cell clearance capability, and resulting in damage visible on agarose gel. After 30 s of
405 treatment, population viability was significantly reduced in tandem with sustained leakage,
406 while significant DNA damage was only evident for *L. monocytogenes*.
407 Little DNA damage was noted for *E. coli* strains, even after 30 s treatment. The plasma
408 reactive species may interact with the multi-layered Gram negative cell membrane and the
409 polysaccharide chains and compromise membrane integrity, thus DNA damage may not be

410 the primary mechanism of action for short treatment times at high voltage. Further increasing
411 treatment time could cause more adverse effects on nucleic acids resulting in irreversible
412 DNA damage with loss of cell culturability. The PCR results reveal multi-site DNA strand
413 breakage. Cell viability could be maintained with the low level DNA damage observed with
414 the very short plasma treatment time of 5 s (Fig. 4); specifically, activities related to multi-
415 copy genes may be unaffected. Increasing the treatment time up to 30 s at high voltage, the
416 predominant effect of our system was related to the target cell; where membrane damage may
417 be the primary effect but for *L. monocytogenes* intracellular components were major targets.
418 Recent studies highlighted activation of repair systems of plasma treated bacteria in addition
419 to up or down regulation of specific genes under ACP stress (Roth *et al.* 2010, Sharma *et al.*
420 2009). To understand in detail the ACP effects on intracellular targets, investigations on
421 regulatory factors of ACP treated bacteria could elucidate the interaction between reactive
422 species and cell response.
423 Overall, there was a strong effect of the ACP process parameters of working gas mixture and
424 treatment time on inactivation of *E. coli* and *L. monocytogenes*. The working gas ratios were
425 associated with different bactericidal efficacies. We have found using a high voltage of 70
426 kV_{RMS} that the reactive species generated in a very short treatment time of 5 s had significant
427 effects on cell integrity. Extending treatment time to 30 s, caused significant bacterial
428 reduction with mode of action dependent on bacterial type.
429

430 **Acknowledgements**
431 The research leading to these results has received funding from the European Community's
432 Seventh Framework Program (FP7/2207-2013) under grant agreement number 285820.

References:

Boxhammer, V., Morfill, G.E., Jokipii, J.R., Shimizu, T., Klämpfl, T., Li, Y.F., Köritzer, J., J Schlegel J. and Zimmermann, J.L. (2012). Bactericidal action of cold atmospheric plasma in solution. *New J Phys* **14**(11): 1-18. doi:10.1088/1367-2630/14/11/113042.

Braoudaki, M. and Hilton, A.C. (2004) Low level of cross-resistance between triclosan and antibiotics in *Escherichia coli* K-12 and *E. coli* O55 compared to *E. coli* O157. *FEMS Microbiol Lett* **235**(2),305-309.

Cooper, M., Fridman, G., Fridman, A. and Joshi, S. G. (2010) Biological responses of *Bacillus stratosphericus* to floating electrode-dielectric barrier discharge plasma treatment. *J Appl Microbiol* **109**(6), 2039-2048. doi: 10.1111/j.1365-2672.2010.04834.x.

Deng, S., Ruan, R., Mok, C.K., Huang, G., Lin, X. and Chen, P. (2007) Inactivation of *Escherichia coli* on almonds using nonthermal plasma. *J Food Sci* **72**(2), M62-66.

Dobrynin, D., Friedman, G., Fridman, A. and Starikovskiy, A. (2011a) Inactivation of bacteria using DC corona discharge: role of ions and humidity. *New J Phys* **13**,103033. doi:10.1088/1367-2630/13/10/103033.

Dobrynin, D., Wasko, K., Friedman, G., Fridman, A. and Fridman, G. (2011b) Cold plasma sterilization of open wounds: live rat model. *J Plasma Med* **1**(2), 109-114.

Dobrynin, D., Fridman, G., Friedman, G. and Fridman, A. (2009) Physical and biological mechanisms of direct plasma interaction with living tissue. *New J Phys* **11**,115020. doi:10.1088/1367-2630/11/11/115020

Ermolaeva, S.A., Varfolomeev, A.F., Chernukha, M.Y., Yurov, D.S., Vasiliev, M.M., Kaminskaya, A.A., Moisenovich, M.M., Romanova, J.M., Murashev, A.N., Selezneva, I.I., Shimizu, T., Sysolyatina, E.V., Shaginyan, I.A., Petrov, O.F., Mayevsky, E.I., Fortov, V.E., Morfill, G.E., Naroditsky, B.S. and Gintsburg, A.L. (2011) Bactericidal effects of non-

457 thermal argon plasma in vitro, in biofilms and in the animal model of infected wounds. *J Med*
458 *Microbiol* **60**(1),75-83.
459 Eto, H., Ono, Y., Ogino, A. and Nagatsu, M. (2008) Low-temperature internal sterilization of
460 medical plastic tubes using a linear dielectric barrier discharge. *Plasma Process Polym*
461 **5**(3),269-274.
462 Fridman, G., Brooks, A.D., Balasubramanian, M., Fridman, A., Gutsol, A., Vasilets, V.N.,
463 Ayan, H. and Friedman, G. (2007) Comparison of direct and indirect effects of non-thermal
464 atmospheric-pressure plasma on bacteria. *Plasma Process Polym* **4**(4), 370-375.
465 Ghomi, H., Safa, N., Ramezani, K. and Karimi, M. (2009) Bacterial inactivation by DBD
466 plasma in atmospheric pressure. *19th International Symposium on Plasma Chemistry*
467 *(ISPC19)*, Bochum.
468 Hong, Y. F., Kang, J. G., Lee, H. Y., Uhm, H. S., Moon, E. and Park, Y. H. (2009)
469 Sterilization effect of atmospheric plasma on *Escherichia coli* and *Bacillus subtilis*
470 endospores. *Lett Appl Microbiol* **48**, 33-37.
471 Hosein, A.M. (2010) *Survival of Escherichia coli O157:H7 in acidified foods: a predictive*
472 *modeling approach*. Unpublished PhD thesis. North Carolina State University.
473 Hury, S., Vidal, D.R., Desor, F., Pelletier, J. and Lagarde, T. (1998) A parametric study of the
474 destruction efficiency of *Bacillus* spores in low pressure oxygen-based plasmas. *Lett Appl*
475 *Microbiol* **26**(6),417-421.
476 Imlay, J.A. (2013) The molecular mechanisms and physiological consequences of oxidative
477 stress: lessons from a model bacterium. *Nature Reviews Microbiology* 11, 443-454. doi
478 :10.1038/nrmicro3032
479 ISO 11290-2. (1998). Amendment 1. 2004. Modification of the enumeration medium.
480 Geneva: ISO.

Joshi, S.G., Cooper, M., Yost, A., Paff, M., Ercan, U.K., Fridman, G., Friedman, G., Fridman, A. and Brooks, A.D. (2011) Nonthermal dielectric-barrier discharge plasma-induced inactivation involves oxidative DNA damage and membrane lipid peroxidation in *Escherichia coli*. *Antimicrob Agents Chemother* **55**(3),1053-1062.

Kayes, M.M., Critzer, F.J., Kelly-Wintenberg, K., Roth, J.R., Montie, T.C. and Golden, D.A. (2007) Inactivation of foodborne pathogens using a one atmosphere uniform glow discharge plasma. *Foodborne Pathog Dis* **4**, 50-59.

Klämpfl, T.G., Isbary, G., Shimizu, T., Li, Y.F., Zimmermann, J.L., Stolz, W., Schlegel, J., Morfill, G.E. and Schmidt, H.U. (2012) Cold atmospheric air plasma sterilization against spores and other microorganisms of clinical interest. *Appl Environ Microbiol* **78**, 5077-5082

Kvam, E., Davis, B., Mondello, F. and Garner, A.L. (2012) Nonthermal atmospheric plasma rapidly disinfects multidrug-resistant microbes by inducing cell surface damage. *Antimicrob Agents Chemother* **56**(4): 2028-2036. doi: 10.1128/AAC.05642-11

Laroussi, M. and Leipold, F. (2004) Evaluation of the roles of reactive species, heat, and UV radiation in the inactivation of bacterial cells by air plasmas at atmospheric pressure. *Int J Mass Spectrom* **233** (1-3), 81-86.

Laroussi, M., Mendis, D.A. and Rosenberg, M. (2003) Plasma interaction with microbes. *New J Phys* **5**, 41.1-41.10.

Lee, K., Paek, K., Ju, W. and Lee, Y. (2006) Sterilization of bacteria, yeast, and bacterial endospores by atmospheric-pressure cold plasma using helium and oxygen. *J Microbiol* **44**,269-275.

Lerouge, S, Wertheimer, M.R., Marchand, R, Tabrizian, M. and Yahia, L. H. (2000) Effect of gas composition on spore mortality and etching during low-pressure plasma sterilization. *J Biomed Mater Res* **51**,128–135.

505 Liang, Y. D., Wu, Y., Sun, K., Chen, Q., Shen, F. X., Zhang, J., Yao, M. S., Zhu, T. and
506 Fang, J. (2012) Rapid inactivation of biological species in the air using atmospheric pressure
507 nonthermal plasma. *Environ Sci Technol* **46** (6), 3360-3368. doi: 10.1021/es203770q
508 Liu, X.H., Hong, F., Guo, Y., Zhang, J. and Shi, J.J. (2013) Sterilization of *Staphylococcus*
509 *aureus* by an atmospheric non-thermal plasma jet. *Plasma Sci Technol* **15**(5), 439.
510 doi:10.1088/1009-0630/15/5/09
511 Ma, Y., Zhang, G., Shi, X., Xu, G. and Yang, Y. (2008) Chemical mechanisms of bacterial
512 inactivation using dielectric barrier discharge plasma in atmospheric air. *IEEE Trans Plasma*
513 *Sci* **36**(4), 1615-1620.
514 Machala, Z., Jedlovský, I., Chládeková, L., Pongrác, B., Giertl, D., Janda, M., Šikurová, L.
515 and Polčic, P. (2009) DC discharges in atmospheric air for bio-decontamination-
516 spectroscopic methods for mechanism identification. *Eur Phys J D* **54**(2),195-204
517 Maurer, L.M., Yohannes, E., Bondurant, S.S., Radmacher, M. and Slonczewski, J.L. (2005)
518 pH regulates genes for flagellar motility, catabolism and oxidative stress in *Escherichia coli*
519 K-12. *J Bacteriol* **187**(1), 304-319.
520 Misra, N.N., Tiwari, B.K., Raghavarao, K.S.M.S. and Cullen, P.J. (2011) Nonthermal plasma
521 inactivation of food-borne pathogens. *Food Eng Rev* **3**(3-4), 159-170.
522 Müller, S. and Zahn, R.J. (2007) Air pollution control by non-thermal plasma. *Contrib*
523 *Plasma Phys* **47**(7), 520-529.
524 Parvulescu V. I., Magureanu M. and Lukeš P. (2012) *Plasma Chemistry and Catalysis in*
525 *Gases and Liquids*. Wiley-VCH: Weinheim, Germany, ISBN-13: 978-3-527-33006-5.
526 Purevdorj, D., Igura, N., Ariyada, O., Hayakawa, I. (2003). Effect of feed gas composition of
527 gas discharge plasmas on *Bacillus pumilus* spore mortality. *Lett Appl Microbiol* **37**(1), 31-34.

528 Roth, S., Feichtinger, J. and Hertel, C. (2010) Characterization of *Bacillus subtilis* spore
529 inactivation in low pressure, low temperature gas plasma sterilization processes. *J Appl*
530 *Microbiol* **108**(2),521-531.
531 Sensenig, R., Kalghatgi, S., Fridman, G., Shereshevsky, A., Brooks, A., Vasilets, V., Gutsol,
532 A., Fridman, A. and Friedman, G. (2008) Induction of apoptosis in melanoma cells by non-
533 thermal atmospheric plasma discharge. *SSO's 61st Annual Cancer Symposium*. Chicago,
534 USA. March 13-16.
535 Sharma, A., Collins, G. and Pruden, A. (2009) Differential gene expression in *Escherichia*
536 *coli* following exposure to nonthermal atmospheric pressure plasma. *J Appl Microbiol* **107**
537 (5),1440-1449
538 Shigenaga, M. K., Lee, H. H., Blount, B. C.,Christen, S., Shigeno, E. T., Yip, H. and Ames,
539 B. N. (1997) Inflammation and NO_x-induced nitration: Assay for 3-nitrotyrosine by HPLC
540 with electrochemical detection. *Proc Natl Acad Sci U S A* **94**(7), 3211-3216.
541 Takamatsu, T., Ichikawa, M., Hirai, H., Sasaki, R., Shibata, M., Miyahara, H., Matsumoto, Y.
542 and Okino, A. (2011) 'Sterilization effect of various gas non-thermal plasma'. *IEEE Int Conf*
543 *on Plasma Science (ICOPS)*, Chicago, IL.
544 Venezia, R.A., Orrico, M., Houston, E., Yin, S.M. and Naumova, Y.Y. (2008) Lethal activity
545 of nonthermal plasma sterilization against microorganisms. *Infect Control Hosp Epidemiol*
546 **29**(5), 430-436.
547 Virto, R., Mañas, P., Álvarez, I., Condon, S. and Raso, J. (2005) Membrane damage and
548 microbial inactivation by chlorine in the absence and presence of a chlorine-demanding
549 substrate. *Appl Environ Microbiol* **71**(9), 5022-5028. doi: 10.1128/AEM.71.9.5022-5028.
550 Zhang, M., Oh, J.K., Cisneros-Zevallos, L. and Akbulut, M. (2013) Bactericidal effects of
551 nonthermal low-pressure oxygen plasma on *S. typhimurium* LT2 attached to fresh produce
552 surfaces. *Journal of Food Engineering* **119**(3),425–432.

553 Ziuzina, D., Patil, S., Cullen, P.J., Keener, K.M. and Bourke, P. (2013) Atmospheric cold
554 plasma inactivation of *Escherichia coli* in liquid media inside a sealed package. *J Appl*
555 *Microbiol* **114**(3),778-787. doi: 10.1111/jam.12087.
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579

580 **Table 1: Designed primers for PCR**

Organism		5'-3' Sequence	PCR product length
E. coli ATCC 25922 *E. coli* NCTC 12900	Forward	CAG GCC TAA CAC ATG CAA GT	1410 bp
	Reverse	CGA AGG TTA AGC TAC CTA CTT	
L. monocytogenes NCTC 11994	Forward	TAAAGAGAGT TTGATCCTGG C	1418 bp
	Reverse	CCT ACC GAC TTC GGG TGT T	

581

582

583 **Table 2: Effect of voltage levels on ACP inactivation efficacy for *E. coli* ATCC 25922**
584

Voltage (kV_{RMS})	Gas mixtures	Mode of Plasma Exposure					
		Direct			Indirect		
		Initial cell density (Log CFU ml^{-1})	Reduction (Log CFU ml^{-1})	SD*	Initial cell density (Log CFU ml^{-1})	Reduction (Log CFU ml^{-1})	SD*
56	1*	8.0	1.8^{a}	0.6	8.0	1.6^{a}	0.5
	2*	7.7	1.0^{a}	0.2	7.7	1.2^{a}	0.4
70	1*	7.9	3.4^{b}	0.4	7.9	ND*^{c}	0.1
	2*	7.7	2.8^{d}	0.2	7.7	3.2^{d}	0.7

585

586 Different letters indicate a significant difference at the 0.05 level between voltage level and between

587 gas types

588 *SD: standard deviation

589 ND*: Under detection limit

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

591 *Gas mix 1 : Air

592 Gas mix 2 : 90% N_2+10% O_2

593

594

595 **Table 3: Effect of gas mixtures on ACP inactivation efficacy**
596

Organism	Gas mixtures	Mode of Plasma Exposure					
		Direct			Indirect		
		Initial cell density (Log CFU ml^{-1})	Reduction (Log CFU ml^{-1})	SD*	Initial cell density (Log CFU ml^{-1})	Reduction (Log CFU ml^{-1})	SD*
E. coli ATCC 25922	1	7.9	3.4^{a}	0.4	7.9	ND*^{d}	0.1
	2	7.7	2.8^{b}	0.2	7.7	$3.2^{a, b}$	0.7
	3	8.0	6.6^{c}	0.1	8.0	ND*^{d}	0.1
E. coli NCTC 12900	1	7.9	1.8^{ab}	0.2	7.9	1.6^{a}	0.1
	2	8.0	1.4^{a}	0.4	8.0	1.8^{b}	0.1
	3	8.0	2.1^{b}	0.5	8.0	ND*^{c}	0.1
L. monocytogenes NCTC 11994	1	8.3	ND*^{a}	0.7	8.3	ND*^{a}	0.1
	2	8.2	4.1^{b}	0.1	8.2	ND*^{a}	0.1
	3	8.2	ND*^{a}	0.0	8.2	ND*^{a}	0.0

597

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

600 *SD: Standard Deviation

601 ND *: Under detection limit

602 Experimental conditions: 70 kV_{RMS} 30 s treatment, 24 h post-treatment storage

603 *Gas mix 1: Air

604 Gas mix 2: 90% N_2+10% O_2,

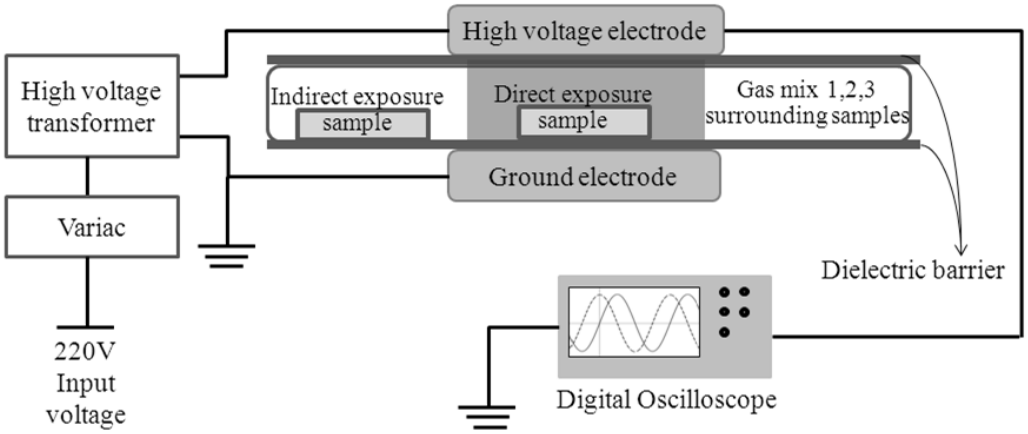
605 Gas mix 3: 65% O_2 + 30% CO_2+5% N_2

606

607

608

609



610

611 **Fig.1: A schematic diagram of the experimental plasma device.**

612

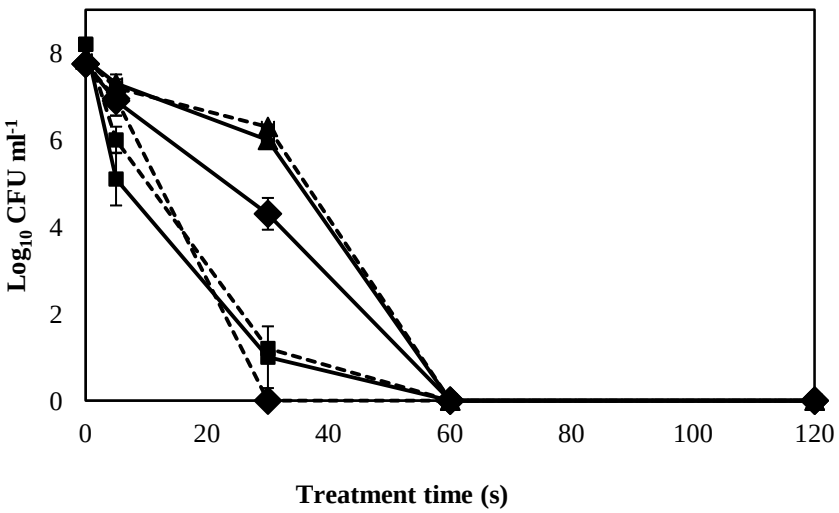


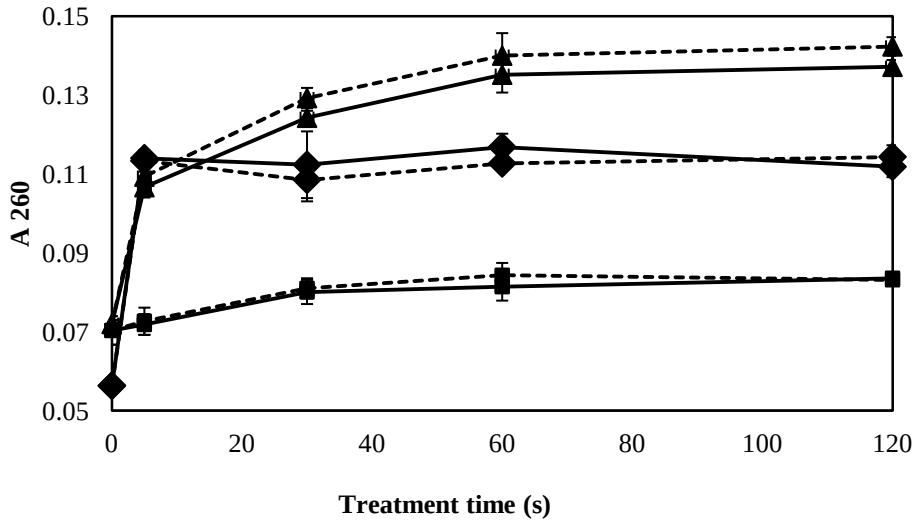
Fig. 2: ACP inactivation of bacterial strains in PBS

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

621



622

623 **Fig. 3: Absorbance 260 after ACP treatment in PBS**

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

625 ◆*E. coli* ATCC 25922; ▲*E. coli* NCTC 12900; ■*L. monocytogenes* NCTC 11994

626 Solid line: direct exposure; Dotted line: indirect exposure

627

628

629

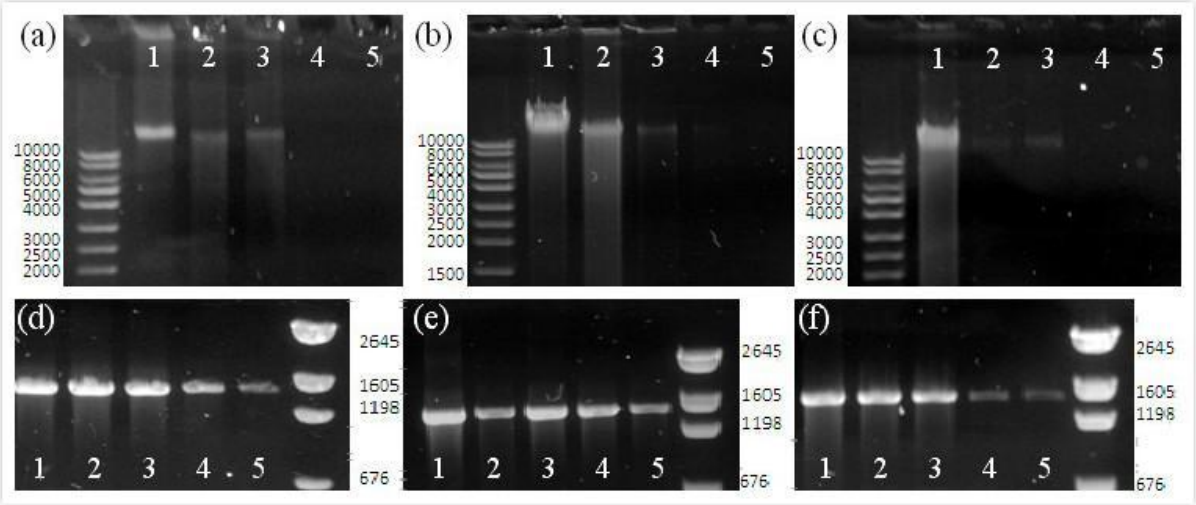


Fig. 4: Agarose gel electrophoresis showing genomic DNA and PCR amplified products of untreated and ACP treated samples

Voltage: 70 kV_{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