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

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24 *Running Title: Bacterial inactivation by Atmospheric Cold Plasma*

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29 **Abstract**

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

35 Methods and Results:  $10^8$  CFU ml<sup>-1</sup> *E. coli* ATCC 25922, *E. coli* NCTC 12900 and *L.*  
36 *monocytogenes* NCTC11994 were ACP treated in 10ml phosphate buffered saline (PBS).  
37 Working gas mixtures used were; Air (gas mix 1), 90% N<sub>2</sub>+10% O<sub>2</sub> (gas mix 2) and 65%  
38 O<sub>2</sub>+30% CO<sub>2</sub>+5% N<sub>2</sub> (gas mix 3). Greater reduction of viability was observed for all strains  
39 using higher voltage of 70 kV<sub>RMS</sub>, and with working gas mixtures with higher oxygen content  
40 in combination with direct exposure. Indirect ACP exposure for 30 s inactivated below  
41 detection level both *E. coli* strains. *L. monocytogenes* inactivation within 30 s was  
42 irrespective of the mode of exposure. Leakage was assessed using A<sub>260</sub> absorbance and DNA  
43 damage was monitored using PCR and Gel electrophoresis. Membrane integrity was  
44 compromised after 5 s, with noticeable DNA damage also dependent on the target cell after  
45 30 s.

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

50 Significance and Impact of study: Using atmospheric air as working gas resulted in useful  
51 inactivation by comparison with high nitrogen or high oxygen mixes. The mechanism of  
52 inactivation was a function of treatment duration and cell membrane characteristics, thus  
53 offering potential for optimised process parameters specific to the microbial challenge.

54 Key words: DBD-ACP, *Escherichia coli*, *Listeria monocytogenes*, voltage level, cell  
55 integrity, DNA damage

## 56 **Introduction**

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

69 ACP has been proved effective for microbial inactivation (Deng *et al.* 2007; Joshi *et al.* 2011;  
70 Ziuzina *et al.* 2013). Plasma discharge results in the generation of a wide range of reactive  
71 species responsible for the antimicrobial effects. Depending on the cell envelope differences,  
72 different inactivation responses were observed in plasma sterilization studies, where Gram-  
73 positive bacteria were found to be more resistant than Gram-negative bacteria (Lee *et al.*  
74 2006; Ermlaeva *et al.* 2011). In contrast other studies indicated no significant differences in  
75 the effect of plasma treatment between Gram positive and Gram negative bacteria (Kayes *et*  
76 *al.* 2007; Venezia *et al.* 2008).

77 The inactivation efficacy of ACP is governed by system and process variables including  
78 power input, mode of exposure, duration of exposure and gas composition, as well as

79 features of the target such as microbial cell type ( Deng *et al.* 2007; Fridman *et al.* 2007;  
80 Ghomi *et al.* 2009; Takamatsu *et al.* 2011; Liu *et al.* 2013). Higher system voltage and  
81 extended treatment time have been associated with greater inactivation efficacy (Deng *et al.*  
82 2007; Ghomi *et al.* 2009; Joshi *et al.* 2011; Liu *et al.* 2013). With regard to mode of  
83 exposure, the magnitude of the field generated with direct voltage gap between electrodes can  
84 cause sheer stress to cells, and energetic ions can directly affect cells subjected to direct  
85 exposure (Dobrynin *et al.* 2009; Dobrynin *et al.* 2011a). The diffusion of recombined or  
86 longer lived species through the medium and the target may lead to different inactivation  
87 patterns in association with mode of exposure (direct/ indirect) and post treatment storage  
88 time (Ziuzina *et al.* 2013). Therefore, in this study we assessed the effect of the system  
89 parameters of high voltage levels and mode of exposure in conjunction with the gas  
90 composition.

91 Working gas type may influence the range and type of reactive species formed with an  
92 expected significant effect on microorganisms (Lerouge *et al.* 2000; Purevdorj *et al.* 2003;  
93 Zhang *et al.* 2013). Using air as a working gas, the reactive species generated could include  
94 reactive oxygen species (ROS), reactive nitrogen species (RNS), ultraviolet (UV) radiation,  
95 energetic ions and charged particles. ROS can play the most crucial role in the inactivation  
96 of microbes (Joshi *et al.* 2011). Hydrogen peroxide, singlet and atomic oxygen have a strong  
97 oxidative effect on microbes as well as ozone (Dobrynin *et al.* 2009), which can all be  
98 generated using plasma discharge in air and oxygen-containing mixtures. Dobrynin *et al.*  
99 (2009) concluded that oxygen was required for fast and effective inactivation of bacteria,  
100 however, their study compared a range of single gas types with air. In contrast, Boxhammer  
101 *et al.* (2012) analysed the relative contribution of ROS and RNS produced by ACP in air and  
102 concluded that the bactericidal effect was related to a combination of oxidative and  
103 nitrosative effects. Discharging plasma in an air or nitrogen containing gas mixture can also

104 generate reactive nitrogen species (NO<sub>x</sub>). However, it was the combined application of NO  
105 and H<sub>2</sub>O<sub>2</sub> that yielded a higher inactivation effect on *E. coli* than a treatment with NO or  
106 H<sub>2</sub>O<sub>2</sub> alone (Boxhammer *et al.* 2012). Another NO<sub>x</sub> species, peroxyxynitrite, which can be  
107 rapidly endogenously formed due to the reaction of nitric oxide and superoxide, can damage  
108 proteins, lipids and DNA as reported in Shigenaga *et al.* (1997). Thus, the type and range of  
109 reactive species generated can influence the mechanism of inactivation and this range of  
110 reactive species may vary with the working gas composition employed.

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

## 123 **MATERIALS AND METHODS**

### 124 ***Bacteria types and Growth Conditions***

125 Three bacteria types were used in this study. *E. coli* ATCC 25922 and *L. monocytogenes*  
126 NCTC 11994, were obtained from microbiology stock culture of the School of Food Science  
127 and Environmental Health, Dublin Institute of Technology. *E. coli* NCTC 12900, (non-  
128 toxigenic O157:H7) was obtained from National Collection of type cultures of the Health

129 Protection Agency (HPA, UK). Strains were selected to present both Gram positive and  
130 Gram negative foodborne challenges and to facilitate comparison with other studies. Strains  
131 were maintained as frozen stocks at -70 °C in the form of protective beads, which were plated  
132 onto tryptic soy agar (TSA, Scharlau Chemie) and incubated overnight at 37 °C to obtain  
133 single colonies before storage at 4 °C.

#### 134 ***Preparation of Bacterial Cell Suspensions***

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

#### 142 ***ACP system configuration***

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

#### 152 ***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<sub>2</sub>+ 10% O<sub>2</sub> (gas mix 2) and  
160 65%O<sub>2</sub> + 30% CO<sub>2</sub> +5% N<sub>2</sub> (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<sup>-1</sup> for 1 min. Bacterial  
162 samples were then treated with ACP at either 56 kV<sub>RMS</sub> or 70 kV<sub>RMS</sub> 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<sup>-1</sup>. Plates were incubated at 37 °C 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<sup>-1</sup> units.

### 176 ***Cell membrane integrity***



177 Membrane integrity was examined by determination of the release of material absorbing at  
178 260 nm and 280 nm (Virto *et al.* 2005). The UV absorbance at 260 nm and 280 nm ( $A_{260}$  and  
179  $A_{280}$ ) were used to indicate the effect on cell membrane integrity. Untreated (bacterial cells in  
180 PBS) and ACP-treated samples were centrifuged at 13,200 g for 10 min. Untreated controls  
181 determined the release of any intracellular material before ACP treatment. 200  $\mu$ L  
182 supernatant of each sample was transferred into microtitre plate wells and measured by  
183 Synergy<sup>TM</sup> HT Multi-Mode Microplate Reader (BioTek Instruments Inc.) at 260 nm and 280  
184 nm.

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

186 Plasma treated aliquots were precipitated by ethanol with a final concentration of 70% for 5  
187 min and centrifuged at 13,200 g for 10 min to obtain the pellet. Genomic DNA was then  
188 extracted from the pellet by Wizard Genomic DNA Purification Kit (Promega) as per  
189 manufacturer's instructions. The amount of DNA was quantified by measuring the  
190 absorbance at 260 nm. The genomic DNA amplification of conserved bacterial regions i.e.  
191 16S rRNA was performed using the primers listed in Table 1. All reactions were performed  
192 with GoTaq Colorless Mastermix (Promega). 25  $\mu$ L PCR reaction system was used that  
193 contained, 12.5  $\mu$ L Mastermix, 2  $\mu$ L of each primer (0.2 nmol), 2  $\mu$ L of genomic DNA as  
194 template (0.2 ng) and sterilized water to make final volume up to 25  $\mu$ L. Amplification  
195 programme was conducted as: initial denaturation step at 95 °C for 5 min, followed by 25  
196 cycles at 95 °C 45 s for denaturation, at 51 °C 30 s annealing, at 72 °C 90 s extension, and 72  
197 °C 10 min for final extension.

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

202 ***Statistical Analysis***

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

207 **RESULTS**

208 ***The effect of voltage level on DBD-ACP inactivation efficiency***

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

219 ***The effect of gas mixtures on DBD-ACP inactivation efficiency***

220 In order to assess the effect of gas mixtures on ACP inactivation efficacy, the higher voltage  
221 level was used against bacterial strains studied. With direct exposure of ACP generated in  
222 either in gas mix 1, 2 or 3, the population of *E. coli* ATCC 25922 was reduced by 3.4, 2.8 and  
223 6.6 log cycles, respectively ( $p \leq 0.05$ ). However, indirect exposure in gas mix 1 and 3 resulted  
224 in greater inactivation rates whereas only 3.2 log cycles reduction were recorded when gas  
225 mix 2 was utilised (Table 3 ). While in the case of non-toxicogenic strain *E. coli* NCTC 12900,  
226 inactivation below detection was achieved only after indirect exposure in gas mix 3. The

227 other gas mixtures were less effective. *L. monocytogenes* population was below detection  
228 level after indirect exposure of ACP generated in all three gas mixtures tested. With direct  
229 ACP exposure only, gas mixes 1 and 3 were more effective than the gas mix 2 for  
230 inactivation of *L. monocytogenes* (Table 3).

### 231 *Ozone measurements*

232 Ozone concentrations were measured immediately after plasma exposure with GASTEC gas  
233 tubes. Ozone concentrations of 1280 and 1000 ppm was noted immediately after direct ACP  
234 treatment in gas mix 1 or 2, respectively. Indirect ACP treatment recorded slightly increased  
235 concentrations of 1440 ppm and 1367 ppm, respectively. Higher concentrations of 2000 and  
236 4000 ppm were noted after direct or indirect ACP treatment in gas mix 3, respectively. No  
237 ozone concentrations were recorded for stored samples after 24 h of post-treatment storage.

### 238 *Effect on cell membrane integrity*

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

250 Figure 3 presents the release of intracellular components (nucleic acid) absorbing at 260 nm  
251 following treatment. The results obtained at 280 nm (protein) were similar (data not shown).

252 For both *E. coli* strains, the absorption curves showed similar trends. A sharp increase in  
253 absorbance followed by a steady stage was recorded, indicating the cell integrity was  
254 compromised within 5 s of ACP treatment. While a similar trend was observed for the  
255 absorbance of *L. monocytogenes* NCTC 11994, the leakage recorded even after 120 s  
256 treatment was significantly less than that for *E. coli* strains within 5 s treatment.

### 257 ***DNA Damage***

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

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

### 274 **DISCUSSION**

275 In order to ensure system efficacy, ACP was tested against different types of bacteria which  
276 can demonstrate different responses against plasma stress as noted previously (Hury *et al.*

277 1998; Laroussi *et al.* 2003, Kvam *et al.* 2012). Applied voltage level had an impact on ACP  
278 antimicrobial efficacy, where at the higher voltage level greater microbial inactivation was  
279 achieved. The energy of ACP discharge is decided by applied voltage and frequency, thus  
280 generating different amounts of reactive species which influence inactivation (Deng *et al.*  
281 2007; Liu *et al.* 2013). Liu *et al.* (2013) studied the relationship between reactive species  
282 generation using helium as working gas and atmospheric non thermal plasma jet voltage level  
283 over time and found that four kinds of active species,  $N_2^+$ , OH, He and O, increased gradually  
284 with increasing applied voltage, which they attributed as responsible for the increasing  
285 inactivation efficacy. In the preliminary stages of our study, higher operating voltage resulted  
286 in higher inactivation efficacy, which could also be attributed to the concentration of  
287 generated reactive species influencing the inactivation rate.

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

299 Besides voltage level, the working gas utilised for ACP discharge had a major effect on  
300 inactivation. Overall gas mix 3 was associated with greater inactivation ( $p < 0.05$ ). No  
301 significant differences were observed between gas mix 1 and 2 effects overall ( $p > 0.05$ ).  
302 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<sup>·</sup>) and ozone (O<sub>3</sub>). 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<sup>·</sup> radicals, H<sub>2</sub>O<sub>2</sub> and hydronium ions H<sub>3</sub>O<sup>+</sup>  
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

360 5 s in association with a large spike in the material leaking from the cell. However, for  
361 *Listeria*, up to 3 log reductions were noted after 5 s but with a much smaller spike cell  
362 leakage recorded. The possible explanation could be that the short ACP exposure of 5 s  
363 results in reversible damage with the likelihood of activation of a cell response system for  
364 repairing the damage (Dobrynin *et al.* 2009). Nevertheless, when treatment time was  
365 increased, bacterial populations were reduced to undetectable levels for all strains. The  
366 diffusion of generated reactive species into the cell results in either irreparable damage to cell  
367 membrane and/ or major cell constituents.

368 The inactivation efficacy was also related to anti-oxidative activities of the target bacteria.  
369 Compared to *E. coli* ATCC 25922, the non-toxicogenic *E. coli* NCTC 12900 has been reported  
370 to have a stronger resistance to acid stress, multi-drug resistance and higher rate of mutations,  
371 which has cross protective effect against a wide range of environmental stresses including  
372 oxidative stress (Braoudaki and Hilton 2004; Maurer *et al.* 2005; Hosein 2010,). These  
373 characteristics could impact the resistance of *E. coli* NCTC 12900 resulting in different  
374 responses yielding greater resistance to ACP stress by comparison with *E. coli* ATCC 25922.

375 Comparing the results of inactivation, *L. monocytogenes* was more sensitive to ACP  
376 treatment than the two *E. coli* strains studied. In the case of *L. monocytogenes*, the diffusion  
377 of ROS and RNS across the membrane would cause a severe irreversible damage of  
378 macromolecules including DNA, making the bacterial cells susceptible to ACP treatment.

379 Therefore, we performed further investigations to assess ACP effects on genomic DNA  
380 damage and amplified DNA products.

381 In our study using high voltage plasma, the extent of genomic DNA damage was dependent  
382 on type of bacteria and treatment time. Thus indicating that the concentration of ACP  
383 generated reactive species increased with time, resulting in time dependent genomic DNA  
384 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 al.*  
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<sub>RMS</sub> 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

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

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

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

Voltage (kV <sub>RMS</sub> )	Gas mixtures	Mode of Plasma Exposure					
		Direct			Indirect		
		Initial cell density (Log CFU ml <sup>-1</sup> )	Reduction (Log CFU ml <sup>-1</sup> )	SD*	Initial cell density (Log CFU ml <sup>-1</sup> )	Reduction (Log CFU ml <sup>-1</sup> )	SD*
56	1*	8.0	1.8 <sup>a</sup>	0.6	8.0	1.6 <sup>a</sup>	0.5
	2*	7.7	1.0 <sup>a</sup>	0.2	7.7	1.2 <sup>a</sup>	0.4
70	1*	7.9	3.4 <sup>b</sup>	0.4	7.9	ND* <sup>c</sup>	0.1
	2*	7.7	2.8 <sup>d</sup>	0.2	7.7	3.2 <sup>d</sup>	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<sub>2</sub>+10% O<sub>2</sub>

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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 <sup>-1</sup> )	Reduction (Log CFU ml <sup>-1</sup> )	SD*	Initial cell density (Log CFU ml <sup>-1</sup> )	Reduction (Log CFU ml <sup>-1</sup> )	SD*
<i>E. coli</i> ATCC 25922	1	7.9	3.4 <sup>a</sup>	0.4	7.9	ND* <sup>d</sup>	0.1
	2	7.7	2.8 <sup>b</sup>	0.2	7.7	3.2 <sup>a, b</sup>	0.7
	3	8.0	6.6 <sup>c</sup>	0.1	8.0	ND* <sup>d</sup>	0.1
<i>E. coli</i> NCTC 12900	1	7.9	1.8 <sup>ab</sup>	0.2	7.9	1.6 <sup>a</sup>	0.1
	2	8.0	1.4 <sup>a</sup>	0.4	8.0	1.8 <sup>b</sup>	0.1
	3	8.0	2.1 <sup>b</sup>	0.5	8.0	ND* <sup>c</sup>	0.1
<i>L. monocytogenes</i> NCTC 11994	1	8.3	ND* <sup>a</sup>	0.7	8.3	ND* <sup>a</sup>	0.1
	2	8.2	4.1 <sup>b</sup>	0.1	8.2	ND* <sup>a</sup>	0.1
	3	8.2	ND* <sup>a</sup>	0.0	8.2	ND* <sup>a</sup>	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<sub>RMS</sub> 30 s treatment, 24 h post-treatment storage

603 \*Gas mix 1: Air

604 Gas mix 2: 90% N<sub>2</sub>+10% O<sub>2</sub>,

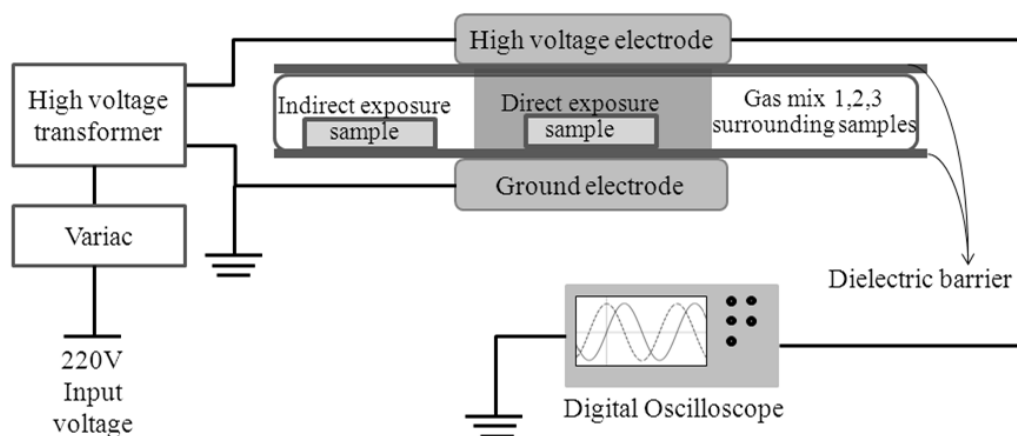
605 Gas mix 3: 65% O<sub>2</sub> + 30% CO<sub>2</sub>+5% N<sub>2</sub>

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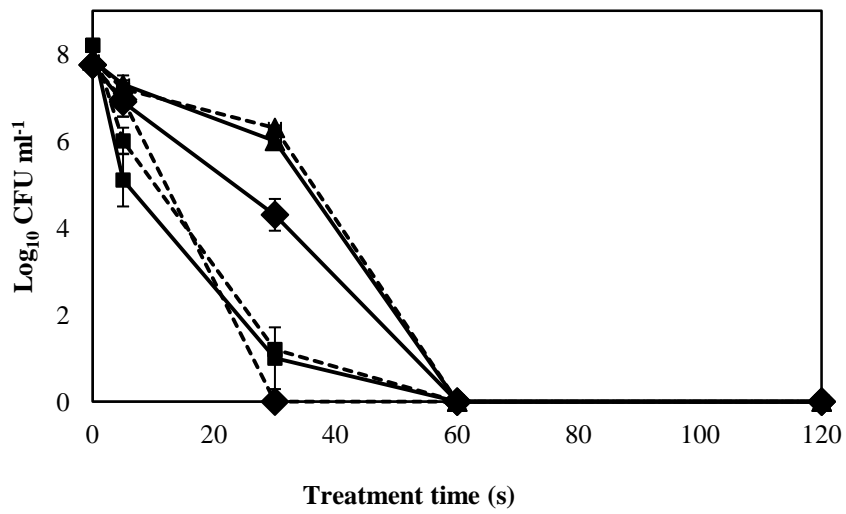


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611 **Fig.1: A schematic diagram of the experimental plasma device.**

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615 **Fig. 2: ACP inactivation of bacterial strains in PBS**

616 Experimental conditions: Voltage: 70 kV<sub>RMS</sub>; Treatment time: 0~120 s; Post treatment storage time:

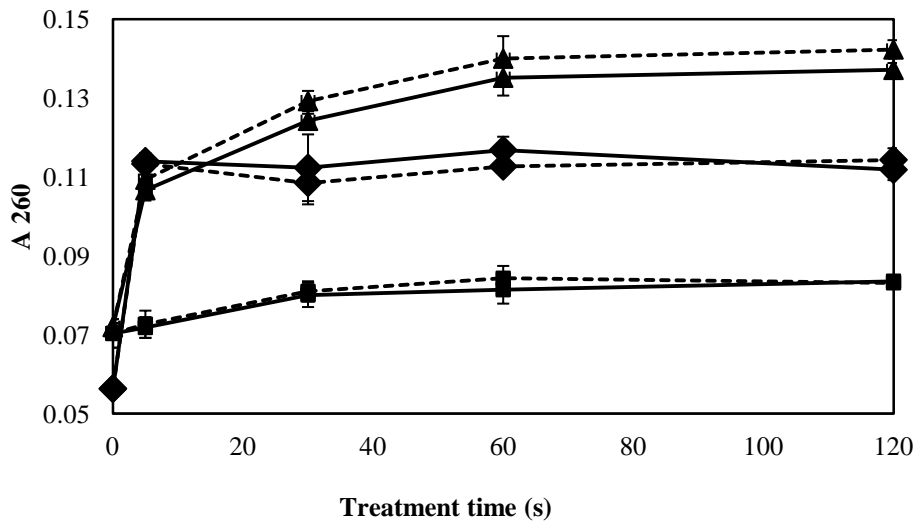
617 24 h; Gas mix: Air

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

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

620

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622

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

624 Voltage: 70 kV<sub>RMS</sub>; 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

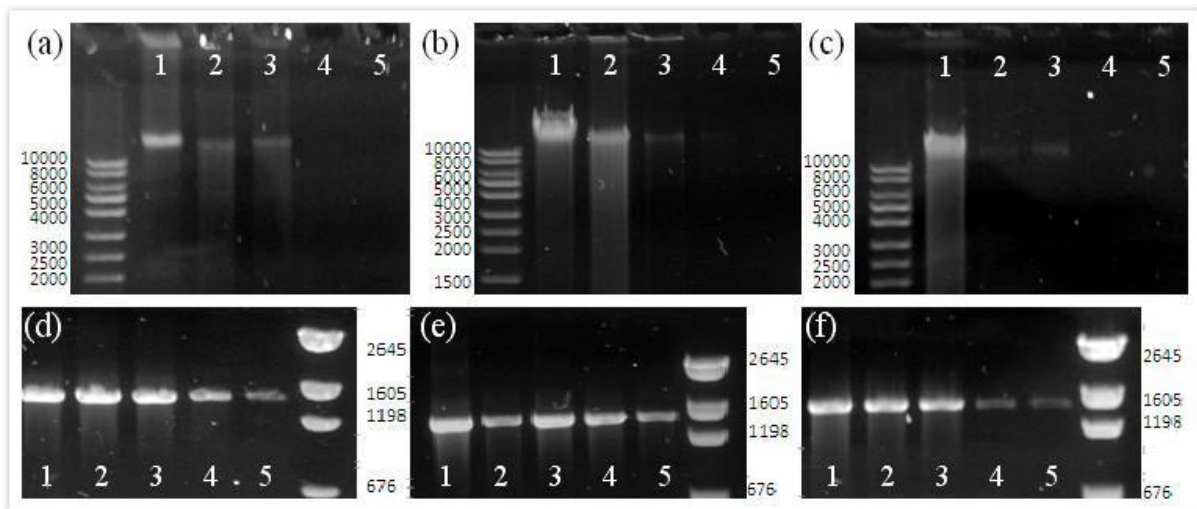
626 Solid line: direct exposure; Dotted line: indirect exposure

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

635 Voltage: 70 kV<sub>RMS</sub>; Treatment time: 0~30 s; Post treatment storage time: 24 h; Gas mix: Air

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

638 16S rRNA PCR results of (d) *E. coli* ATCC 25922; (e) *E. coli* NCTC 12900; (f) *L. monocytogenes*  
639 NCTC 11994

640 Lane 1: Non plasma treatment control; 2: 5 s directly treated samples; 3: 5 s indirectly treated samples;  
641 4: 30 s directly treated samples; 5: 30 s indirectly treated samples

642

643