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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

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Authors

Lu Han, Sonal Patil, Kevin M. Keener, Patrick Cullen, and Paula Bourke

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

3 Lu H.¹, Patil S.¹, Keener, K.M.², Cullen, P.J.¹, & Bourke, P.^{1*}.

4

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

9

10

11

12

13 *Corresponding Author

14 Paula Bourke

15 School Food Science and Environmental Health

16 Dublin Institute of Technology

17 Dublin 1, Ireland

18 paula.bourke@dit.ie

19 00 353 1 4027594

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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⁻¹ *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% N₂+10% O₂ (gas mix 2) and 65%
38 O₂+30% CO₂+5% N₂ (gas mix 3). Greater reduction of viability was observed for all strains
39 using higher voltage of 70 kV_{RMS}, 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 A₂₆₀ 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_x). However, it was the combined application of NO
105 and H₂O₂ that yielded a higher inactivation effect on *E. coli* than a treatment with NO or
106 H₂O₂ alone (Boxhammer *et al.* 2012). Another NO_x 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⁻¹ 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_{RMS} or 70 kV_{RMS} 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% N₂+ 10% O₂ (gas mix 2) and
160 65%O₂ + 30% CO₂ +5% N₂ (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 °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⁻¹ 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 μ L
182 supernatant of each sample was transferred into microtitre plate wells and measured by
183 SynergyTM 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 μ L PCR reaction system was used that
193 contained, 12.5 μ L Mastermix, 2 μ L of each primer (0.2 nmol), 2 μ L of genomic DNA as
194 template (0.2 ng) and sterilized water to make final volume up to 25 μ 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_{RMS}, 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_{RMS} achieved significantly greater inactivation effects
215 compared to lower voltage level tested ($p \leq 0.05$). The indirect plasma exposure at 70 kV_{RMS},
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-toxigenic 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[·]) and ozone (O₃). 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[·] radicals, H₂O₂ and hydronium ions H₃O⁺
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_{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

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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	

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% N₂+10% O₂

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*
<i>E. coli</i> 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
<i>E. coli</i> 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
<i>L. monocytogenes</i> 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% N₂+10% O₂,

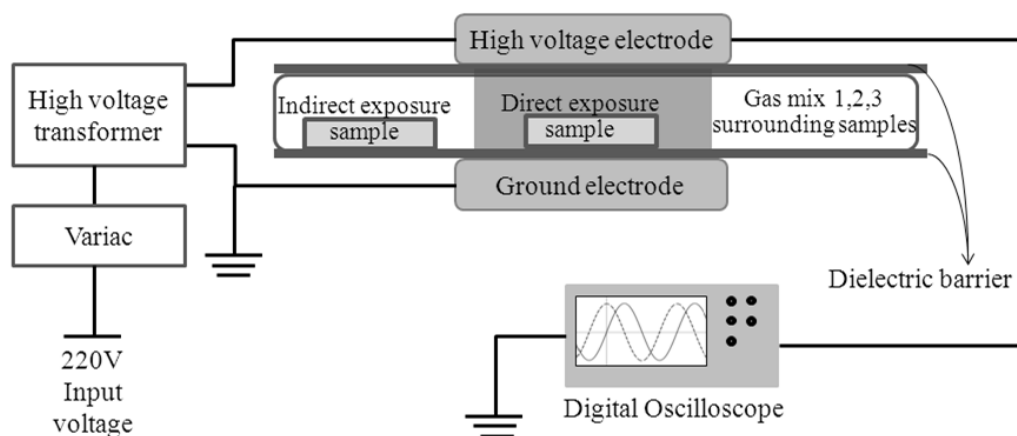
605 Gas mix 3: 65% O₂ + 30% CO₂+5% N₂

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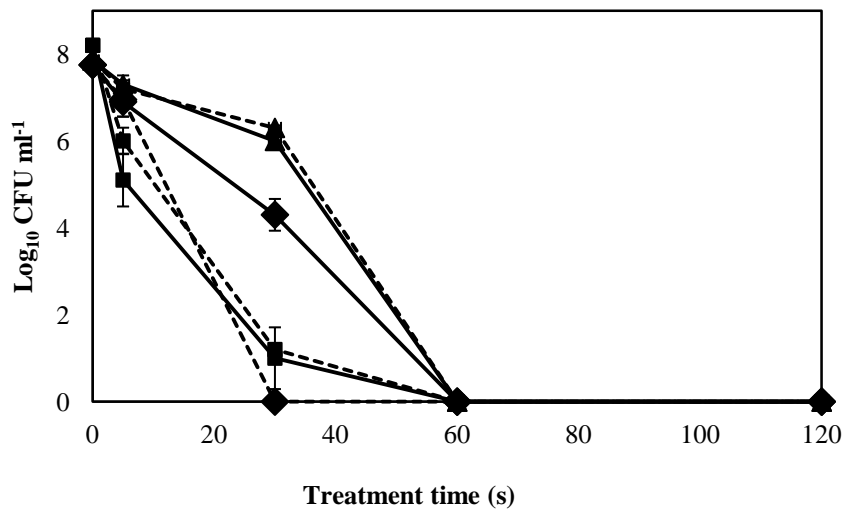


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611 **Fig.1: A schematic diagram of the experimental plasma device.**

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

616 Experimental conditions: Voltage: 70 kV_{RMS}; Treatment time: 0~120 s; Post treatment storage time:

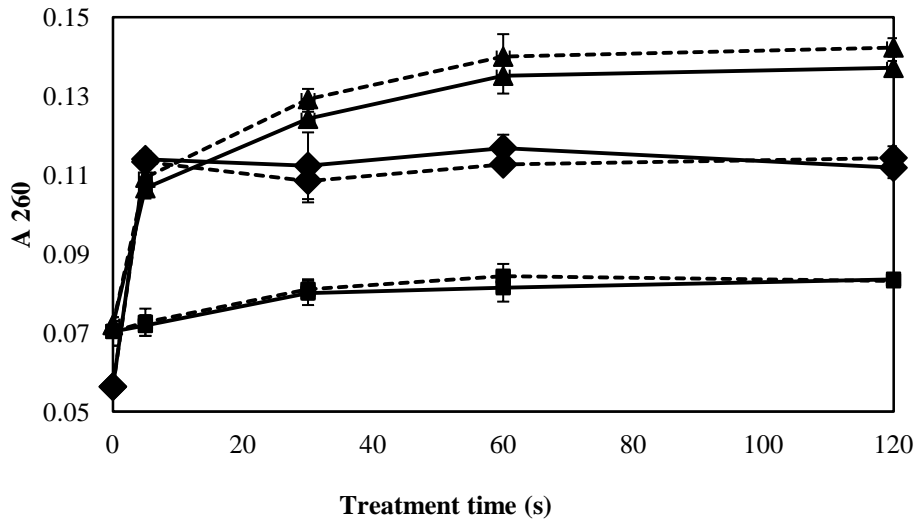
617 24 h; Gas mix: Air

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

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

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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

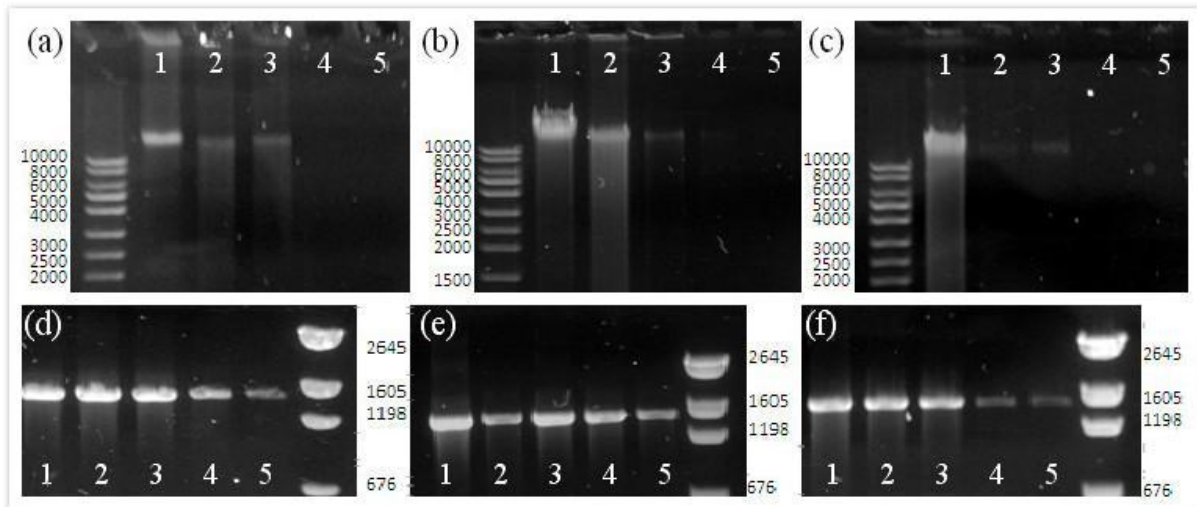
626 Solid line: direct exposure; Dotted line: indirect exposure

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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_{RMS}; 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