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Atmospheric Cold Plasma Inactivation of Escherichia Coli, Salmonella Enterica Serovar Typhimurium and Listeria Monocytogenes Inoculated on Fresh Produce

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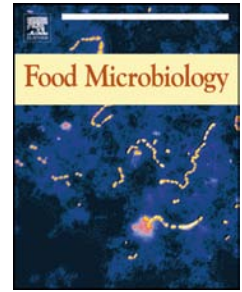
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1 **Atmospheric Cold Plasma inactivation of *Escherichia coli*, *Salmonella enterica* serovar**
2 **Typhimurium and *Listeria monocytogenes* inoculated on fresh produce**

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12 **Abstract**

13 Atmospheric cold plasma (ACP) represents a potential alternative to traditional methods for
14 non-thermal decontamination of foods. In this study, the antimicrobial efficacy of a novel
15 dielectric barrier discharge ACP device against *Escherichia coli*, *Salmonella enterica*
16 Typhimurium and *Listeria monocytogenes* inoculated on cherry tomatoes and strawberries,
17 was examined. Bacteria were spot inoculated on the produce surface, air dried and sealed
18 inside a rigid polypropylene container. Samples were indirectly exposed (i.e. placed outside
19 plasma discharge) to a high voltage (70kV_{RMS}) air ACP and subsequently stored at room
20 temperature for 24 h. ACP treatment for 10, 60 and 120 s resulted in reduction of *Salmonella*,
21 *E. coli* and *L. monocytogenes* populations on tomato to undetectable levels from initial
22 populations of 3.1, 6.3, and 6.7 log₁₀ CFU/sample, respectively. However, an extended ACP
23 treatment time was necessary to reduce bacterial populations attached on the more complex

24 surface of strawberries. Treatment time for 300 s resulted in reduction of *E. coli*, *Salmonella*
25 and *L. monocytogenes* populations by 3.5, 3.8 and 4.2 log₁₀ CFU/sample, respectively, and
26 also effectively reduced the background microflora of tomatoes.

27 Highlights:

28 A key advantage of this in-package non-thermal decontamination approach is the possibility
29 to eliminate of post-processing contamination of the produce, thus increasing microbiological
30 food safety and extension of produce shelf life. Inactivation was dependent on fresh produce
31 surface features.

32 Key words: Atmospheric cold plasma, decontamination efficacy, pathogenic bacteria, fresh
33 produce, ozone.

34 1. Introduction

35 The benefits associated with consumption of fresh produce maintain a high consumer demand
36 for a wide range of pre-packed ready to use products. Nevertheless, fresh produce may
37 contribute to the transmission of bacterial, parasitic and viral pathogens (Abadias *et al.* 2008).
38 In recent years, foodborne human illnesses resulting from contaminated fresh produce have
39 been widely reported globally. Most reporting countries identified *Escherichia coli* O157:H7,
40 *Listeria monocytogenes* and *Salmonella* spp. as the target pathogens capable of causing
41 severe human infection and deaths (Rangel *et al.* 2005; Raybaudi-Massilia *et al.* 2009;
42 Olaimat and Holley 2012; CDC, 2012). A wide range of fresh fruit and vegetable products
43 have been implicated in foodborne infections, such as lettuce, sprouted seed, melon,
44 tomatoes, radish, pepper, basil and other mixed salads (Fernandez *et al.* 2013; Olaimat and
45 Holley 2012). Pathogens, such as *E. coli* O157:H7 and *Salmonella* may reside in protected
46 sites on surface of the fresh produce and be able to survive for long periods of time beyond
47 the expected shelf-life (Olaimat and Holley 2012). Flessa *et al.* (2005) reported that *L.*
48 *monocytogenes* is capable of survival on the surface of fresh intact or cut strawberries

49 throughout the shelf life of the fruit and can survive on frozen strawberries for periods of 4
50 weeks. A health hazard to the consumers may also arise due to the possible presence of
51 microbial toxins as a consequence of produce contamination with spoilage bacteria (Issa-
52 Zacharia *et al.* 2010).

53 Raw fruits and vegetables can become contaminated while growing or during harvesting,
54 postharvest processing, storage or distribution (Cevallos-Cevallos *et al.* 2012). How bacteria
55 attach and the strength of attachment has not been well understood, but once attached to the
56 surface of fresh produce it is difficult to remove the pathogens by washing (Berger *et al.*
57 2010; Warning and Datta 2013). Conventional postharvest washing and sanitising treatments
58 are not highly effective for produce, often resulting in less than 2 log unit reductions of
59 pathogens (Niemira 2012). Moreover, some low pH based preservation techniques may
60 contribute to the bacterial adaption to acidic environment and subsequently increase their acid
61 resistance (Roberts and Wiedmann 2005). Disinfection can become less effective when
62 microorganisms are attached to produce surface include biofilm formation, concentration
63 reduction of sanitizer near produce surface and accessibility of sanitizer to cells attached to
64 rough surfaces (Wang *et al.* 2012). Pathogens can also attach to surface through interaction
65 with epiphytic microflora and may be further protected by internalising which itself
66 dependant on many produces phyllosphere characteristics (Erickson 2012).

67 Non-thermal antimicrobial treatments of fruits, vegetables and other food produce have been
68 the subject of much research. Atmospheric cold plasma (ACP) technology is a relatively new
69 approach aiming to improve microbiological safety in conjunction with maintenance of
70 sensory attributes of the treated foods. A key process advantage is the minimal water usage.
71 However, apart from issues associated with water mediated decontamination, it is likely that
72 many of the features associated with minimal processing and phyllosphere of produce that
73 impact on traditional washing decontamination, may also interact with the optimum

74 application of ACP. The antimicrobial efficacy and design of ACP systems including
75 producer gas composition, electrode configuration as well as the type of bacteria and
76 substrate varies widely among research studies (Fernandez *et al.* 2013, Niemira 2012;
77 Noriega *et al.* 2011; Niemira and Sites 2008). The use of indirect plasma in conjunction with
78 utilisation of closed chambers for decontamination of meat produce have been highlighted in
79 recent studies conducted by Rod *et al.* (2012) and Frohling *et al.* (2012b). Our previous study
80 also demonstrated the antimicrobial efficiency of indirect ACP exposure, where *E. coli* in a
81 sealed package was readily inactivated within seconds (Ziuzina *et al.* 2013). However, there
82 are limited numbers of reports based on in-package plasma decontamination of fresh fruits
83 and vegetables (Fan *et al.* 2012; Klockow and Keener 2009). Therefore, the objective of this
84 study was to evaluate the efficacy of indirect ACP generated inside a sealed package against
85 *E. coli*, *Salmonella* and *L. monocytogenes* inoculated on cherry tomatoes and strawberries
86 and to evaluate its potential to reduce background microflora present on cherry tomatoes and
87 strawberries in order to increase the produce shelf life.

88 **2. Materials and methods**

89 **2.1. Bacterial strains and inocula preparation**

90 Three bacterial strains were used in this study. *Escherichia coli* NCTC 12900 was obtained
91 from National Collection of type cultures of the Health Protection Agency (HPA, UK),
92 *Salmonella enterica* Typhimurium ATCC 14028 and *Listeria monocytogenes* NCTC 11994
93 were obtained from the microbiology stock culture of the School of Food Science and
94 Environmental Health of the Dublin Institute of Technology. Stock cultures were maintained
95 at -70°C in the form of protective beads (Technical Services Consultants Ltd, UK). One
96 protective bead of each culture was streaked onto separate tryptic soy agar (TSA,
97 ScharlauChemie, Spain), incubated at 37°C for 24 h and further maintained at 4°C. A single
98 isolated colony of each culture was inoculated in tryptic soy broth without glucose (TSB-G,

99 ScharlauChemie, Spain) and incubated at 37°C for 18 h. The cells were harvested by
100 centrifugation at 10,000 rpm for 10 min, washed twice in sterile phosphate buffered solution
101 (PBS, Oxoid LTD, UK) and finally resuspended in PBS, resulting in concentration of 8-9
102 Log₁₀ CFU/ml, which were further used as the working inoculum. The concentration of
103 inoculum was confirmed by plating appropriate dilutions on TSA, followed by incubation at
104 37°C for 24 h for *E. coli* and *Salmonella* and 48 h for *L. monocytogenes*.

105 **2.2. Preparation of produce**

106 Whole fresh cherry tomatoes and strawberries (Class 1, Origin: Spain) were purchased from
107 the local supermarket and stored at 4°C until use. The tomatoes were 2±0.5 cm in diameter
108 and 5-15 g in weight. Strawberries weight was approximately 10-20 g. The same produce
109 cultivar was used for each experiment. Cherry tomatoes were sterilized with 70% of ethanol
110 (Klerwipe 70/30, Shield Medicare LTD, Farnham, UK) in order to reduce the background
111 microbial load before surface inoculation of respective bacterial strain. Sterilized samples
112 were then washed with sterile deionized water to remove any remaining ethanol residue and
113 allowed to dry in the laminar flow safety cabinet at 23°C for 1 h prior to inoculation (Mattson
114 *et al.* 2011). In order to assess ACP treatment efficacy for reduction of the background
115 microflora, unsterilized tomatoes were also used.

116 **2.3. Fresh produce inoculation procedure**

117 For inoculation, tomatoes and strawberries were placed with the blossom end down on sterile
118 petri dishes. The samples were spot-inoculated with bacteria applying either 50 µl or 100 µl
119 of a culture on the tomato or strawberry surface, respectively (Das *et al.* 2006; Mahmoud *et*
120 *al.* 2007). The droplets were deposited in several different locations, ensuring that the
121 inoculum did not flow to the side of the samples. Inoculated samples were dried for 1 h in
122 laminar flow safety cabinet to allow the attachment of bacteria on the surface of produce
123 prior to the ACP treatment.

124 **2.4. Experimental design**

125 The ACP system utilised was a dielectric barrier discharge system previously described in
126 Ziuzina *et al.* (2013), with a maximum high voltage output of 120 kV at 50 Hz. The distance
127 between the two 15 cm diameter aluminium disk electrodes was 40 mm which was equal to
128 the height of the polypropylene container (310 x 230 x 40 mm) utilised as both a sample
129 holder and as a dielectric barrier.

130 Inoculated samples (four of either tomatoes or strawberries) were aseptically transferred on
131 one of the corner of the container so as to expose the samples to indirect ACP discharge
132 (Fig.1). The distance between the samples and centre of the electrodes was within the range
133 from 140 mm to 160 mm. In order to evaluate ACP treatment efficacy against background
134 microflora, uninoculated samples were used. After product loading, each container was
135 sealed within a high barrier polypropylene film (Cryovac, B2630, USA) and placed between
136 the aluminum electrodes of the transformer. The inoculated and uninoculated samples were
137 treated with 70 kV_{RMS} for 30 s - 300 s in air and at atmospheric pressure. All samples were
138 subjected to a post-treatment storage time of 24 h at room temperature. In order to evaluate
139 any possible effect of storage on the bacterial growth, inoculated control samples were stored
140 for 24 h under similar conditions. All experiments were performed in duplicate and replicated
141 three times to ensure reproducibility of the experimental data and are reported as log₁₀
142 CFU/sample.

143 **2.5. Microbiological analysis**

144 For microbiological analysis, inoculated untreated control samples (to estimate initial
145 attached bacterial population), inoculated untreated samples stored for 24 h (to assess the
146 effect of storage on microbial growth), uninoculated untreated control samples (to determine
147 initial background microflora), and either inoculated or uninoculated ACP treated samples
148 were analyzed. The samples were aseptically transferred into separate sterile stomacher bags

149 (BA6041, Seward LTD, UK) with 10 ml of sterile MRD and hand rubbed for 2-3 min. The
150 resulting suspension was serially diluted in MRD. The surviving *E. coli*, *Salmonella* and *L.*
151 *monocytogenes* populations were determined by agar overlay method (Mahmoud 2010).
152 Briefly, aliquots of an appropriate dilution were surface plated on TSA, incubated for 2-4 h,
153 and overlaid with the appropriate selective media: Sorbitol MacConkey agar (SMAC,
154 ScharlauChemie, Spain) supplemented with Cefixime-Tellurite (CT, Oxoid LTD, England)
155 for *E. coli*, Xylose Lysine Deoxycholate agar (XLD, ScharlauChemie, Spain) for *Salmonella*,
156 and polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM, ScharlauChemie,
157 Spain) supplemented with PALCAM Listeria Selective Supplement (Oxoid LTD, England)
158 for *L. monocytogenes*. Plates were then incubated for 24-48 h at 37°C.
159 Surviving background microflora of the uninoculated samples was evaluated using non-
160 selective media TSA for estimation of aerobic mesophilic bacteria and Potato Dextrose agar
161 (PDA, ScharlauChemie, Spain) for estimation of yeasts and moulds, with further incubation
162 of agar plates at 37°C and 25°C, for 48 h and 5 days, respectively. The limit of detection for
163 bacterial recovery on food samples was 1.0 Log₁₀ CFU/sample.

164

165 **2.6. Ozone measurements**

166 Ozone concentration inside the sealed package was measured using Gastec ozone detector
167 tubes (Product #18M, Gastec Corporation, Japan). Measurements were taken immediately
168 after plasma treatment and after 24 h of post treatment storage.

169

170 **2.7. Scanning Electron Microscopy (SEM)**

171 Attachment of different bacteria, namely *E. coli* and *L. monocytogenes*, attached on tomato
172 and strawberry samples was observed using SEM. Inoculated strawberry samples were
173 prepared as described by Gratao *et al.* (2008) with minor modifications. Briefly, the samples

174 were spot inoculated with either bacterium and dried under laminar flow at 23°C. The tissue
175 from the inoculated sites of the fruit was excised forming 1 cm in diameter and 1 mm of
176 thickness pieces. The cells were fixed in ice-cold 2.5% glutaraldehyde in 0.05 M sodium
177 cacodylate buffer (pH7.4) (SCB) for 2 h. The cells were washed with the same buffer three
178 times and fixed in 1% osmium tetroxide for 2 h at 4°C. After 2 h of fixation, bacterial cells
179 were washed with SCB followed by three washes with distilled water. The samples were
180 dehydrated using increasing concentrations of ethanol (30%, 50%, 70%, 80%, 95%, and
181 99.5%) and freeze dried (Labconco, FreeZone 6; Mason Technology, Dublin, Ireland). In
182 order to prevent surface charging by the electron beam, the samples were sputter-coated with
183 gold particles using Emitech K575X Sputter Coating Unit resulting in a coating of 10 nm
184 after 30 s. The samples were examined visually using a FEI Quanta 3D FEG Dual Beam
185 SEM (FEI Ltd, Hillsboro, USA) at 5 kV.

186 **2.8. Statistical Analysis**

187 Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, USA). The
188 surviving population of *E. coli*, *Salmonella* and *L. monocytogenes* and ozone concentration
189 following ACP treatment were subjected to analysis of variance (ANOVA). Means were
190 compared according to the method of Fisher's Least Significant Difference-LSD at the 0.05
191 level.

192 **3. Results**

193 Generally, indirect ACP treatment with subsequent 24 h of storage effectively reduced the
194 numbers of microorganisms on either produce surface studied. On cherry tomatoes,
195 treatments for 10 s, 60 s, and 120 s reduced populations of *Salmonella*, *E. coli* and *L.*
196 *monocytogenes* to undetectable levels, respectively. However, an extended treatment time of
197 300 s was necessary to reduce bacterial populations attached on the more complex surface of
198 strawberries.

199 3.1. Inactivation of bacteria on cherry tomatoes

200 The influence of ACP treatments on viability of *E. coli*, *Salmonella* and *L. monocytogenes* is
201 represented in Figure 2. Tomato samples were inoculated with an average of $3.1 \pm 0.6 \log_{10}$
202 CFU/sample for *E. coli*, $6.3 \pm 0.6 \log_{10}$ CFU/sample for *Salmonella* and $6.7 \pm 0.6 \log_{10}$
203 CFU/sample for *L. monocytogenes*. After treatment for 10 s and above *Salmonella*
204 populations on tomato were undetectable. Treatment for 45 s reduced populations of *E. coli*
205 and *L. monocytogenes* by 2 ± 1.2 and $4.5 \pm 0.2 \log_{10}$ CFU/sample, respectively. Further
206 increasing treatment time from 45 s to 60 s reduced populations of *L. monocytogenes* by 5.1
207 $\pm 0.5 \log_{10}$ CFU/sample and reduced populations of *E. coli* to undetectable levels. Populations
208 of *L. monocytogenes* were reduced to levels below detection limits after extended treatment
209 for 120 s.

210 3.2. Inactivation of bacteria on strawberries

211 Reductions of *E. coli*, *Salmonella* and *L. monocytogenes* inoculated on strawberries are
212 represented on Figure 3. The average initial attached population of *E. coli*, *Salmonella* and *L.*
213 *monocytogenes* was 4.4 ± 1.7 , 6.6 ± 1.2 and $7.3 \pm 0.3 \log_{10}$ CFU/sample, respectively. After 60
214 s and 120 s of ACP treatment, populations of *E. coli* were reduced by 1.2 ± 1.6 and 1.6 ± 0.1
215 \log_{10} CFU/sample, respectively, with significantly different reductions of $3.5 \pm 0.7 \log_{10}$
216 CFU/sample achieved after treatment for 300 s ($P \leq 0.05$). Similarly, populations of
217 *Salmonella* were reduced by 1.7 ± 0.1 and $3.8 \pm 0.4 \log_{10}$ CFU/sample after ACP exposure for
218 120 s and 300 s, respectively. No significant difference in antimicrobial efficacy of ACP
219 treatments for either 120 s or 300 s against *L. monocytogenes* was observed where average
220 reductions of approximately $4.2 \pm 0.5 \log_{10}$ CFU/sample were recorded. No changes were
221 noticed in the levels of bacterial populations attached on the untreated control tomato or
222 strawberries samples after storage for 24 h.

223 3.3. Inactivation of background microflora on produce

224 The reductions of background microflora on cherry tomatoes and strawberries due to indirect
225 ACP treatments are represented on Figure 3.

226 An average of initial background microflora on cherry tomatoes was $5 \pm 0.1 \log_{10}$
227 CFU/sample (Fig. 4a). After 60 s of ACP treatment the aerobic mesophilic counts were
228 reduced by $3 \pm 0.7 \log_{10}$ CFU/sample while yeasts and moulds were reduced by $2.5 \pm 0.6 \log_{10}$
229 CFU/sample. Further increase in treatment time to 120 s resulted in reductions of yeasts and
230 moulds to undetectable levels while population of mesophilic bacteria was reduced of by 4.2
231 $\pm 0.8 \log_{10}$ CFU/sample. Mesophilic bacteria were not detected when the treatment time was
232 increased to 300 s. Untreated and stored for 24 h samples showed no changes in the growth
233 levels of background microflora on tomato samples.

234 Lower reduction levels of spoilage microorganisms by ACP treatment were observed in the
235 case of strawberry samples (Fig. 4b). Significant decrease in mesophilic counts was observed
236 after 60 s of ACP treatment, resulting in reductions by $1.6 \pm 0.9 \log_{10}$ CFU/sample ($P \leq 0.05$)
237 from the control $3.6 \pm 0.3 \log_{10}$ CFU/sample. Populations of mesophilic bacteria did not
238 decrease further when treatment time was extended from 60 s to either 120 s or 300 s.
239 Populations of yeasts and moulds initially present on strawberries were $5.5 \pm 0.1 \log_{10}$
240 CFU/sample. These levels decreased by $1.0 \pm 0.8 \log_{10}$ CFU/sample after 120 s of ACP
241 treatment. Extending the treatment time from 120 s to 300 s resulted in an additional 0.4 ± 0.4
242 log reduction in the population of yeasts and moulds. It should be noted that the levels of
243 mesophilic bacteria of untreated control strawberry samples increased by $1.8 \pm 1.0 \log_{10}$
244 CFU/sample during 24 h storage, whereas populations of yeasts and moulds remained the
245 same.

246 **3.4. Ozone generation**

247 Generation of ozone inside the sealed package containing either cherry tomatoes or
248 strawberry samples as a function of ACP treatment time is represented in Figure 5. The ozone

249 concentration inside the package containing cherry tomatoes increased gradually with
250 increasing the treatment time. All ACP treatment times studied resulted in significant
251 increase of ozone concentration ($P \leq 0.05$) with maximum concentration of 5600 ppm
252 achieved after 300 s of treatment. However, no significant difference in ozone concentration
253 generated during the treatment of strawberry samples was observed. ACP treatment for 60 s
254 resulted in an average of 2800 ppm, and further increasing treatment time from 60 s to 120
255 and 300 s resulted in an average of 3200 and 3500 ppm of ozone, respectively.

256 3.5. Scanning Electron Microscopy (SEM)

257 In order to examine if the complex substrate surface features had any effect on the bacterial
258 adherence, and thus effect antimicrobial efficacy of ACP treatment, SEM analysis of
259 untreated *E. coli* and *L. monocytogenes* inoculated on produce surface was conducted. Figure
260 6(a,b) represents the surface of strawberry and tomato, respectively, inoculated with *L.*
261 *monocytogenes* where strong bacterial attachment in the form of clusters was noticed. On the
262 contrary, only a small amount of individually attached bacterial cells of *E. coli* on the rough
263 surface of strawberry was found (Fig. 6c).

264 4. Discussion

265 The indirect ACP treatment showed better inactivation efficacy against inoculated challenge
266 bacteria and background microflora present on the surface of the two different products
267 tested. Cherry tomatoes were selected as they have been associated with recent foodborne
268 illness outbreaks and represent common raw food ingredients of commercial salads.
269 Strawberries are also popular fruits and consumed raw. Moreover, these produce types
270 present different surface decontamination challenges to the ACP system, i.e. tomato surface
271 which is smooth, and the more complex surface of strawberry - uneven with numerous seeds.
272 In general, higher inactivation rates due to ACP treatment were achieved for bacteria
273 inoculated on smooth surface of tomatoes. *Salmonella* and *E. coli* were more rapidly

274 inactivated on tomato than *L. monocytogenes*. Among the three bacteria studied, *Salmonella*
275 was the most sensitive to ACP, where 10 s of treatment time reduced bacterial population to
276 undetectable levels. For tomato, increasing treatment time enhanced the inactivation efficacy
277 of ACP in the case of *E. coli* and *L. monocytogenes*. Increasing treatment time from 45 s to
278 60 s inactivated *E. coli* populations present on tomatoes, whereas inactivation to undetectable
279 levels of *L. monocytogenes* was obtained only after an extended treatment time of 120 s. It is
280 reported that Gram positive bacteria are more resistant to ACP treatments than Gram negative
281 (Montie *et al.* 2000; Lee *et al.* 2006; Ermolaeva *et al.* 2011; Frohling *et al.* 2012a), which was
282 also clearly demonstrated in the current study. *Salmonella* and *E. coli* are Gram negative
283 bacteria with a thinner outer membrane compared to the Gram positive *L. monocytogenes*.
284 The thicker membrane of the Gram positive bacteria may present a barrier to the diffusion of
285 plasma reactive species through the bacterial cell wall, thus impacting antimicrobial efficacy.
286 On the contrary, Fan *et al.* (2012) revealed greater sensitivity of Gram positive *L. innocua*
287 than Gram negative *Salmonella* and *E. coli* inoculated on tomato surface. Interestingly, other
288 comparative studies reported similar susceptibility between Gram positive and Gram negative
289 bacteria to ACP with respect to inactivation (Kostov *et al.* 2010; Olmez and Temur 2010;
290 Klampfl *et al.* 2012). Clearly, the target cell characteristics are important factors for
291 inactivation efficacy, but no clear trend is apparent and complex interactions with the system,
292 process, surface or medium may also impact on efficacy in combination with cell type.

293 In this study we observed that the difference in the initial levels of the attached bacterial
294 populations complicates the comparison of the bacterial sensitivity to the ACP treatments
295 based on bacterial cell membrane characteristics. It is widely accepted that high initial
296 bacterial concentration may affect inactivation efficacy of plasma treatment. The study
297 conducted by Fernandez *et al.* (2012) clearly demonstrated that increasing the concentration
298 of *S. Typhimurium* from 5 to 8 log₁₀ CFU/filter reduced the inactivation efficiency of ACP,

299 suggesting that the initial concentration of microorganisms present on foods plays an
300 important role in the efficacy of plasma treatment. In the present work, the lower initial
301 populations of *E. coli* attached on tomatoes surface did not necessarily contribute to the
302 increased ACP bactericidal characteristics. Within 45 s of treatment populations of *E. coli*
303 were reduced by 2 log from the initial 3.1 log₁₀ CFU/sample, whereas this treatment time
304 resulted in the reductions of *L. monocytogenes* populations by 4.5 log from the initial 6.7
305 log₁₀ CFU/sample, and only 10 s was required to reduce *Salmonella* by 6.3 log₁₀ CFU/sample.
306 This indicates the importance of the mechanisms and strengths of bacterial attachment with
307 respect to a decontamination procedure. It has also been demonstrated that the resistance to
308 ACP may also vary between bacteria species. Despite the higher inoculation levels on tomato
309 surface, *Salmonella* appeared to be more sensitive than *E. coli*. Similar results were achieved
310 in the research conducted by Niemira and Sites (2008) where *Salmonella* Stanley was more
311 sensitive to ACP than *E. coli* inoculated on both agar and apple surfaces.

312 The influence of the produce type on the overall antimicrobial efficacy of ACP was observed
313 when results are compared with the strawberry decontamination study. Treatment for 120 s
314 significantly reduced *L. monocytogenes* inoculated on strawberries. Increasing treatment time
315 to 300 s did not yield any further reductions of bacteria. However, after 300 s of treatment, a
316 proportional reduction of *E. coli* and *Salmonella* was achieved. Strawberry surface is more
317 porous than the surface of tomato. Irregularities of the fruit surface may provide many niche
318 areas for bacteria, providing physiological barrier or protection against ACP treatments. This
319 factor probably contributed to the reduced ACP bactericidal effect on Gram negative bacteria
320 on strawberries by comparison with tomatoes.

321 The influence of the complexity of the produce surface structure on inactivation efficacy of
322 ACP was observed when treatments were evaluated for the reduction of background
323 microflora naturally present on the produce. The causative agents of microbial spoilage in

324 fruits and vegetables can be bacteria (*Erwinia* spp., *Enterobacter* spp., *Propionibacterium*
325 *chlohexanicum*, *Pseudomonas* spp., and lactic acid bacteria) as well as moulds and yeasts
326 (*Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., and *Saccharomyces* spp., *Cryptococcus*
327 spp., *Rhodotorula* spp.) (Raybaudi-Massilia *et al.* 2009). In recent study conducted by Jensen
328 *et al.* (2013), 34 different species from 23 different genera for bacteria and 22 different
329 species from 9 different genera for yeasts were identified in strawberry samples. Despite this
330 potential diversity of indigenous microflora, an ACP treatment time of 120 s significantly
331 reduced the numbers on smooth surface of tomatoes in our study. However, again ACP was
332 not very effective for the reduction of background microflora on more complex surface of
333 strawberries, although tomato and strawberries tend to share similar bacterial communities
334 (Leff and Fierer 2013).

335 Current information available for characterisation of ACP suggests that plasma is a source of
336 heat, UV radiation, charged particles and reactive oxygen and nitrogen based species (ROS
337 and RNS, respectively) with a main role given to the ROS as prime plasma disinfectants
338 (Laroussi and Leipold 2004; Laroussi 2009). In this study, it was demonstrated that
339 increasing the treatment time resulted in increased antimicrobial efficacy of ACP against
340 bacteria inoculated on produce. Moreover, the inoculated samples were indirectly exposed to
341 plasma, i.e., at some distance to the plasma discharge (~160 mm from the centre of the
342 plasma discharge). In case of indirect treatment the charged particles and the short-lived
343 species would not be expected to play a role due to their potential to recombine before
344 reaching the sample (Laroussi 2009). Therefore, ozone was expected to be one of the key
345 factors contributing to antimicrobial efficacy of ACP treatments. It has been demonstrated
346 earlier that considerable reductions of bacteria by indirect ACP occurred within seconds
347 when extended post treatment storage was applied, suggesting diffusion of the reactive
348 species into liquids during post-treatment storage, thereby affecting microbial cells (Ziuzina

349 *et al.* 2013). Extended 24 h post treatment storage time was also employed in the current
350 study. It is likely that 24 h post treatment storage time facilitated ACP action on the bacterial
351 cells by retaining generated reactive species within closed container, thus, promoting
352 diffusion of the species inside the product tissue.

353 In the current work, as the treatment time increased, a significant increase in the ozone
354 generated by plasma inside the package containing produce was noted ($P \leq 0.05$). However, it
355 was also observed that the produce type influenced the concentration of ozone, where lower
356 ozone levels were recorded for strawberry samples. Strawberries surface exhibit numerous
357 pores, likely making the surface contact area larger than the area of tomato surface. This
358 surface area differential may contribute to the increased dissolution rate of ozone generated
359 inside the strawberry package, with subsequent reduced antimicrobial efficacy of ACP with
360 regard to the all bacteria tested.

361 Considering the lower ozone concentrations and the consequent lower reductions of the
362 challenge bacteria and background microflora on strawberries, it is likely that protection by
363 more complex produce structures could be a critical parameter determining plasma treatment
364 efficacy. Similarly, Fernandez *et al.* (2013) demonstrated that antimicrobial efficacy of
365 plasma was influenced by produce surface features with higher bacterial reduction levels
366 achieved on microbial filters than on more complex biotic surfaces.

367 As mentioned earlier, in this study, variations between initial populations of bacteria were
368 apparent, with *Salmonella* and *L. monocytogenes* more readily attaching on the surface of
369 either produce than *E. coli*. Regardless of the different surface features of the produce
370 studied, SEM images confirmed the larger populations of *L. monocytogenes* adherent cells in
371 addition to clusters of cells present. Despite the irregular nature of strawberry surface, which
372 would probably facilitate bacterial attachment, *E. coli* populations visualised by SEM on the
373 fruit surface were still less dense by comparison with *L. monocytogenes* images. A possible

374 explanation for the lower levels of attached *E. coli* is the presence and interaction with
375 naturally existing indigenous epiphytic bacteria. Depending on the types of epiphyte present
376 the survival of pathogens can be either enhanced or inhibited (Erickson 2012). For example,
377 Cooley *et al.* (2006) demonstrated that one epiphyte *Enterobacter asburiae* isolated from
378 lettuce inhibited colonisation of *E. coli*, whereas another epiphyte *Wausteria paucula* had the
379 opposite effect; enhancing *E. coli* survival.

380 Other factors that may affect microbial attachment to fresh produce are the different
381 morphology and chemistry of the produce as different fruits and vegetables offer different
382 microniches for the attachment, penetration and proliferation of bacteria (Keeratipibul *et al.*
383 2011). Motility of microorganisms facilitates pathogen entry into wounds, stomata and other
384 existing fruit surface openings (Deering *et al.* 2012). We observed in SEM images that
385 bacterial cells were likely adhered inside the natural crevices of produce surface or close to
386 these regions. Naturally existing crack and pits on the surface of produce provide bacteria
387 opportunity to internalise. Internalisation through the naturally existing opening is widely
388 described in literature and considered as one of the major route of pathogens entry to plant
389 tissue (Deering *et al.* 2012). Incidences of internalisation dependent upon concentration of
390 bacteria, their location on the plant, age, integrity and stages of plant development, as well as
391 indigenous agonistic/antagonistic bacteria present on plant have been reported (Erickson
392 2012; Shi *et al.* 2009). This study indicated that the decontaminating effect of ACP is a
393 function of produce type and the contaminating pathogen. The produce surface has an
394 influence on pathogen attachment, with the potential for internalisation particularly
395 associated with minimally processed fresh produce. Therefore the depth to which the plasma
396 generated chemical species are able to diffuse through a tissue in order to affect internalised
397 cells or those within a biofilm requires further investigation to elucidate how that diffusion
398 capability of ACP can be effectively harnessed. Overall, the results of this study indicated

399 that bacterial attachment and increased survivability on more complex surfaces following
400 ACP treatments should be considered as very important factors influencing treatment design.

401 **Conclusion**

402 In summary, the high voltage indirect ACP treatment was highly efficient for
403 decontamination of fresh produce inside a sealed package. Short treatment times of 10, 60 s
404 and 120 s resulted in reductions to undetectable levels of *Salmonella*, *E. coli* and *L.*
405 *monocytogenes*, respectively on cherry tomatoes. However, treatment times of up to 300 s
406 were required to attain substantial reductions on strawberry surfaces. Similarly,
407 yeasts/moulds and mesophiles on tomato surface were not detected after 120 to 300 s,
408 respectively. Thus, it can be concluded that ACP treatment with 24 h post treatment storage
409 can eliminate microorganisms on fresh produce surfaces inside a sealed package. In order to
410 achieve optimum decontamination efficiency by ACP, factors including type of produce,
411 their inherent surface characteristics, bacterial type, the strength and the nature of their
412 attachment as well as the diffusion capacity of the plasma species, to holistically address the
413 food safety issues associated with fresh produce, should be considered.

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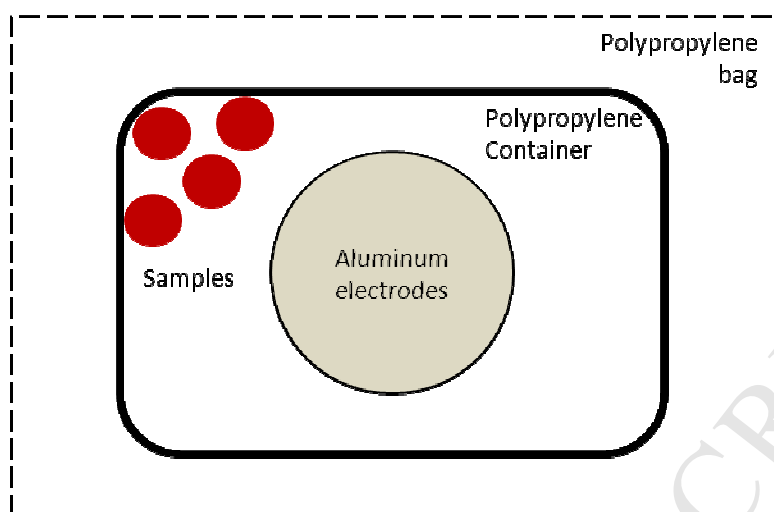
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564 **Figures:**



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566 Fig.1: Schematic diagram of samples distributed within polypropylene container with respect
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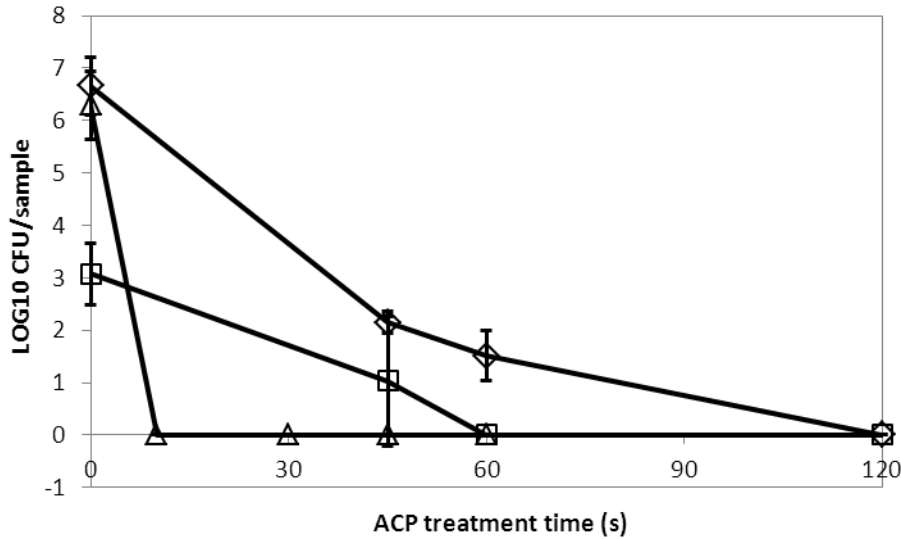
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580 Fig.2: ACP inactivation efficacy against *E. coli* (□), *Salmonella* (△) and *L. monocytogenes* (◇)
581 inoculated on cherry tomatoes. Vertical bars represent standard deviation. Limit of detection
582 1.0 log₁₀ CFU/sample.

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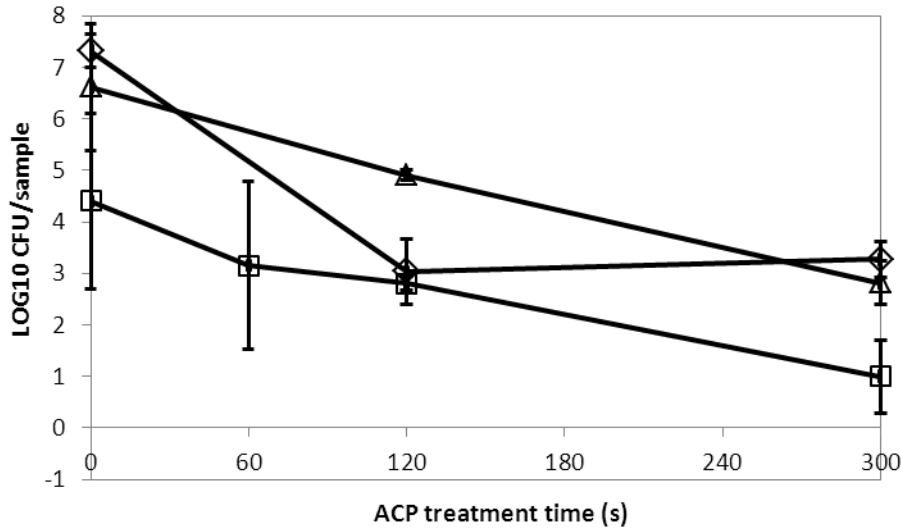
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598 Fig.3: ACP inactivation efficacy against *E. coli* (□), *Salmonella* (△) and *L. monocytogenes* (◇)
599 inoculated on strawberries. Vertical bars represent standard deviation. Limit of detection 1.0
600 log₁₀ CFU/sample.

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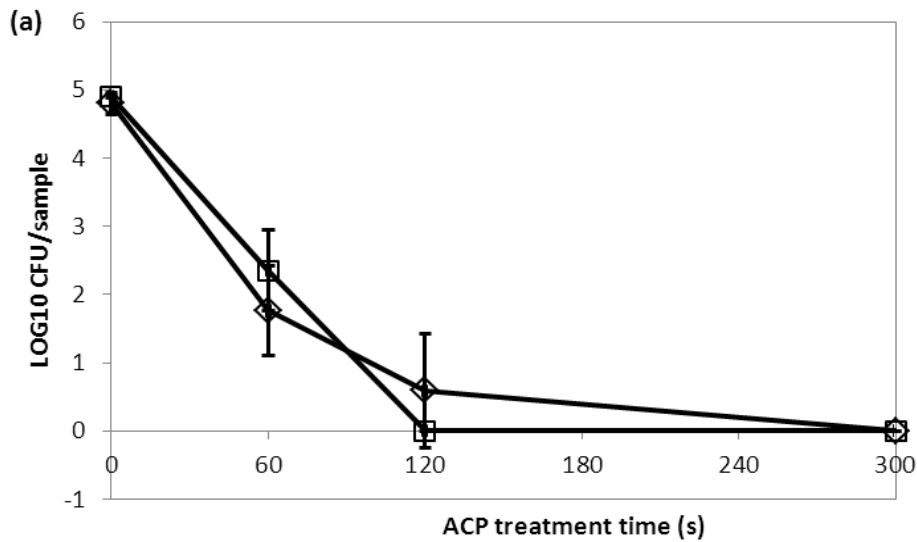
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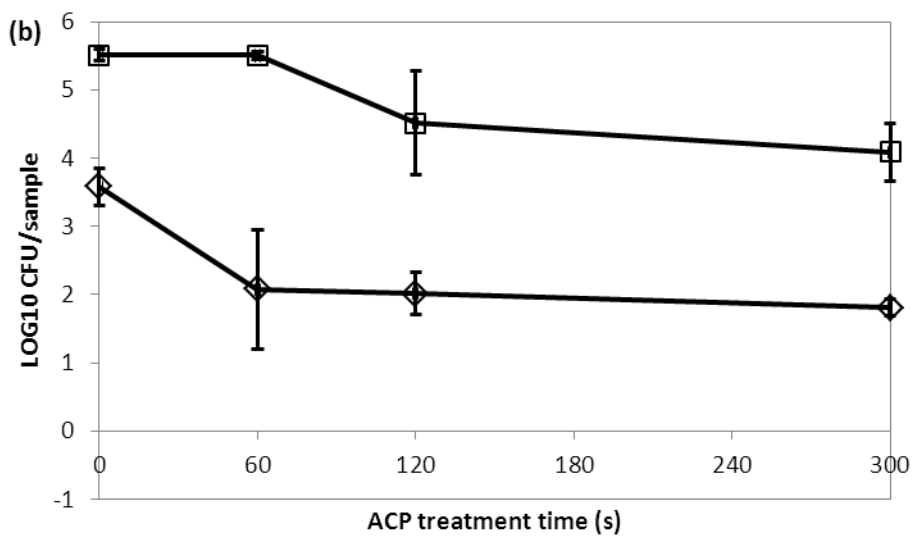
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617 Fig.4: ACP inactivation efficacy against aerobic mesophilic bacteria (\diamond) and yeasts and
 618 moulds (\square) on (a) cherry tomatoes and (b) strawberries. Vertical bars represent standard
 619 deviation. Limit of detection 1.0 log_{10} CFU/sample.

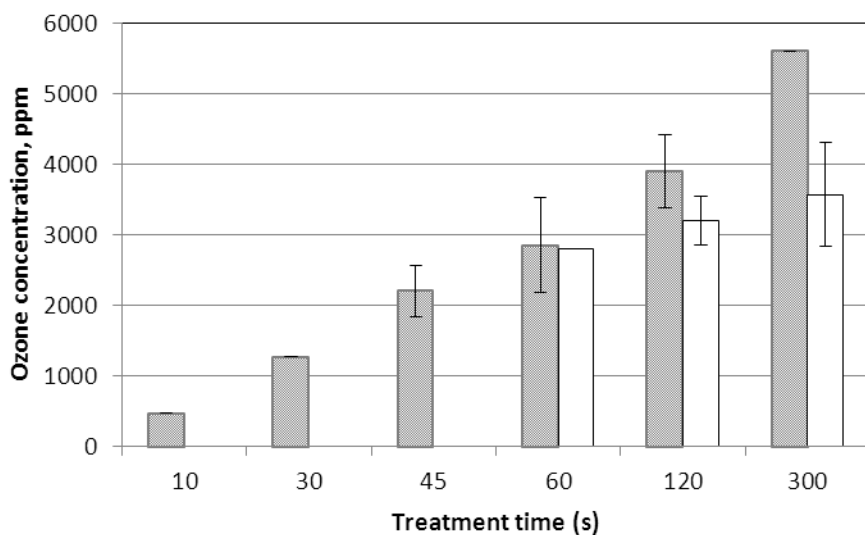
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626 Fig.5: Generation of ozone inside a sealed package during ACP treatment of either inoculated
627 or uninoculated samples of cherry tomatoes (■) and strawberries (□). Vertical bars represent
628 standard deviation.

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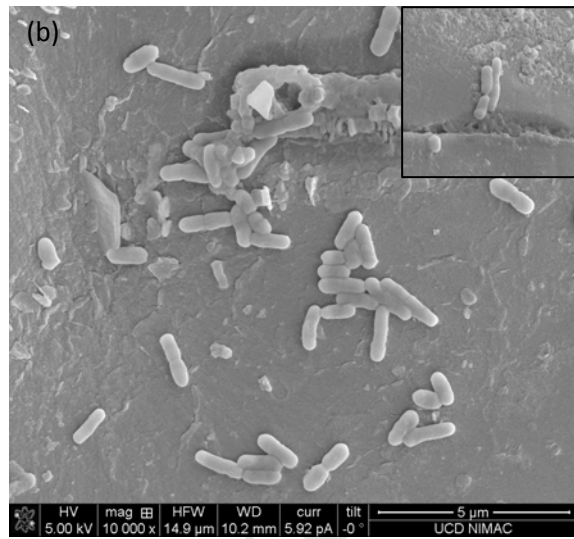
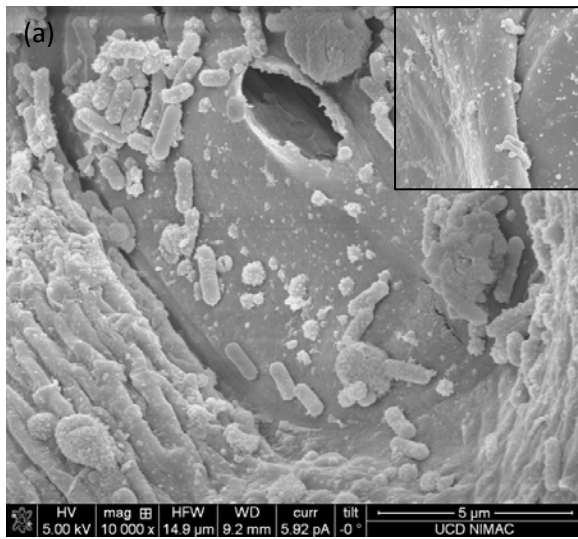
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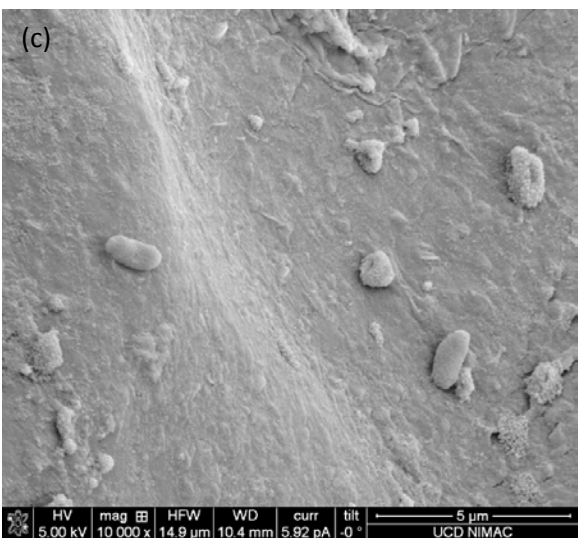
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658 Fig.6: SEM images of untreated *L. monocytogenes* on (a) strawberries and (b) cherry

659 tomatoes, and (c) *E. coli* inoculated on strawberry.

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YFMIC 2105 Food Microbiology

Atmospheric Cold Plasma inactivation of *Escherichia coli*, *Salmonella enterica* serovar

Typhimurium and *Listeria monocytogenes* inoculated on fresh produce

Highlights

- In this study antimicrobial efficacy of ACP against *Escherichia coli*, *Salmonella enterica* Typhimurium and *Listeria monocytogenes* inoculated on cherry tomatoes and strawberries was evaluated.
- A key advantage of this high voltage level treatment for in-package non-thermal decontamination approach is the possibility to eliminate post-processing contamination of the produce.
- This approach has potential to provide both increased microbiological food safety and extension of produce shelf life.
- Inactivation was however, dependent on fresh produce surface features and pathogen type.