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# Microbiological Interactions With Cold Plasma

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# 1 **Microbiological Interactions with Cold Plasma**

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7 ***Running Title: Cold Plasma Microbiology***

## 8 **Summary**

9 There is a diverse range of microbiological challenges facing the food, healthcare and clinical  
10 sectors. The increasing and pervasive resistance to broad-spectrum antibiotics and health  
11 related concerns with many biocidal agents drives research for novel and complementary  
12 antimicrobial approaches. Biofilms display increased mechanical and antimicrobial stability  
13 and are the subject of extensive research. Cold plasmas (CP) have rapidly evolved as a  
14 technology for microbial decontamination, wound healing and cancer treatment, owing to the  
15 chemical and bio-active radicals generated known collectively as reactive oxygen (ROS) and  
16 nitrogen species (RONS). This review outlines the basics of CP technology and discusses  
17 interactions with a range of microbiological targets. Advances in mechanistic insights are  
18 presented and applications to food and clinical issues are discussed. The possibility of tailoring  
19 CP to control specific microbiological challenges is apparent. This review focuses on  
20 microbiological issues in relation to food and health care associated human infections, the role  
21 of CP in their elimination and the current status of plasma mechanisms of action.

22 **Keywords:** Cold plasma technology, Microbiological interactions, Mechanism of action, Anti-  
23 microbial resistance, Biofilms, Food, Healthcare.

## 24 **What is cold plasma?**

25 Plasma is commonly referred to as the fourth state of matter where increases in the material's  
26 energy levels converts its state from solid to liquid to gas and ultimately to an ionised state of  
27 the gas, "plasma", which exhibits unique properties. Cold plasma (CP) is comprised of several  
28 excited atomic, molecular, ionic, and radical species, co-existing with numerous reactive  
29 species, including electrons, positive and negative ions, free radicals, gas atoms, molecules in  
30 the ground or excited state and quanta of electromagnetic radiation (UV photons and visible  
31 light). Depending on the generation conditions, plasma can be classified into low-,  
32 atmospheric- or high-pressure and also subdivided into thermal and non-thermal plasmas.  
33 Furthermore, non-thermal plasma or CP can be generated from either atmospheric pressure  
34 therefore called atmospheric CP (ACP), or low pressure, where both plasmas generate similar  
35 reactive species and same electron densities range, thus possess similar microbial inactivation  
36 mechanisms (Zhang *et al.* 2013). Thermal plasma can be generated by heating the gas to high  
37 temperatures, which may exceed several thousands of Kelvins, where all the constituent  
38 chemical species, electrons and ions exist in a thermodynamic equilibrium (Moreau *et al.* 2008;  
39 Wan *et al.* 2009; Misra *et al.* 2011; Banu *et al.* 2012; Niemira 2012; Scholtz *et al.* 2015). In  
40 contrast, CP are characterised by non-equilibrium, where cooling of the ions and uncharged  
41 molecules is significantly more effective than that of energy transfer from electrons resulting  
42 in the gas remaining at a low temperature (Niemira 2012; Scholtz *et al.* 2015).

## 43 **Common types of plasma devices**

44 The application of a strong electromagnetic field to a neutral gas that induces ionisation is the  
45 most commonly used method of generating CP (Banu *et al.* 2012). CP may be obtained by a  
46 diversity of electrical discharges, such as corona discharge, micro hollow cathode discharge,  
47 gliding arc discharge, one atmospheric uniform glow discharge, dielectric barrier discharge,  
48 atmospheric pressure plasma jet and plasma needle. The type of plasma source will generally

49 influence the technological application along with the composition and abundance of the  
50 chemical species produced (Nehra *et al.* 2008; Scholtz *et al.* 2015). For environmental,  
51 biological and biomedical applications the dielectric barrier discharge (DBD) and plasma jet  
52 are the two most commonly used forms of CP generation (Fig. 1). This is primarily due to their  
53 simple design and the possibility of reconfiguration to suit many types of targets and treatment  
54 requirements.

### 55 **Mechanisms of action of cold plasma**

56 The chemical composition of CP is complex, and multiple different reactive agents are  
57 expected to play a role, independently or in synergy, in inactivation of microbial targets. In  
58 general, the composition and thus the efficacy of CP will depend on the device design and  
59 system operating parameters, such as gas composition, flow rate, moisture, temperature,  
60 voltage and frequency (Dobrynin *et al.* 2009; Wan *et al.* 2009; Ehlbeck *et al.* 2011).  
61 Atmospheric air CP is an excellent source of electrons and positive and negative ions, free  
62 radicals, stable conversion products (e.g. ozone), excited atoms and molecules, and ultraviolet  
63 radiation (UV) photons (Stoffels *et al.* 2008). The majority of reactive species produced by the  
64 commonly used plasma sources include electronically and vibrationally excited oxygen O<sub>2</sub> and  
65 nitrogen N<sub>2</sub>; active forms of oxygen molecules and atoms, i.e. reactive oxygen species (ROS),  
66 such as atomic oxygen O, singlet oxygen <sup>1</sup>O<sub>2</sub>, superoxide anion O<sub>2</sub><sup>-</sup> and ozone O<sub>3</sub>; reactive  
67 nitrogen species (RNS), such as atomic nitrogen N, excited nitrogen N<sub>2</sub>(A), nitric oxide NO•;  
68 if humidity is present H<sub>2</sub>O<sup>+</sup>, OH<sup>-</sup> anion, OH• radical or H<sub>2</sub>O<sub>2</sub> are also generated (Scholtz *et al.*  
69 2015). The exact mechanisms of CP mediated bacterial inactivation are still under  
70 investigation, but several generated products have been demonstrated to play a role. These  
71 products include ROS, RNS, UV radiation and charged particles within a plasma gas phase.  
72 Among the ROS, ozone, atomic oxygen, singlet oxygen, superoxide, peroxide, and hydroxyl

73 radicals, are considered to be involved in bacterial inactivation (Joshi *et al.* 2011; Alkawareek  
74 *et al.* 2012).

75 Most bacteria, particularly, anaerobes are considered to be very sensitive to ROS species  
76 (Stoffels *et al.* 2008). The diffusion of oxygen species or oxygen containing radicals (nitric  
77 oxide) through a bacteria cell wall causes local damage possibly by oxidation of cytoplasmic  
78 membrane, protein and DNA strands (Gallagher *et al.* 2007). Joshi *et al.* (2011) reported that  
79 singlet oxygen and hydrogen peroxide species were responsible for membrane lipid  
80 peroxidation, as ROS scavengers significantly reduced the oxidative damage of *E. coli* DNA.  
81 Moreover, the inactivation efficacy of RNS can be stimulated with the presence of ROS, which  
82 indicated the importance of oxygen blend in working gases (Boxhammer *et al.*, 2012).  
83 Sureshkumar *et al.* (2010) demonstrated that adding 2% oxygen to nitrogen gas resulted in the  
84 formation of nitric oxides, which significantly enhanced the inactivation effect. The presence  
85 of these reactive species was confirmed by optical emission spectroscopy.

86 Bombardment on the cell wall by charged particles, electrons and ions can break chemical  
87 bonds, cause erosion through etching, formation of lesions and openings in the membranes,  
88 inducing further penetration of plasma toxic compounds inside a bacterial cell (Gallagher *et al.*  
89 2007; Moreau *et al.* 2008). Inactivation through erosion is believed to be easier to achieve in  
90 Gram-negative bacteria, due to the vulnerability of the cell wall, compared with Gram-positive  
91 species with a thicker membrane structure (Stoffels *et al.* 2008). However, the intracellular  
92 damage was more obvious in Gram-positive bacteria as a result of higher intracellular ROS  
93 level (Han *et al.* 2015). Another significant role in the mechanical disruption of bacterial cell  
94 membrane is the effect of charged particles, which is widely classified in literature as direct  
95 and indirect (Dobrynin *et al.* 2009). Indirect treatment design employs distance or metal mesh  
96 to avoid direct contact of charged particles with samples. The charged particles do not largely  
97 participate in treatment but recombine before reaching the sample (Laroussi 2009). With direct

98 contact, charged particles could accumulate on surface and cause electrostatic stress. This could  
99 lead to morphology changes by overcoming the tensile strength of cell membrane (Mendis *et al.*  
100 *al.* 2000; Laroussi *et al.* 2003). Cell membrane perforation caused by etching will enhance the  
101 diffusion of secondary reactive species that might be formed in the plasma discharge inside the  
102 cell. Etching, as a result of reaction between the excited atoms/molecules and radicals and  
103 organic materials causes breakdown of bonds, particularly for hydrocarbon compounds. This  
104 in turn will lead to the formation of molecular fragments and volatile compounds emanating  
105 from the cells, causing morphological changes, ranging from reduction in cell size to the  
106 appearance of deep channels in the cell, up to complete cellular destruction. Atomic oxygen  
107 and ozone easily react with these open bonds, which facilitates a faster etching of molecules  
108 (Ermolaeva *et al.* 2011; Fricke *et al.* 2012). This erosion effect leading from the cleavage of  
109 chemical bonds can also lead to the demise of microbial support structures such as biofilms.  
110 Graves (2014) proposed a model, which emphasised the importance of the biological systems  
111 adaptive response, thus recognising that a biological systems response may occur over a longer  
112 time and space scale than the initial exposure to plasma reactive species. Figure 2 further  
113 illustrates the complexity of microbial inactivation mechanisms with plasma reactive species.  
114 Despite the extensive research on the antimicrobial effects of CP, it is necessary to consider  
115 this technology in tandem with the nature of the microbial contamination presented in foods,  
116 their processing environments as well as clinical and healthcare situations to elucidate how the  
117 mechanisms and mode of delivery may be optimized to provide effective alternative  
118 antimicrobial technologies.

### 119 **Cold plasma for food safety applications**

120 Bacterial pathogens are considered a critical food safety issue, followed by foodborne viruses,  
121 bacterial toxins, pesticide residues and mycotoxins (van Boxtael *et al.* 2013). Most reporting  
122 countries identify *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. as

123 the target pathogens of concern (Raybaudi-Massilia *et al.* 2009; Olaimat and Holley 2012). In  
124 addition the bacterial capacity for biofilm formation, internalisation of contaminating cells  
125 within a host tissue or structure and/or formation of highly resistant spores often complicate or  
126 negate food disinfection processes (Fig. 3). CP technology has been demonstrated as a potential  
127 alternative to conventional methods attributable to its non-thermal nature, its proven potential  
128 to enhance microbiological safety and maintain quality characteristics of a wide range of foods  
129 within fast processing times. CP has been studied for decontamination of many food groups  
130 including fresh fruits and vegetables, meat and meat products, milk and dairy products, egg  
131 and egg products, seafood, fruit juices, powdered products, nuts, cereals and grains.  
132 Advantages that broaden the scope for food processing include reduced water usage, lack of  
133 chemical residue and use of atmospheric air as a working gas. The compatibility with other  
134 food processing unit operations aids the development of large-scale systems for different  
135 commodities. The interactions between CP treatment, the effector molecules and  
136 microorganisms are complex and depend on numerous system, process and target parameters.  
137 These include plasma device, voltage level, frequency, working gas, gas flow rate, humidity  
138 level, distance between the target and plasma emitter, type of product, surface characteristics  
139 and volume in addition to the type, concentration and physiological state of microorganisms.  
140 This complexity makes comparisons in reported efficacies difficult (Fig. 4).

#### 141 **Inactivation of food borne pathogenic microorganisms**

142 The mode of exposure and type of system configuration significantly impact on antimicrobial  
143 efficacy. Hertwig *et al.* (2015) compared direct plasma treatment using a radio frequency argon  
144 plasma jet to a remote treatment using a microwave generated air plasma for effects on  
145 *Salmonella* inoculated on whole black pepper with higher bactericidal effects achieved using  
146 remote air plasma. In air plasmas, both reactive nitrogen and reactive oxygen species are  
147 generated, which directly impact on microorganisms and can lead to their inactivation.

148 Reactive nitrogen species can accumulate on the microbial surface and easily diffuse through  
149 cell membranes, causing a decrease of intracellular pH. The intracellular pH plays a major role  
150 in cell function and affects enzyme activity, reaction rates, protein stability and structure of  
151 nucleic acids (Hertwig *et al.* 2015). It has been demonstrated that using a contained ACP  
152 system, which facilitates the post treatment retention of reactive species can enhance the anti-  
153 microbial efficacy for decontamination of fresh foods (Ziuzina *et al.* 2014). Kim *et al.* (2013)  
154 established that the distance between samples and plasma emitter as well as position of meat  
155 samples during plasma exposure played a crucial role in inactivation efficiency of treatment  
156 against *S. Typhimurium*. A distance of 20 mm using double sided treatments for 2.5 min of  
157 chicken breasts had greater inactivation than a single-side treatment for 5 min with similar  
158 patterns observed for pork loin.

159 The main advantages of low pressure CP generation approach are the possibility to avoid  
160 arcing (as a result of the use of lower voltage levels for generation of plasma), which may  
161 damage fragile surfaces such as fresh produce surfaces and suitability for the treatment of pre-  
162 packed produce using a low pressure vacuum (Zhang *et al.* 2013). In the decontamination of  
163 shell eggs, Mok and Song (2013) used air generated low-pressure discharge plasma and  
164 achieved a 6 log reduction of *Salmonella Typhimurium* after 5 min of treatment. In this study,  
165 the working gas used for generation of plasma played a vital role in inactivation effects, with  
166 the highest inactivation achieved using air following by oxygen and nitrogen. Importantly, the  
167 bacterial reductions were achieved with no denaturation of either the egg white or yolk. In a  
168 study conducted by Ragni *et al.* (2010), inoculated shell eggs were treated in a plasma after-  
169 glow chamber generated using resistive barrier discharge. Reductions by up to 4.5 and 3.5 log  
170 units were observed for *S. Enteritidis* and *S. Typhimurium*, respectively, using air with higher  
171 moisture contents (65%) and treatment time of 90 min. This demonstrates the critical role of  
172 humidity level for achieving effective microbiological control with CP. The enhanced effect of



173 increased relative humidity (RH) on the efficiency of inactivation was attributed to the presence  
174 of oxygen reactive species (ROS) as detected in the discharge emission spectra. An increase in  
175 OH radical irradiance in the emission spectrum using a humid atmosphere, which mainly  
176 results from the direct dissociation of water molecules by electron impact and is a function of  
177 the concentration of water vapour, was recorded. Although a considerable treatment time was  
178 required to significantly reduce *Salmonella* populations on eggs, treatment using a plasma  
179 after-glow chamber also provides gentle conditions during processing, thus minimizing  
180 changes in egg quality traits (Ragni *et al.* 2010).

181 In line with the demands of modern consumption, the control of pathogens on heat sensitive  
182 fresh foods drives research in non-thermal approaches. CP research has focused on the  
183 microbiological safety of fresh produce as they remain a major vehicle for transmission of food  
184 borne diseases. CP has been investigated for control of *Salmonella* on lettuce, spinach,  
185 tomatoes, apples and strawberries. Fernandez *et al.* (2013), found that the inactivation rate of  
186 *S. Typhimurium* was independent of growth phase or growth temperature and that 15 min of  
187 ACP treatment was required to achieve 2.72 log reductions of viability of cells on lettuce using  
188 a nitrogen plasma jet system. The authors reported an effect of produce surface with reduced  
189 inactivation efficiency reported for strawberry and potato by comparison with lettuce.  
190 Scanning electron microscopy (SEM) studies have revealed that different food surface  
191 characteristics such as the convolutions of strawberry surfaces and the walls of the eukaryotic  
192 cells of potato tissue, could obscure bacterial cells and create physical barriers that are mitigate  
193 the efficacy of ACP inactivation, whereas smooth surfaces such as cherry tomatoes facilitated  
194 rapid inactivation times (Fernandez *et al.* 2013; Ziuzina *et al.* 2014). In contrast, Zhang *et al.*  
195 (2013), found that the antimicrobial efficacy of 10 minute low-pressure oxygen plasma  
196 treatment was unaffected by product surface interactions. The authors reported that treatment  
197 time and plasma energy density were critical for high inactivation rates against *S. Typhimurium*

198 inoculated on spinach (rough hydrophobic), lettuce (smooth hydrophilic), tomato (smooth  
199 hydrophobic) and potato (rough hydrophilic) surfaces. Higher plasma energy densities can give  
200 rise to higher intensities of UV irradiation, UV photons, and plasma reactive species thereby  
201 enhancing bactericidal properties of treatment. However, care should be taken when longer  
202 treatments are applied as fresh food quality characteristics may be altered (Zhang *et al.* 2013).  
203 In 2013, in the EU, 6,043 confirmed cases of verocytotoxigenic *E. coli* (VTEC) infections  
204 resulting in 13 deaths were reported (EFSA, 2015). Enterohemorrhagic *E. coli* O157:H7 is  
205 recognised as the most predominant serotype, causing severe illness in humans. Prieto-Calvo  
206 *et al.* (2016) reported that strains of the serotype O157 were in general more resistant to food-  
207 related stresses, such as acid, alkaline, heat, high hydrostatic pressure, UV and ACP, than  
208 strains of other serotypes when they had a functional RpoS (a global regulator of the general  
209 stress response in Gram-negative bacteria such as VTEC). Applying a high voltage AC  
210 atmospheric corona discharge system to milk reduced suspended *E. coli* by almost 4 log cycle  
211 (54%) after 20 min of plasma application, regardless of the fat content of the milk and no viable  
212 cells were detected after 6 weeks (Gurol *et al.* 2012).

213 Klockow and Keener (2009), exposed whole spinach leaves inoculated with *E. coli* to 5 min of  
214 in package DBD plasma, where employing a post-treatment storage time for 24 h yielded  
215 optimum inactivations ranging from 3–5 log CFU/leaf. Bermudez-Aguirre *et al.* (2013)  
216 reported the effect of treatment time (30 s to 10 min) and voltage level (3.95 kV up to 12.83  
217 kV) using an argon plasma needle array reactor on *E. coli* populations on a range of produce  
218 surfaces. Combining higher voltage level and extended treatment time was more effective in  
219 microbial inactivation (1.6 log) when associated with lower initial bacterial counts and  
220 smoother substrate surface (tomatoes, followed by lettuce, were easier to disinfect than carrots).  
221 SEM analysis showed the major structural damage to *E. coli* cells, with disruption and loss of  
222 thin cell membrane surrounding the cytoplasmic content, perforations on the membrane and

223 surface and inner components of the cell due to the action of ACP electric field and other  
224 charged particles, thus promoting cellular death. A correlation between increasing surface  
225 complexity and a reduced ACP antimicrobial efficiency was further established by Butscher *et*  
226 *al.* (2016). In this work, *E. coli* inoculated on alfalfa, onion, radish and cress seeds was exposed  
227 to argon plasma generated in an atmospheric pressure pulsed DBD system. While 10 min of  
228 treatment (longest treatment duration at 10 kHz, 8 kV, 500 ns pulses) caused the reduction of  
229 *E. coli* on onion seeds by 1.4 log, the identical treatment conditions resulted in a 3.4 log  
230 reduction of cells on cress seeds. SEM analysis illustrated the multiple cracks in onion seeds,  
231 which may shelter microorganisms and protect them from the surface concentrated effects of  
232 dry plasma treatment. Similarly, complex surface properties significantly affected  
233 decontamination efficacy of ACP against *E. coli* inoculated on fresh produce (Ziuzina *et al.*  
234 2014, Baier *et al.* 2015). With regards to moisture content, 17% was found to be an optimum  
235 for the decontamination of seeds, as compared to either 8 or 30% (Butscher *et al.* 2016). This  
236 was attributed to the chemistry reactions generated in the liquid phase, which can result in the  
237 formation of more stable secondary reactive species and the acidification of the milieu with  
238 combined lethality to microorganisms. Different strain responses to plasma treatment have  
239 been reported. Argon plasma treatment for 1 minute reduced *E. coli* O157:H7 levels on the  
240 surface of corn salad leaves by 3.3 log, whereas 2 min of treatment was required to reduce *E.*  
241 *coli* O104:H4 to similar levels (Baier *et al.* 2016).

242 Generally, these studies indicate that CP can effect good inactivation of *E. coli* for liquid and  
243 solid food products. Whilst complex surface characteristics pose a major challenge to the  
244 decontamination efficacy of plasma, improvements are possible through retention of active  
245 species using in package design as well as optimisation of parameters such as treatment  
246 duration, treatment regime, discharge moisture content and plasma inducer gas composition.

247 *Listeria* spp. are ubiquitous, tolerant to extreme conditions such as low pH, low temperature  
248 and high salt conditions, and are found in a variety of food and environmental matrices  
249 (Jeyaletchumi *et al.* 2010) often as a post processing contaminant. Song *et al.* (2009)  
250 demonstrated >8 log CFU/g reductions using air ACP against a three-strain cocktail of *L.*  
251 *monocytogenes* inoculated on sliced cheese in association with an input power of 150 W and  
252 treatment time of 2 min. The efficacy of treatment was largely influenced by the food  
253 characteristics examined, with only 1.73 log CFU/g reductions achieved when bacteria was  
254 inoculated on ham. Besides AC voltage and excitation frequency, Noriega *et al.* (2011)  
255 investigated the effect of the presence of oxygen in the carrier gas on inactivation efficacy of  
256 ACP against *L. innocua* inoculated on chicken muscle and skin. Higher voltage and frequency  
257 levels and the presence of oxygen in the carrier gas resulted in the greatest inactivation  
258 efficiency, where > 3 log reduction was achieved after 4 min of treatment on muscle, however,  
259 8 min of treatment was required to achieve 1 log reduction on skin. SEM images of chicken  
260 muscle and skin revealed surface features wherein bacteria could effectively be protected from  
261 the chemical species generated within the gas plasma. In contrast, no significant effects of  
262 treatment time and power intensity on decontamination effects of treatment was found by Rod  
263 *et al.* (2012) when ACP was evaluated against *L. innocua* inoculated on sliced ready to eat  
264 meat product, bresaola. Applying multiple treatments with a 10 min interval increased  
265 inactivation in line with increasing the number of treatments. The reported results involving  
266 different foods clearly indicate that the inactivation effect of plasma treatment on *Listeria* is  
267 dependent not only on plasma treatment conditions but also on the type of foods and their  
268 inherent surface characteristics, which has to be always considered to achieve efficient  
269 microbial inactivation with plasma systems.

270 The target cell characteristic is also an important factor to be considered for the achievement  
271 of efficient decontamination with plasma technology. Han *et al.* (2015) proposed a model

272 where the mechanisms of action against Gram-positive and Gram-negative microorganisms  
273 differed. Ziuzina *et al.* (2014) demonstrated that Gram-negative *Salmonella* and *E. coli* were  
274 more rapidly inactivated on tomato than Gram-positive *L. monocytogenes* and among the three  
275 bacteria studied, *Salmonella* was the most sensitive to ACP.

276 In contrast, there was no clear pattern of sensitivity between Gram-negative *E. coli* and  
277 *Salmonella* isolates inoculated on almonds reported by Niemira *et al.* (2012). The sensitivity  
278 of Gram-negative bacteria to ACP treatment was also demonstrated by Niemira *et al.* (2008)  
279 where maximal reduction of 3.7 log was obtained after 3 min of treatment *Salmonella* Stanley  
280 on apples which was greater than those obtained for *E. coli* (3.4 log CFU/ml). *E. coli* inoculated  
281 on radicchio leaves was significantly reduced after 15 min CP treatment, however, 30 min of  
282 plasma treatment was necessary to achieve a significant reduction of *L. monocytogenes* counts  
283 (Pasquali *et al.* 2016). Jayasena *et al.* (2015) investigated the effect of bacterial cell wall  
284 structure on inactivation efficacy of an oxygen/nitrogen plasma generated on flexible thin-layer  
285 DBD, and found higher reductions for Gram-negative pathogens. Min *et al.* (2016) reported  
286 higher resistance for *Salmonella* to in-package DBD ACP treatment as compared to *E. coli*, *L.*  
287 *monocytogenes* or Tulane virus (TV) inoculated on lettuce (~6 log CFU/g lettuce). In this work,  
288 5 min of treatment at 34.8 kV resulted in reduction of *E. coli*, *Salmonella*, *L. monocytogenes*,  
289 and TV by 1.1, 0.4, 1.0 log CFU/g, and 1.3 log PFU/g, respectively, without modifications of  
290 moisture or gas in the packages. Moreover, extended post treatment storage did not induce  
291 further reductions in contrast with the previous reports. Some studies report no clear patterns  
292 of sensitivity to plasma between Gram-positive and Gram-negative species. Kim *et al.* (2011)  
293 reported effective microbial reduction using helium/oxygen gas mixture for the three  
294 pathogenic microorganisms inoculated on bacon. The initial counts (7-8 Log CFU/g) of *E. coli*,  
295 *L. monocytogenes*, and *S. Typhimurium* were reduced to 4.80, 5.79, and 6.46 log CFU/g after  
296 plasma treatment at 125W for 90 s. Again, increasing the input power and plasma treatment

297 time provided higher inactivation levels for *E. coli*, *L. monocytogenes* and *S. Typhimurium*,  
298 regardless of gas composition used for generation of plasma. Likewise, *E. coli*, *L.*  
299 *monocytogenes*, and *S. Typhimurium* counts were each reduced by approximately 2.4 log CFU/  
300 mL following plasma treatment for 10 min.

### 301 **Cold plasma control of bacterial biofilms**

302 Many human pathogens grow predominantly as biofilms rather than in planktonic mode  
303 (Giaouris *et al.* 2013; Sharma *et al.* 2014). Bacterial biofilms are broadly described as a  
304 microbially derived sessile community characterized by cells that are attached to a substratum  
305 or to each other and are embedded in a matrix of extracellular polymeric substances (EPS), and  
306 exhibit an altered phenotype with respect to growth rate and gene transcription (Giaouris *et al.*  
307 2013). Formation of bacterial biofilms on food contact surfaces, on food processing equipment  
308 and in potable water distribution systems contributes to food spoilage, cross-contamination of  
309 food products and spread of foodborne pathogens (Kim and Wei 2012), and therefore represent  
310 a major challenge in food industry (Borges *et al.* 2013). Moreover, biofilms are more resistant  
311 to various environmental stresses and the actions of applied antimicrobial treatment.

312 With regard to biofilm susceptibility to the mechanisms of CP, Jahid *et al.* (2014a)  
313 demonstrated that 15 s of ACP treatment reduced planktonic populations of *Aeromonas*  
314 *hydrophila* by > 5 log. However, 5 min of treatment was necessary to significantly reduce  
315 bacterial biofilm populations associated with lettuce. Similarly, *Salmonella*, *L. monocytogenes*  
316 and *E. coli* suspended in lettuce broth were undetectable after 30 s of in-package ACP  
317 treatment, however, 5 min of treatment was required to significantly reduce bacterial  
318 populations when cells were either attached on the surface or grown as biofilms on lettuce  
319 (Ziuzina *et al.* 2015). Rapid inactivation of *Salmonella* biofilms attached on glass surface was  
320 achieved with plasma jet emitter operating at 1 atm using filtered air as the feed gas. CP reduced  
321 biofilms by up to 1.57, 1.82 and 2.13 log CFU/mL after 5, 10 and 15 s of treatment, (Niemira

322 *et al.* 2014). A submerged or underwater DBD plasma reactor has been used to inactivate  
323 biofilms of key food-borne pathogens, such as *E. coli*, *Cronobacter sakazakii* and  
324 *Staphylococcus aureus* generated on stainless steel surface with reductions by 5.50, 6.88 and  
325 4.20 log CFU/coupon, respectively, obtained after 90 min of treatment. The ATR-FTIR  
326 measurement showed gradual reduction of carbohydrates, proteins, and lipid and DNA peak  
327 regions with increased plasma exposure time (Khan *et al.* 2016). Furthermore, Gabriel *et al.*  
328 (2016) addressed the influence of different surface features on the bacterial attachment and  
329 therefore biofilm formation and susceptibility to treatment. *Pseudomonas aeruginosa* biofilms  
330 were developed on different types of stainless steel, such as 316 and 304 with different finishes  
331 namely, mirror, hairline and 2B surfaces. Variations in D-values were observed between  
332 surface finishes within a specific stainless steel type. However, significant variations were not  
333 observed between the same surface finish of different steel types. A 5-log reduction in the  
334 population was observed in a relatively short treatment times of ~ 90 s (Gabriel *et al.* 2016).  
335 Although with different range of processing times required to achieve significant inactivation  
336 of biofilms of foodborne pathogens, these studies demonstrated that CP could be an alternative  
337 technology for effective decontamination of materials within food processing environment.  
338 The major mechanisms for CP mediated biofilm inactivation reported to date are illustrated in  
339 Figure 5 and include alterations in cell membrane integrity, destruction of EPS, cells and  
340 cellular components, reduction of biofilm thickness, reduced culturability and metabolic  
341 activity of cells. Air DBD ACP treatment for 5 min significantly altered biofilm structures of  
342 *E. coli* and *P. aeruginosa* formed on polycarbonate membranes, changing the healthy cells  
343 interconnected by self-produced EPS matrices to irregularly shaped cell fragments. This  
344 corresponded to >5 log reductions in biofilms developed in 96 well plate model (Ziuzina *et al.*  
345 2014, 2015). Alkawareek *et al.* (2012) demonstrated marked susceptibility of *P. aeruginosa*  
346 biofilms in vitro to plasma jet treatment operating in a helium oxygen mixture after minutes of

347 treatment. In this work, confocal scanning laser microscopy (CLSM) demonstrated that vast  
348 majority of cells within biofilm of 40 - 80  $\mu\text{m}$  thickness were non-viable after 3 min of  
349 treatment. Pei *et al.* (2012) also reported that plasma generated ROS were able to penetrate to  
350 the bottom layer of a 25.5 $\mu\text{m}$ -thick *Enterococcus faecalis* biofilm and produce a strong  
351 bactericidal effect.

352 These studies indicate the ability of the plasma reactive species to penetrate deeply into the  
353 biofilm and inactivate the cells within and / or that secondary reactive products were formed at  
354 the biological or liquid interface that mediate an antimicrobial effect. Within 5 min of in  
355 package ACP DBD treatment, *P. aureuginosa* biofilm thickness went from 23 to 6  $\mu\text{m}$  (Ziuzina  
356 *et al.* 2014). Similarly, *Candida albicans* biofilm, with a thickness of 10 to 20  $\mu\text{m}$ , was  
357 completely removed within 5 min of argon/oxygen plasma treatment (Fricke *et al.* 2012).  
358 Severe damage and etching effect of plasma on *Candida albicans* biofilms were also observed  
359 by Koban *et al.* (2010) and Sun *et al.* (2012) whereas effects on biofilms of Gram-positive and  
360 Gram-negative bacteria were reported by Lee *et al.* (2009).

361 Research to date proposing anti-biofilm mechanisms of action of CP has often used biofilms  
362 developed on abiotic surfaces in response to clinical manifestation or industrial surface  
363 biofouling. In the case of biofilm formation on food surfaces, another important factor that has  
364 potential to further elevate resistance to antimicrobial agents is the internalisation of bacterial  
365 pathogens. Bacterial internalization may occur through entering plant natural openings (e.g.  
366 hydathodes, stomata, lenticels) or physically damaged sites during processing and is dependent  
367 on time, temperature, light, pressure, produce surface characteristics and the native endophytic  
368 microbial community (Kroupitski *et al.* 2009; Golberg *et al.* 2011; Deering *et al.* 2012; Gu *et*  
369 *al.* 2013a, b; O'Beirne *et al.* 2014). In a comparative study of several decontamination  
370 approaches, 200 ppm chlorine, 2% citric, lactic, or malic acids, 32 Hz ultra-sonication, 390  
371  $\text{mJ}/\text{cm}^2$  ultraviolet-C, and 750  $\text{mJ}/\text{cm}^2$  cold oxygen plasma were compared for the reduction of



372 *L. monocytogenes* biofilms formed on lettuce and cabbage surfaces. The highest reduction was  
373 achieved using ACP regardless of the produce used. This further suggests that plasma reactive  
374 species could penetrate or degrade the biofilm matrix, leading to cell inactivation (Srey *et al.*  
375 2014). However, cells that had internalized into vegetable stomata could not be reached.  
376 Fernandez *et al.* (2013) also suggested that vegetable stomata and convolutions on the product  
377 surface can play a significant role in protecting microbial cells from the action of ACP  
378 generated reactive species. The antimicrobial potential of in-package ACP treatment with  
379 subsequent 24 h of storage was demonstrated as an effective approach for inactivating  
380 *Salmonella*, *L. monocytogenes* and *E. coli* biofilms formed on lettuce (Ziuzina *et al.* 2015).  
381 Moreover, plasma treatment was challenged with bacteria internalised in lettuce tissue and  
382 SEM analyses showed that cold plasma treatment in conjunction with 24 h of post treatment  
383 storage had detrimental effects on surface attached cells. However, high remaining  
384 concentrations of cells were noted inside the stomata. Jahid *et al.* (2015) also reported  
385 increased resistance of *Salmonella* Typhimurium bacterial biofilms on lettuce leaves to plasma  
386 due to internalization and extensive colonization in produce stomata wells. These findings  
387 highlight the advantages of in package treatment design, which mitigates against  
388 recontamination or cross contamination events by surviving microorganisms protected by  
389 biofilms and/or within complex structures.

390 In response to the diversity of microbiological challenges, the forms they can take and  
391 antimicrobial resistance, CP devices and approaches are being developed which may be dry or  
392 liquid mediated. There is a need for standardised surface-food-microbial systems to facilitate  
393 adequate comparison of antimicrobial efficacy of different CP systems. Moreover, because a  
394 majority of persistent bacterial infections are associated with biofilms, biofilm resistance  
395 towards antimicrobial treatments, including plasma treatment, should form part of the studies  
396 where antimicrobial effect of novel decontamination technology is investigated.

## 397 **Clinical applications of cold plasma**

398 The past two decades have witnessed a rapid expansion in the potential applications of CP to  
399 controlling infection within the clinical setting. Primarily, these studies have been aimed at  
400 controlling bacterial pathogens, but CP exposure has been shown to rapidly and effectively  
401 inactivate a wide range of infectious agents. In particular, the ability of plasma to eradicate  
402 bacterial biofilms efficiently has been demonstrated by a number of groups. Biofilms represent  
403 a particular challenge in the healthcare setting, since they exhibit highly elevated tolerance to  
404 antimicrobial challenge (Olson *et al.* 2002 Ceri *et al.* 2010), are implicated in medical device-  
405 and health care associated infections (Revdiwala *et al.*, 2012) and act as reservoirs of infection  
406 in the environment (Hall-Stoodley and Stoodley, 2009). Biofilms also represent a major  
407 virulence characteristic in acute and chronic infections, where they are the predominant mode  
408 of growth (Wolcott and Erlich 2008). Recently, ACP has also been shown to rapidly inactivate  
409 biofilms of antibiotic resistant bacteria such as the so-called ESKAPE pathogens (Flynn *et al.*  
410 2015) and *Burkholderia cenocepacia* (Alshraideh *et al.* 2016), however the effects of plasma  
411 can be highly variable, with biomass and catalase production playing significant roles  
412 mediating biofilm tolerance to plasma exposure. ACP exposure has also been shown, for the  
413 first time, to induce formation of plasma-resistant persister cells in *Pseudomonas aeruginosa*  
414 biofilms (Mai-Prochnow *et al.* 2015), attributed to the production of the redox-active antibiotic  
415 pigment, phenazine. Whilst the above studies indicate an ability of bacteria, particularly in the  
416 biofilm mode of growth, to develop tolerance to plasma exposure, a number of studies have  
417 focused specifically on the effects of plasma interaction with antibiotic resistant bacteria.  
418 Bayliss and co-workers described the restoration of antibiotic sensitivity in MRSA following  
419 cold plasma exposure, and suggest the possibility of combined treatment with plasma exposure  
420 and conventional antibiotics as a mechanism to reinstate sensitivity to and circumvent  
421 antibiotic resistance (Bayliss *et al.* 2013). Plasma exposure appears to lead to rapid disinfection

422 of multidrug resistant bacterial via induction of cell surface damage, indicating a physical  
423 mechanism of bactericidal activity (Kvam *et al.* 2012). Recently, cold atmospheric plasma  
424 treatment has been shown to eradicate both vancomycin resistant enterococci (VRE) and high  
425 level gentamicin resistant (HLGR) enterococci, however efficacy was dependent on degree of  
426 resistance and membership of special resistance groups of clinical-outbreak importance (Napp  
427 *et al.* 2016).

#### 428 **Control of quorum sensing-mediated virulence**

429 Although the mechanism of action of plasmas vary according to the unique chemical  
430 environment created by different types of plasma generation device, and whilst the precise  
431 mechanism of action is still not completely understood in each case, a number of cellular targets  
432 have been identified which interact with plasma components and lead to loss of cell viability  
433 (Alkawareek *et al.* 2014). Despite this, the interactions of cold plasma with molecular  
434 components of cell signalling pathways and their downstream virulence factors has only  
435 recently been described. Quorum sensing is a cell density-dependent cell-cell signaling  
436 mechanism employed by bacteria to regulate group behaviours at a community level, including  
437 biofilm formation, antimicrobial tolerance and resistance and virulence (Williams 2007). The  
438 first demonstration of the ability of plasma to interfere with quorum sensing-controlled  
439 virulence factors, by Ziuzina and co-workers (2015), such as pyocyanin and elastase (lasB)  
440 described a high voltage DBD ACP with samples treated 'in pack'. Following short exposures,  
441 pyocyanin production was significantly inhibited and lasB activity reduced after 300 seconds  
442 exposure. Supporting these observations, Flynn and colleagues demonstrated the ability of  
443 plasma exposures to directly disrupt quorum sensing molecules utilized by Gram negative  
444 bacteria, the acylhomoserine lactones (AHLs), and reduce downstream bioluminescence and  
445 pigment production in reporter strains and significantly reduced production of pycyanin and  
446 pyoverdinin, reducing virulence of *P. aeruginosa* in an in vivo model (Flynn *et al.* 2016). The

447 ability to modulate bacterial virulence with short exposures opens the potential for cold plasma  
448 treatments to be employed in an anti-virulence, rather than an antimicrobial/bactericidal,  
449 context which may reduce the likelihood of resistance development.

#### 450 **Cold plasma and sporicidal activity**

451 Bacterial endospores (or spores), dormant structures formed by members of the Genera  
452 *Bacillus* and *Clostridium*, exhibit highly elevated tolerance to environmental stresses, allowing  
453 them to survive for prolonged periods in a dormant state (Kennedy 1994; Leggett *et al.* 2012).  
454 Evolutionary adaptations, which facilitate long-term dormancy in the environment also impart  
455 significant resistance to disinfectants, chemical sterilants, thermal inactivation and desiccation  
456 (Setlow 2006; Leggett *et al.* 2012). Bacterial spores therefore pose particular challenges in the  
457 food industry, pharmaceutical manufacturing environments and healthcare settings, where they  
458 represent persistent sources of product contamination. The mechanisms of intrinsic resistance  
459 to chemical disinfectants/sterilants are primarily due to their impermeable outer layers and low  
460 water content (Leggett *et al.* 2016) and, given the production of a highly oxidizing environment  
461 produced by cold plasmas, similar to oxidizing disinfectants like sodium hypochlorite,  
462 hydrogen peroxide and peracetic acid, similar resistance profiles are observed when assessing  
463 the sporicidal effects of plasma exposure. Amongst the first reports of spore inactivation by  
464 atmospheric pressure, cold plasma described the inactivation of endospores of *Bacillus*  
465 *stearothermophilus* and *Bacillus subtilis* on solid surfaces, fabrics, filter paper and powder  
466 culture media using One Atmosphere Uniform Glow Discharge Plasma (OAUGDP) device at  
467 room temperature (Kelly-Wintenberg *et al.* 1998). The authors reported variable sensitivity to  
468 plasma exposures, with seven minute exposures reducing *B. stearothermophilus* by  $\geq 3 \log_{10}$   
469 reductions in CFU, whilst 5 minutes exposure reduced *B. subtilis* viable spore counts by  $\geq 5$   
470  $\log_{10}$  reductions in CFU.

471 Van Bokhorst-van de Veen *et al.* (2014) tested nitrogen plasma biocidal activity against  
472 *Bacillus cereus*, *Bacillus atrophaeus* and *G. stearothermophilus* spores and compared ACP  
473 efficacy to heat, hypochlorite, hydrogen peroxide, and UV treatment. Plasma treatment of 20  
474 min reduced spores of *B. cereus*, *G. stearothermophilus*, and *B. atrophaeus* by 3.7, 4.2, and 4.9  
475 log units respectively. Spores of different bacteria varied in their degree of inactivation by  
476 applied heat, hypochlorite, hydrogen peroxide, and UV treatments, whereas similar  
477 inactivation results were obtained for spores treated with ACP. Distinct morphological changes  
478 included the appearance of rough spore surfaces from the etching action of ACP treatment. Lee  
479 and co-workers also described the sporocidal activity of a helium/oxygen ACP system. Their  
480 data indicated that the sterilizing effects of their plasma system was due to reactive oxygen  
481 radicals and not UV, and reported a D-value of 14 minutes which was not correlated to initial  
482 spore density (Lee *et al.* 2006).

483 Recently, the application of a high voltage (70kV<sub>RMS</sub>) DBD ACP system to inactivation of  
484 *Bacillus atrophaeus* within a sealed package was described. Rapid direct and indirect (in-  
485 package) sporocidal activity was demonstrated, with 60s exposures bringing about reductions  
486 of  $\geq 6$  log<sub>10</sub> reductions (direct) and 2.1 or 6.3 log<sub>10</sub> reduction of spore viability, depending on  
487 gas types used for plasma generation. Sporocidal activity was critically influenced by relative  
488 humidity and plasma-generated reactive species other than ozone were found to be critical to  
489 inactivation efficiency (Patil *et al.* 2014). The sporocidal activity of nitrogen ACP is not based  
490 on UV-C radiation only. To distinguish between lethal effects of emitted UV-light and reactive  
491 species, Reineke *et al.* (2015) exposed UV-sensitive mutant spore strains of *B. subtilis* to jet  
492 argon plasmas with different UV emission intensities and a significant impact of UV-light on  
493 the first phase of spore inactivation was confirmed. The sporocidal effects of pure argon plasma  
494 were comparable with high UV emission plasma against *B. atrophaeus* and *B. subtilis* spores,  
495 confirming that spore inactivation is dominated by the action of UV photons if the UV intensity

496 is high enough. Cold plasmas generated in air have demonstrated efficacy against *Clostridium*  
497 *difficile* spores on hospital surfaces (vapor permeable mattress sections and stainless steel)  
498 (Claro *et al.* 2015).

499 Schnabel *et al.* (2012) evaluated plasma treatment of *B. atrophaeus* spores inoculated on  
500 different seeds. The surface structure of investigated seeds played an important role in  
501 sporicidal action of ACP. Depending on seed surface characteristics, 15 min of treatment  
502 reduced the number of spores by > 6 log units. Hertwig *et al.* (2015) reported reductions by 2.4  
503 and 2.8 log for *B. subtilis* and *B. atrophaeus* spores inoculated on whole black pepper,  
504 respectively, after 30 min of exposure to plasma afterglow and by 0.8 and 1.3 log, respectively,  
505 after 15 min exposure to direct plasma jet treatment. SEM analysis demonstrated modification  
506 of the external shape of spores, which was attributed to the decomposition of organic material  
507 by etching and photo-desorption, which are associated with chemical bond breakage leading to  
508 the formation of volatile compounds. Butscher *et al.* (2015) employed a low-pressure fluidized  
509 bed plasma reactor for decontamination of *B. amyloliquefaciens* on wheat grains with > 2 log  
510 units reductions in 30s at power input of 900 W. Spore elimination required an hour of plasma  
511 treatment which raised the surface temperature of grains to 90°C. Butscher *et al.* (2016) later  
512 reported the influence of substrate shape and surface properties on efficacy of atmospheric  
513 pressure DBD-generated pulsed plasma inactivation of *Geobacillus stearothermophilus*. While  
514 10 min of treatment yielded ~ 5 log reductions on polypropylene granules, the maximum spore  
515 inactivation on wheat grains was 3 log units after 60 min of treatment. Thus, there are  
516 considerable gaps in knowledge for rapid plasma control of spores within biological matrices,  
517 that do not compromise other desirable or essential elements of that matrix.

### 518 **Virucidal activity of cold plasma**

519 Cold plasmas have shown significant promise in replacing conventional disinfectant  
520 approaches for the inactivation of viruses. Initially, CP was shown to rapidly inactivate

521 bacteriophages, often employed as a facile surrogate model for evaluating the virucidal activity  
522 of chemical disinfectants against human, animal and plant pathogenic viruses (Alshraiedeh *et*  
523 *al.* 2013). Venezia and colleagues described the antimicrobial activity of a commercially  
524 available system (PlasmaSol apparatus) against a range of bacteria, spores and viruses. The  
525 authors report 4-6 log<sub>10</sub> reductions in PFU ml<sup>-1</sup> of temperate and lytic bacteriophages after 10  
526 minutes exposure (Venezia *et al.* 2008). Interestingly, a separate study examining the virucidal  
527 activity of a novel dielectric barrier discharge reactor, Yasuda and co-workers reported rapid  
528 inactivation of lambda phage infectivity by up to 6 log<sub>10</sub> reductions after only 20 seconds  
529 (Yasuda *et al.* 2010). The *E. coli* MS2 bacteriophage has been validated as a convenient,  
530 representative surrogate for human norovirus in establishing the virucidal activity of biocides  
531 in chemical disinfectant efficacy tests (Maillard *et al.* 1994; Pinto *et al.* 2010). Alshraiedeh and  
532 colleagues reported the virucidal efficacy of a handheld, helium/oxygen, KHz driven  
533 atmospheric pressure non thermal plasma jet (described in Alkawareek *et al.* 2012) against  
534 MS2 bacteriophage. MS2 bacteriophage was rapidly inactivated, with inactivation rate constant  
535 increasing with increasing oxygen percentages in the feed gas, up to 0.75%. Up to 3 log<sub>10</sub>  
536 reductions in PFU ml<sup>-1</sup> were recorded after 3 seconds, with > 7 log<sub>10</sub> reductions in PFU ml<sup>-1</sup>  
537 after 9 minutes exposure (Alshraiedeh *et al.* 2013). A cold oxygen plasma, described by Terrier  
538 *et al.* (2009) was also shown to efficiently inactivate nebulized respiratory viruses human  
539 parainfluenza virus 3 (hPIV-3), respiratory syncytial virus (RSV) and influenza virus A  
540 (H5N2), reducing the titre of each by up to 6.5, 3.8 and 4 log<sub>10</sub> TCID<sub>50</sub> ml<sup>-1</sup>, respectively,  
541 within the allocated treatment time. The ability of CP to inactivate norovirus (foodborne  
542 outbreak strain) in faecal samples has recently been demonstrated (Ahlfeld *et al.* 2015). Such  
543 field testing of virucidal activity of CP in clinical samples supports the potential application of  
544 CP systems to efficiently disinfect virally contaminated surfaces and fomites, reducing the  
545 potential risk of onward transmission of infectious agents.

## 546 **Anti-protozoal activity of cold plasma**

547 Whilst the antimicrobial efficacy of CP is now well established in terms of antibacterial,  
548 antifungal, antiviral and sporocidal activity, the anti-protozoal activity has received relatively  
549 little attention. However, studies are emerging which indicate that CP exposure yields moderate  
550 reductions in protozoal viability. Recently, a pulsed-gas plasma-discharge (PPGD) system was  
551 evaluated for its ability to inactivate the enteric protozoal pathogen *Cryptosporidium parvum*,  
552 a common cause of water-borne disease (cryptosporidiosis) in humans (Hunter & Syed, 2001).  
553 The environmentally stable oocysts exhibit resistance to chemical disinfectants, such as  
554 chlorine, hypochlorous acid and ozone (Pereira *et al.* 2008; Rowan 2011). Therefore, alternative  
555 methods for decontamination of waste and drinking water are urgently required. Hayes *et al.*  
556 (2013) report for the first time the inactivation of *C. parvum* oocytes by pulsed electric  
557 discharges into gas injected liquids, which results in generation of ozone, hydrogen peroxide  
558 and UV light. In this study a 4 log<sub>10</sub> reduction in *C. parvum* oocyte viability was achieved after  
559 32 minutes of PPGD exposure (Hayes *et al.* 2013). Heaselgrave and co-workers also reported  
560 the inactivation of trophozoites and cysts of the protozoan *Acanthamoeba polyphagia* and  
561 *Acanthamoeba castellannii* using ACP generating apparatus (ambient air plasma).  
562 *Acanthamoeba* spp. are ocular pathogens which are etiological agents of *Acanthamoeba*  
563 keratitis (AK), a potentially sight limiting corneal infection, sometimes associated with contact  
564 lens use (Lorenzo-Morales *et al.* 2015). Trophozoites of *A. polyphagia* and *A. castellannii* were  
565 highly susceptible to plasma inactivation, exhibiting complete inactivation after 1 and 2  
566 minutes exposure, respectively. Furthermore, for the more disinfectant resistant cyst stage of  
567 both species, 4-minute exposures led to complete inactivation (Heaselgrave *et al.* 2016). These  
568 studies indicate that, whilst variations in inactivation efficiency for protozoa depend on plasma  
569 generating system parameters and test protozoan/life cycle stage, CP may have promise in  
570 controlling protozoal infections and contamination across a broad range of applications.



571 **Concluding comments and future directions**

572 There are recent advances, which further the understanding of the antimicrobial mechanisms  
573 of CP generated reactive species across the range of microbiological challenges. These  
574 mechanistic insights can drive successful adoption of CP technology. There is strong potential  
575 for CP to address some of the most critical issues including antimicrobial resistance and  
576 sustainability. The range of mechanisms of action in addition to the possibility of synergistic  
577 action with known biocidal or anti-biotic agents suggests there is scope to enhance activity  
578 against resistant strains, or even reinstate antibiotic sensitivity. The diversity of application  
579 devices and technologies available allows flexibility in application although comparisons can  
580 be difficult to make. The liquid mediated effects of CP generated reactive species, which are  
581 linked to the stable secondary forms of the reactive species are chemically and biochemically  
582 quantifiable, opening up avenues for quantifiable dosage regimens. The flexibility of adoption  
583 for safety as well as spoilage concerns drives research associated with foods using plasma  
584 processed air or liquids where the efficacy required to comply with microbiological criteria for  
585 sensitive foods can be attained. From a contamination control perspective, a unique advantage  
586 can be offered with in package generation of plasma reactive species, as this approach mitigates  
587 post processing contamination and cross contamination events. However, it is important that  
588 the demonstrated efficacy is considered in tandem with establishing the human and  
589 environmental safety of the approach to drive regulatory acceptance and compliance.

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903

#### 904 **List of figure legends**

905 Figure 1: Schematic diagram of a) DBD-CP: 1 – power supply, 2 – electrodes, 3 – dielectric  
906 barrier, 4 – plasma discharge, 5 – sample; b) Plasma Jet: 1 – power supply, 2 – high voltage  
907 electrode, 3 – tube electrode, 4 – nozzle, 5 – ring electrode, 6 – gas inlet, 7 – plasma  
908 discharge, 8 - sample. Adapted from Lu et al (2012).

909 Figure 2: Mechanisms of cold plasma generated reactive species with respect to complexity  
910 of microbiological challenges.

911 Figure 3: Microbial challenges associated with disinfection

912 Figure 4: Parameters influencing plasma treatment decontamination efficacy

913 Figure 5: Cold plasma mechanisms of action on biofilms. Lee et al (2009), Alkawareek et al  
914 (2012), (Fricke et al 2012), Pei et al (2012), Sun et al (2012) and Ziuzina et al 2014, 2015).