2017

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Recommended Citation

Bourke, P., Zuizina, D., Han, L., Cullen, P.J. & Gilmore, B. (2017). Microbiological Interactions with cold plasma. *Journal of Applied Microbiology*, vol. 123, no. 2, pg. 308-324. doi:10.1111/jam.13429.

Microbiological Interactions with Cold Plasma

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Running Title: Cold Plasma Microbiology

Summary

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

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

What is cold plasma?

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

Common types of plasma devices

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

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

Mechanisms of action of cold plasma

The chemical composition of CP is complex, and multiple different reactive agents are expected to play a role, independently or in synergy, in inactivation of microbial targets. In general, the composition and thus the efficacy of CP will depend on the device design and system operating parameters, such as gas composition, flow rate, moisture, temperature, voltage and frequency (Dobrynin *et al*. 2009; Wan *et al*. 2009; Ehlbeck *et al.* 2011). Atmospheric air CP is an excellent source of electrons and positive and negative ions, free radicals, stable conversion products (e.g. ozone), excited atoms and molecules, and ultraviolet radiation (UV) photons (Stoffels *et al*. 2008). The majority of reactive species produced by the commonly used plasma sources include electronically and vibrationally excited oxygen O_2 and nitrogen N_2; active forms of oxygen molecules and atoms, i.e. reactive oxygen species (ROS), such as atomic oxygen O, singlet oxygen $^1O_2$, superoxide anion O^-_2 and ozone O_3; reactive nitrogen species (RNS), such as atomic nitrogen N, excited nitrogen $N_2(A)$, nitric oxide NO•; if humidity is present H_2O^+, OH^- anion, OH• radical or H_2O_2 are also generated (Scholtz *et al*. 2015). The exact mechanisms of CP mediated bacterial inactivation are still under investigation, but several generated products have been demonstrated to play a role. These products include ROS, RNS, UV radiation and charged particles within a plasma gas phase. Among the ROS, ozone, atomic oxygen, singlet oxygen, superoxide, peroxide, and hydroxyl

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

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

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

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

Cold plasma for food safety applications

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

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

Inactivation of food borne pathogenic microorganisms

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

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

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

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

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

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

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

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

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

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

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

et al. 2014). A submerged or underwater DBD plasma reactor has been used to inactivate biofilms of key food-borne pathogens, such as *E. coli*, *Cronobacter sakazakii* and *Staphylococcus aureus* generated on stainless steel surface with reductions by 5.50, 6.88 and 4.20 log CFU/coupon, respectively, obtained after 90 min of treatment. The ATR-FTIR measurement showed gradual reduction of carbohydrates, proteins, and lipid and DNA peak regions with increased plasma exposure time (Khan *et al.* 2016). Furthermore, Gabriel *et al*. (2016) addressed the influence of different surface features on the bacterial attachment and therefore biofilm formation and susceptibility to treatment. *Pseudomonas aeruginosa* biofilms were developed on different types of stainless steel, such as 316 and 304 with different finishes namely, mirror, hairline and 2B surfaces. Variations in D-values were observed between surface finishes within a specific stainless steel type. However, significant variations were not observed between the same surface finish of different steel types. A 5-log reduction in the population was observed in a relatively short treatment times of ~ 90 s (Gabriel *et al*. 2016). Although with different range of processing times required to achieve significant inactivation of biofilms of foodborne pathogens, these studies demonstrated that CP could be an alternative technology for effective decontamination of materials within food processing environment.

The major mechanisms for CP mediated biofilm inactivation reported to date are illustrated in Figure 5 and include alterations in cell membrane integrity, destruction of EPS, cells and cellular components, reduction of biofilm thickness, reduced culturability and metabolic activity of cells. Air DBD ACP treatment for 5 min significantly altered biofilm structures of *E. coli* and *P. aeruginosa* formed on polycarbonate membranes, changing the healthy cells interconnected by self-produced EPS matrices to irregularly shaped cell fragments. This corresponded to >5 log reductions in biofilms developed in 96 well plate model (Ziuzina *et al.* 2014, 2015). Alkawareek *et al.* (2012) demonstrated marked susceptibility of *P. aeruginosa* 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 µm 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 25.5µm-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 µm (Ziuzina
356 *et al.* 2014). Similarly, *Candida albicans* biofilm, with a thickness of 10 to 20 mm, 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 mJ/cm^2 ultraviolet-C, and 750 mJ/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.

Clinical applications of cold plasma

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

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

Control of quorum sensing-mediated virulence

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

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

Cold plasma and sporicidal activity

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

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

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

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

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

Virucidal activity of cold plasma

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

bacteriophages, often employed as a facile surrogate model for evaluating the virucidal activity of chemical disinfectants against human, animal and plant pathogenic viruses (Alshraiedeh *et al.* 2013). Venezia and colleagues described the antimicrobial activity of a commercially available system (PlasmaSol apparatus) against a range of bacteria, spores and viruses. The authors report 4-6 log10 reductions in PFU ml^{-1} of temperate and lytic bacteriophages after 10 minutes exposure (Venezia *et al.* 2008). Interestingly, a separate study examining the virucidal activity of a novel dielectric barrier discharge reactor, Yasuda and co-workers reported rapid inactivation of lambda phage infectivity by up to 6 log10 reductions after only 20 seconds (Yasuda *et al.* 2010). The *E. coli* MS2 bacteriophage has been validated as a convenient, representative surrogate for human norovirus in establishing the virucidal activity of biocides in chemical disinfectant efficacy tests (Maillard *et al.* 1994; Pinto *et al.* 2010). Alshraiedeh and colleagues reported the virucidal efficacy of a handheld, helium/oxygen, KHz driven atmospheric pressure non thermal plasma jet (described in Alkawareek *et al.* 2012) against MS2 bacteriophage. MS2 bacteriophage was rapidly inactivated, with inactivation rate constant increasing with increasing oxygen percentages in the feed gas, up to 0.75%. Up to 3 log_{10} reductions in PFU ml^{-1} were recorded after 3 seconds, with > 7 log_{10} reductions in PFU ml^{-1} after 9 minutes exposure (Alshraiedeh *et al.* 2013). A cold oxygen plasma, described by Terrier *et al.* (2009) was also shown to efficiently inactivate nebulized respiratory viruses human parainfluenza virus 3 (hPIV-3), respiratory syncytial virus (RSV) and influenza virus A (H5N2), reducing the titre of each by up to 6.5, 3.8 and 4 log10 TCID50 ml-1, respectively, within the allocated treatment time. The ability of CP to inactivate norovirus (foodborne outbreak strain) in faecal samples has recently been demonstrated (Ahlfeld *et al.* 2015). Such field testing of virucidal activity of CP in clinical samples supports the potential application of CP systems to efficiently disinfect virally contaminated surfaces and fomites, reducing the potential risk of onward transmission of infectious agents.

Anti-protozoal activity of cold plasma

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

Concluding comments and future directions

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

Acknowledgements: Science Foundation Ireland Grant No 14/IA/2626

Conflict of interest: There is no conflict of interest to declare

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List of figure legends

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

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

Figure 3: Microbial challenges associated with disinfection

Figure 4: Parameters influencing plasma treatment decontamination efficacy

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