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Atmospheric Cold Plasma Interactions with Modified Atmosphere Packaging Inducer Gases for Safe Food Preservation.

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**Atmospheric Cold Plasma Interactions with Modified Atmosphere Packaging Inducer
Gases for Safe Food Preservation.**

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Highlights:

- Atmospheric cold plasma is effective for inactivation of food microbial challenges.
- Both process and target parameters govern inactivation efficacy.
- Oxygen composition in the applied working gas governs inactivation efficacy.
- Nitrogen composition in the applied working gas has synergistic effect with oxygen.

Abstract

Diverse microbiological challenges and pervasive microbial resistance drive technological development in food processing, where increasing process complexity and consumer demand for less processed goods leads to strong demand for effective decontamination. Atmospheric cold plasma (ACP) has wide potential for decontamination application in the food sector. We investigated the effect of Modified Atmosphere Packaging (MAP) gas mixtures on reactive species generated, their efficacy and mechanism of inactivation against *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*. Oxygen levels in the applied working gas had positive interactive effects on ROS generation, in-package inactivation efficacy in conjunction with post-treatment storage time. *Listeria* populations were undetectable after 15s treatment with high Oxygen MAP mix using 24 h post-treatment storage time. However, RNS generation and effect was dependent on the nitrogen content but also on the presence of oxygen. Different modes of interaction of ROS and RNS with Gram positive and Gram negative bacteria were observed.

1. Introduction

Atmospheric cold plasma (ACP) refers to non-equilibrium plasmas generated at near ambient temperatures and pressure. They are composed of particles including free electrons, radicals, positive and negative ions, but are low in collision frequency of gas discharging compared to equilibrium plasma (N. N. Misra, Tiwari, Raghavarao, & Cullen, 2011). ACP technologies are widely studied for sterilization against food and clinical pathogens (Cheng, Sherman, Murphy, Ratovitski, Canady, & Keidar, 2014; Kvam, Davis, Mondello, & Garner, 2012; N. N. Misra, et al., 2011; Sakudo, Misawa, Shimizu, & Imanishi, 2014; Ziuzina, Han, Cullen, & Bourke, 2015). As a non-thermal technology, ACP can be adapted for microbial decontamination and shelf life extension of heat sensitive fresh food products, with additional benefits of low cost, reduced water usage and reduced energy demand (N. Misra, Han, Tiwari, Bourke, & Cullen, 2014).

ACP provides challenging effects against a wide range of microbes through the generation of cell-lethal reactive species (Kvam, et al., 2012; Shintani, Sakudo, Burke, & McDonnell, 2010). Varying process parameters of ACP treatment, including treatment time, post-treatment storage time, applied voltage, applied gas composition and humidity etc., can generate different reactive species profiles. Reactive oxygen and nitrogen species (RONS) are the prominent reactive species in oxygen and nitrogen containing gases (Cheng, et al., 2014; Han, Patil, Keener, Cullen, & Bourke, 2014; Joshi, et al., 2011; Shintani, et al., 2010). ROS play a crucial role in microbicidal actions, with strong oxidative damage on cell envelope, DNA, protein and other essential cell components (Joshi, et al., 2011; Kvam, et al., 2012; Laroussi & Leipold, 2004). Using air ACP; O₃, O atom, singlet oxygen and nitric oxides are reported as the main reactive species, while H₂O₂, OH radicals and HNO_x (x=1,4) are also generated with humid applied gases (Moiseev, et al., 2014; Takamatsu, Kawate, Oshita, Miyahara, Okino, & Fridman, 2013). During ACP discharge, RNS have synergistic

inactivation effects with ROS (Boxhammer, et al., 2012). However, RNS can be rapidly generated endogenously from the reaction of nitric oxide and superoxide during cell metabolism activities and damage proteins, lipids and DNA (Shigenaga, et al. 1997).

Modified atmosphere packaging (MAP) is widely used in the food industry to avoid contamination and weight loss and extend fresh food shelf-life (Kerry, O'grady, & Hogan, 2006; Sivertsvik, Rosnes, & Bergslien, 2002). Nitrogen is the most widely used gas in MAP, as an inert filler gas either to reduce the proportions of the other gases or to maintain pack shape (Kerry, et al., 2006). High levels of oxygen (70–80%) have also been used in MAP to reduce microbial growth in package. Additionally, it is helpful in preserving the bright red colour of fresh meat and maintaining the tenderness and juiciness of meat (Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007; Okayama, Muguruma, Murakami, & Yamada, 1995). The carbon dioxide component is popular in the meat packaging industry for preservation by inhibiting bacterial growth (Sivertsvik, et al., 2002), and maintaining the red colour of meat products. However, because of the high dissociation energy, inert gas CO₂ has low reactive species generation in conjunction with ACP treatment and it represented a negative control in this study (Fridman, 2008). Typically, fresh red meat packaging uses 70% O₂+30% CO₂ for MAP (Sørheim, Nissen, & Nesbakken, 1999) and cooked meats are stored in 70% N₂+30% CO₂ (Smiddy, Papkovsky, & Kerry, 2002). At the same time, low oxygen/high nitrogen (10% O₂+90% N₂) MAP was used for fruit and vegetables, in order to inhibiting respirations and undesirable colour changes (Day, 2000). The in-package treatment design employed in this study has proven efficacy for decontamination of tomatoes, strawberries and fresh meat slices (Han, et al., 2016; N. Misra, Patil, et al., 2014; Ziuzina, Patil, Cullen, Keener, & Bourke, 2014), demonstrating potential for adoption for a range other fresh foods where safe shelf-life extension is required to meet emerging sustainability and innovation demands. In line with other novel technologies, it is important to evaluate on a product basis

as well as understanding the fundamental interactions with existing processes such as MAP working gases. Previous studies have established connections between microbicidal actions with process and system parameters and the reactive species generated (Cheng, et al., 2014; Han, et al., 2014). Besides gas composition, post treatment storage time is a critical parameter influencing ACP inactivation efficacy. During post-treatment storage of samples treated in-package, recombined or longer lived species may contribute to further inactivation (Han, et al., 2014; Ziuzina, Patil, Cullen, Keener, & Bourke, 2013). Moreover, the interaction of microbes with reactive species could be attributed to their structural difference leading to different damaging patterns (Han, et al., 2014; Ziuzina, et al., 2015).

Applications of ACP are under investigation for microbiological and biological control across food, water and environmental sectors, where ACP effects involve both liquid and gas interactions. Understanding the gas liquid interactions of ACP is critical to advance understanding and successful applications in the food sector. Hence, this study investigated the effects of ACP reactive species generated in both gas and liquid phase, as a function of different MAP inducer gas compositions compatible with fresh food processing, treatment time and post-treatment storage times using high voltage ACP. Bacterial interactions were elucidated by comparing the inactivation mechanism of ACP against Gram negative and positive bacteria using intracellular, extracellular and plasma diagnostic tools.

2. Materials and methods

2.1. Bacterial Strains and Growth Conditions

The bacterial strains used were *Escherichia coli* NCTC 12900, *Listeria monocytogenes* NCTC 11994 and *Staphylococcus aureus* ATCC 25923; selected to represent both Gram positive and Gram negative bacteria and to facilitate comparison with other studies.

2.2. Preparation of Bacterial Cell Suspensions

Cells were grown overnight (18 h) by inoculating isolated single colony of respective bacteria in tryptic soy broth without glucose (TSB-G, Scharlau Chemie, Barcelona, Spain), at 37 °C. Cells suspensions of 10^8 CFU ml⁻¹ were prepared in PBS as described in Han, et al. (2014), and 3 ml of bacterial suspensions in PBS were transferred to a sterile 6-well plate prior to ACP treatment.

2.3.ACP system configuration and treatment

The dielectric barrier discharge (DBD) ACP system used in this study is described in Pankaj, Misra, and Cullen (2013) and was operated at high voltage of 70 kV_{RMS} under atmospheric pressure.

Samples in 6-well plates were sealed using a tray sealer with vacuum option and modified atmospheric packaging (MAP, Lavezzini VG600, UK). A tailor-made stainless steel tray mould was manufactured at Holfield Plastics Ltd (Arklow, Ireland) to adapt the packaging machine to our selected trays size (196*154 mm). Two trays were loaded at a time and the packaging conditions were optimised according to the packaging materials selected (30 s 99% vacuum phase; 30 s gas flushing phase; 2.5 s at 115 °C sealing) with

Polyester/BLL/LDPE +anti-fog coating film (thickness 0.06 mm, STEPHENS, Ireland).

Besides air (Gas 3), three MAP gas mixtures were used, 70% N₂+ 30% CO₂ (Gas 1), 90% N₂+ 10% O₂ (Gas 2) and 70% O₂+ 30% CO₂ (Gas 4). The sealed tray acting as a sample holder and another dielectric barrier was placed between two perspex dielectric layers. The distance between the two electrodes was kept constant at 5 cm. Bacterial samples were treated with ACP at 70 kV_{RMS} for 15, 60 and 300 s and subsequently stored at room temperature for 0, 1 or 24 h. All experiments were performed in duplicate and replicated twice.

2.4.Microbiological Analysis

For microbial quantification 1 ml of treated samples was serially diluted in minimum recovery diluent (MRD, Scharlau Chemie, Barcelona, Spain) and 0.1 ml of appropriate dilutions were surface plated on TSA. In order to obtain low microbial detection limits, 1 ml of the treated sample was spread onto TSA plates as described by ISO (1996), incubated at 37 °C for 24 h and counted. Plates with no growth were incubated for up to 72 h and checked for the presence of colonies every 24 h. Results are reported in Log₁₀ CFU ml⁻¹ units.

2.5. Optical emission spectroscopy and ozone measurements

Optical emission spectroscopy (OES) of the discharge within empty tray packages was acquired with an Edmund Optics UV Enhanced Smart CCD Spectrometer with an optical fibre input, optimized for maximum performance in the ultraviolet and visible with a wavelength range of 200-920 nm, and for multichannel operation with ultra-low trigger delay, a gate jitter and spectral resolution of 0.6 nm. The fibre optic was placed directly along a 5mm perforated column in the package side to allow light to cross the centre from the side wall of the polypropylene container. The 5 mm diameter lens collected light from this column across the diameter of the package and focused onto a 200 µm multi-mode fibre. Applied gas mixtures flowed through the container and out from the 5mm lens window during ACP discharging. Data was collected every 20 s for up to 5 min with duplicate analyses performed. In package concentrations of ozone were measured using GASTEC tube detectors (Product # 18M, Gastec Corporation, Kanagawa, Japan) immediately after treatment.

2.6. Detection of reactive oxygen species after ACP treatment

Immediately after ACP treatment, cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate DCFH-DA (Sigma-Aldrich, USA) at a final concentration of 5 µM in PBS for 15 min at 37 °C. Samples of 200 µl were transferred into 96-well fluorescence microplates (Fisher Scientific, UK) and measured by SynergyTM HT Multi-Mode Microplate Reader

(BioTek Instruments Inc.) at excitation and emission wave lengths of 485 and 528 nm. (Joshi, et al., 2011)

To evaluate different types of reactive oxygen species two further probes were used concurrently. The singlet oxygen sensor green reagent SOSGR (Molecular Probes, Life technologies, USA) was first prepared in methanol as 1 mM stock solution. Cell suspensions were incubated with SOSGR at a final concentration of 2 μ M for 15 min at 37 °C immediately after ACP treatment. Samples of 200 μ l were transferred into 96-well fluorescence microplate wells and measured at excitation and emission wave lengths of 485 and 528 nm. (Joshi, et al., 2011)

The presence of hydrogen peroxide was tested with commercial Amplex Red assay kit (Molecular Probes, Life technologies, USA). The assay was performed according to the manufacturer's protocol with control and treated samples immediately after ACP treatment. Fluorescence results were obtained from 100 μ l sample in 96-well fluorescence microplate at excitation and emission wave lengths of 530 and 590 nm. A standard curve was displayed with serial H₂O₂ solutions, where fluorescence signals were converted to hydrogen peroxide concentration of μ M. (Boxhammer, et al., 2012; Joshi, et al., 2011)

2.7.Detection of reactive nitrogen species after ACP treatment

DAF-FM DA, 4-amino-5-methylamino- 2',7'- difluorofluorescein diacetate, is a cell-permeable fluorescent probe for the detection of nitric oxide (NO) which rapidly transforms into water-soluble DAF-FM by cytosolic esterases. However, it is reported to react with ROS at certain levels (Gomes, Fernandes, & Lima, 2005).

Cells were incubated with DAF-FM DA (Sigma-Aldrich, USA) immediately after ACP treatment at a final concentration of 1 μ M in PBS for 15 min at 37 °C. Samples were transferred into 96-well fluorescence microplate wells and measured at excitation and emission wave lengths of 485 and 528 nm.

Since NO and other reactive nitrogen species have a very short life, nitrates and nitrites are measured as evidence of emerging RNS in liquids. A Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, USA) was used for this study. The assay was performed according to the manufacturer's protocol with control and treated samples for all storage times. Absorbance was measured at a wave length of 550 nm. A standard curve was used to convert absorbance results to concentration of μM . (Boxhammer, et al., 2012)

2.8.Statistical Analysis

Statistical analysis was performed using SPSS 22.0 (SPSS Inc., Chicago, U.S.A). Data represent the means of experiments performed in duplicate and replicated at least twice. Means were compared using analysis of variance (ANOVA) using Fisher's Least Significant Difference-LSD at the 0.05 level.

3. Results and discussion

3.1.Inactivation efficacy associated with process and system parameters

Inactivation levels achieved in response to treatment time, post-treatment storage time and applied gas mixtures are shown in Tables 1-3. Exposure to ACP for 15 or 60 s had little effect on *E. coli* using Gas 1, with the 1 log cycle reduction achieved using 15s treatment plus 24 h post treatment storage time representing the highest inactivation with this gas. For Gases 2 and 3, extending treatment time to 300 s plus 24 h post treatment storage time had good inactivation effects, where much lower survival levels were observed at 2.31 ± 0.73 and $4.23 \pm 0.13 \text{ Log}_{10} \text{ CFU ml}^{-1}$ respectively. However, using Gas 4, populations were undetectable after 300 s treatment (Table 1) plus post-treatment storage time (1 or 24 h) ($p < 0.05$).

Similar results were obtained from the inactivation of *S. aureus* (Table 2), with greater effect of post-treatment storage time on inactivation noted. Increasing oxygen content correlated

with reductions ($p < 0.05$), where 300 s treatment plus 24 h post-treatment storage reduced populations to 6.60 ± 0.25 , 4.72 ± 0.73 , 3.54 ± 0.51 Log₁₀ CFU ml⁻¹ or undetectable using the respective gas types 1-4.

Table 3 shows inactivation of *L. monocytogenes*, where much lower survival levels were observed by comparison with *S. aureus*, another Gram + bacteria. Extending treatment time from 15 or 60 s to 300 s enhanced inactivation, where *L. monocytogenes* was not detected using Gas 4 immediately after 300 s treatment, even without post-treatment storage time. By extending post-treatment storage to 24 h, no culturable cells were detected from this high oxygen mix even with only 15 s treatment. While Gases 2 and 3 also achieved inactivation below the detection limit with the longest treatment time of 300 s plus 24 h post-treatment storage, Gas 1 had 6.10 ± 0.13 Log₁₀ CFU ml⁻¹ surviving cells. (Table 3)

The interactive effects of treatment time, post-treatment storage time and gas composition on ACP inactivation efficacy were well elucidated. With all target bacteria, inactivation efficacy positively correlated with both treatment time and oxygen content of MAP gases; while it can be further enhanced with post-treatment storage time (Tables 1-3). Similarly, longer treatment times and higher oxygen content lead to more ROS generation (Cheng, et al., 2014; Han, et al., 2014). The underlying mechanism was further observed as the generation of reactive species in relation to the applied gas. Extended in-package post-treatment storage provided a number of benefits namely the containment and retention of the active component in situ with microbiological target as well as the previously reported reaction time allowing longer lived species act with in-package targets (Han, Patil, Boehm, Milosavljević, Cullen, & Bourke, 2015; Ziuzina, et al., 2014). These benefits could be replicated during standard storage stages or transport or at any stage prior to consumption dependent on the product characteristic demands.

3.2. Reactive species generation in gas phase

239 In-package ozone evolution is shown in Table 4. For Gas 1, ozone measurements were all
240 under the detection limit. No ozone was detected in any treatment condition after the 24 h
241 post-treatment storage time. Ozone concentrations correlated with the increasing inactivation
242 in association with extending the post-treatment storage time, while the exhaustion time of in-
243 package ozone is longer than 1 h.

244 Spectroscopic analysis of the discharge was carried out in 4 gases at 70 kV_{RMS} and over the
245 spectral range of 200–920 nm (Figure 1). Distinct peaks were obtained in the near UV region
246 corresponding to strong spectral emissions from N₂ and N₂⁺ excited molecules. Figure 1 shows
247 the emission intensities during discharge in different gas mixtures. The emission of OH
248 radicals at 309.65 nm (Figure 1 a) from Gas 3 had higher intensities than other gases after 60s
249 discharging, while Gases 1 and 3 had similar intensities during 300 s treatment, which were
250 slightly higher than those from Gas 4. Gas 3 was compressed atmospheric air with high
251 humidity, while Gases 1, 2 and 4 were obtained from dried gas cylinders. Moiseev, et al.
252 (2014) indicated that the humidity inside a package had a quenching effect on ozone
253 generation, resulting in a higher hydroxyl radical density, with a similar effect observed from
254 Figure 1 a, where air showed a higher emission intensity of OH radical during discharging
255 than the other gases. The emission of nitrogen species (the second positive) at 336.65 nm
256 (Figure 1 b) was influenced by the percentage in the gas mixtures. Gas 3 showed higher
257 emission than Gas 1, although they had similar nitrogen percentage. Other peaks of nitrogen
258 species repeated similar patterns (data not shown). Although Gases 1 and 3 had a similar
259 percentage of nitrogen, Gas 3 had higher emission intensities at 336.65 nm and higher
260 detected RNS densities. N₂ molecules have been reported to be resistant to ionization, with
261 high dissociation energy (Fridman, 2008; Shintani, et al., 2010). In our study, the ionisation
262 of N₂ was much higher when mixed with O₂ than with CO₂, while the dissociation energies
263 are similar (Shintani, et al., 2010).

3.3.Reactive species generation in liquid phase

3.3.1. Reactive oxygen species

Figures 2-5 show analysis of reactive species in uninoculated PBS or bacterial cell suspensions immediately post ACP treatment. General ROS densities are represented in Figure 2 as effected by treatment time and gas composition ($p<0.05$). Both of these parameters had a positive effect on total ROS in PBS samples as represented in Figure 2 a, while similar trends were also noticed from cell suspensions in Figure 2 b, c and d. Fluorescence intensities increased in association with the oxygen concentration in applied gas mixtures, where Gas 4 had the highest readings ($p\leq 0.05$). In PBS solution, DCFH DA could be altered by reactive species and transformed to DCFH, which can be further oxidized due to the higher availability of ROS and detected as fluorescence signal. Therefore, fluorescence signal was also detected from PBS solution. The results represent total ROS detected post-discharge, while the levels detected from bacteria suspensions were significantly reduced by their interactions with cell components. At the same time, ROS detected following ACP discharge in Gas 4 were much higher from *S. aureus* and *L. monocytogenes* than *E. coli* (Figure 2, $p<0.05$), although similar inactivation levels in all applied gases were achieved, ref. Tables 1-3. This effect has been attributed to the detected ROS densities and cell damage post ACP exposure (Gaunt, Beggs, & Georghiou, 2006; Han, et al., 2014; Ziuzina, et al., 2015). It indicates the different mechanism of action with Gram positive and negative bacteria, due to the different chemical structure of their cell wall (Laroussi, Mendis, & Rosenberg, 2003; Shintani, 2015). The mechanism of action of reactive species was determined by their targets, which is the cell envelope for Gram negative and intracellular components for Gram positive microbes (Han, et al., 2014). This could also correlate to the generation of H_2O_2 as secondary product in Figure 4. *L. monocytogenes* was the most sensitive target bacteria in our study, where no detectable survival was observed following

exposure to ACP discharge in Gases 2, 3 or 4 with extended treatment time and post-treatment storage time (Tables 1-3). Similar reactive species patterns were observed from *S. aureus* and *L. monocytogenes*, which indicated similar mode of action regardless of sensitivity (Figures 2, 3 and 4). However, membrane damage and cell leakage were observed in both Gram negative and positive bacteria with an exposure time dependent manner by Kvam, et al. (2012), who employed different settings with low voltage but higher frequency. Moreover, PBS has much higher ROS signal than all bacterial samples. This could be attributed to the lack of organic target inside package, slowing down the transformation of short lived species to longer lived species, e.g. H_2O_2 . Generation of singlet oxygen repeated the correlation with oxygen content, which was also observed in PBS and *E. coli* suspensions, but not with the Gram positive bacterial solutions for H_2O_2 (Figures 3 and 4). The effect of treatment time was observed for singlet oxygen in PBS only, but in both PBS and bacterial samples for hydrogen peroxide. In contrast to total ROS results, H_2O_2 concentrations in *E. coli* suspensions were higher than those from PBS samples (Figure 4, $p < 0.05$), while Gram positive samples had significantly lower H_2O_2 concentrations (Figure 4, $p > 0.05$). The cell envelope of *E. coli* consists of lipopolysaccharides, which are easily oxidized and reduce the strong oxidative species to less active species by reaction with O-H bond (such as lipid A) (Arjunan, Sharma, & Ptasinska, 2015; Laroussi, et al., 2003; Yusupov, Bogaerts, Huygh, Snoeckx, van Duin, & Neyts, 2013). These reactions might lead to cell membrane lesion and production of more peroxides, a possible reason for higher signals from *E. coli* samples than PBS. Our previous study showed obvious cell leakage after ACP treatment of Gram negative, but not Gram positive samples (Han, et al., 2014). In addition, H_2O_2 could be consumed in Gram positive bacteria with the presence of cellular catalase (Afanas'ev, 1991).

313 Generation of H_2O_2 in PBS also increased with oxygen content of gas mixtures, but air
314 samples were only slightly lower than those from Gas 4 ($p < 0.05$). The decelerated increase of
315 H_2O_2 concentration might be associated with the generation of other ROS, such as hydroxyl
316 radicals. They are short-lived with high reactivity, cause breakage of DNA strands (Shintani,
317 2015; Shintani, et al., 2010), and might lead to the generation of H_2O_2 which is more stable.
318 However, it has also been reported that H_2O_2 can generate hydroxyl radicals through
319 intracellular redox, such as Fenton reaction (Graves, 2012; Kellogg & Fridovich, 1975), but
320 that this was not reported in reverse. Moreover, DCFH DA was found inapplicable to H_2O_2
321 detection without the presence of sufficient oxidases or peroxidases, which indicates the
322 H_2O_2 concentrations were not included in the fluorescence signal of general ROS (Gomes, et
323 al., 2005). Lower fluorescence signal was obtained in H_2O_2 solutions (up to 30%) with or
324 without cells than ACP treated samples (data not shown). Therefore, H_2O_2 concentrations
325 detected in this study may not be represented in general ROS results, which could explain
326 their different patterns and the conversion between short-lived and long-lived ROS species.
327 In contrast to general ROS and H_2O_2 , similar singlet oxygen signals were found from Gram
328 negative and positive bacteria (Figure 3). Singlet oxygen is a highly reactive species targeting
329 various biological molecules, with strong penetrating effect and relatively short life time
330 (Joshi, et al., 2011; Sousa, Niemi, Cox, Algwari, Gans, & O'Connell, 2011). Singlet oxygen
331 and other ROS (superoxide, hydroxyl radical, H_2O_2 etc.) are cytotoxic species, which could
332 cause damage to bacterial cells through several mechanisms (Beckman, Beckman, Chen,
333 Marshall, & Freeman, 1990; Graves, 2012). These include oxidation of membrane lipids and
334 amino acids in proteins, cross-linking of proteins and oxidation of nucleic acid. The presence
335 of H_2O_2 and nitric oxide could lead to the generation of singlet oxygen and hydroxyl radical
336 and result in more cell damage (Beckman, et al., 1990; Noronha-Dutra, Epperlein, & Woolf,
337 1993). However, the intracellular signal was not achievable with our assay.

3.3.2. Reactive nitrogen species

By comparison with the strong sterilization effect of ROS, the cellular damage with RNS was reported in conjunction with ROS or by inducing oxidative stress (Boxhammer, et al., 2012; Sakudo, et al., 2014). Reactive nitrogen species include nitric oxide (NO), nitrogen dioxide (NO₂), dinitrogen trioxide (N₂O₃), peroxyxynitrite (ONOO⁻), peroxyxynitrous acid (OONOH), and alkylperoxyxynitrite (ROONO) (Arjunan, et al., 2015; Graves, 2012). The main RNS for bactericidal action are NO and NO₂ and their final format after reaction are nitrite and nitrate. RNS detection showed the same trend for both Gram positive and negative strains (Figures 5 and 6).

The highest RNS fluorescence results (Figure 5) were observed from bacterial samples in Gas 2 ($p < 0.05$), which had a higher N₂ percentage (90%) than other applied gases. However, results from PBS samples differed, where those treated in Gas 3 had highest signals ($p < 0.05$), followed by Gas 2 and Gas 1.

Similar to DCFH DA, the diacetate group of DAF-FM DA can be removed by redox reaction before reacting with RNS, especially in PBS. In bacterial suspensions, the amount of free ROS was decreased with the presence of bacterial cells. Therefore, RNS could be clearly detected from cell suspensions. Moreover, ROS have not only been reported to influence the fluorescence of DAF-FM (Balcerczyk, Soszynski, & Bartosz, 2005), but also have reversible or irreversible conversion with RNS (Balcerczyk, et al., 2005; Laroussi, et al., 2004). This could explain the higher detected RNS levels for PBS samples in Gas 3 than Gas 2 (Figure 5 a, $p < 0.05$). Although the package was evacuated prior to refilling with working gases, there was still up to 1% atmospheric air remaining, resulting in slight N₂ content in Gas 4 packages, increasing the RNS signal from treated samples over control in Gas 4 (Figures 5 and 6).

Nitrite and nitrate concentrations in different gas mixtures are presented in Figure 6 for PBS samples only, as the results from PBS and bacterial samples did not show significant differences (data not shown). Similar levels of nitrite and total nitrite/nitrate were detected in MP1 with all post-treatment storage times ($p>0.05$), while obvious different results were obtained from other gas mixtures ($p\leq 0.05$). By extending post-treatment storage time from 0 h to 24 h, a minimal effect on nitrite concentration was observed in Gas 1 for 5 min treated samples (slightly increased from $12.16\pm 0.18\text{ }\mu\text{M}$ to $15.49\pm 0.03\text{ }\mu\text{M}$ with 300 s treatment, $p<0.05$) in Figure 6 a and e, while treatment time had a positive effect ($p<0.05$) shown in Figure 6 a, c and e. Nitrite concentration in Gas 2, 3 and 4 remained very low, indicating that the RNS detected in liquid are dominantly nitrate in these three gases (Figure 6 b, d, f). The presence of oxygen content, even as low as 10%, can oxidize nitrite to nitrate in ACP treatment.

Nitrate concentrations in Gases 2 and 3 increased with extending post-treatment storage time to 24 h ($p<0.05$), from similar levels immediately after 300 s treatment to much higher levels of $28.43\pm 0.45\text{ }\mu\text{M}$ and $22.86\pm 0.63\text{ }\mu\text{M}$ respectively (Figure 6 b, f). Very low nitrite/nitrate concentrations ($<1\text{ }\mu\text{M}$) were found in all treatment conditions in Gas 4, while $2.97\pm 0.13\text{ }\mu\text{M}$ was obtained after treated for 300 s and 24 h storage (Figure 6 f).

Whilst similar nitrite/nitrate levels were detected immediately after ACP exposure, much higher concentrations were observed from Gas 2 and 3 samples than Gas 1 after 24 h post-treatment storage. This confirmed the enhanced N_2 dissociation and generation of RNS in the presence of oxygen in applied gases, indicating RNS action as a long term reaction ($>1\text{ h}$). RNS are short-lived species, but the half-life can be prolonged by converting to peroxynitrite, reacting widely with biomolecules (Graves, 2012; Sakudo, et al., 2014). Reactive species in this study were recorded both immediately after ACP treatment and after 1 or 24 h storage (Table 4, Figure 1-6). After 1 h storage time, inactivation levels were improved by

comparison with unstored samples (Tables 1, 2 and 3, $p \leq 0.05$). During storage, effects on bacterial cells following treatment could be attributed to long-lived reactive species, which can result from conversion of short-lived species. With in-package processing, a retained effect could lead to higher sterilization efficacy with reduced energy consumption. However, levels after 24 h post-treatment storage were slightly higher than 1 h stored samples in some cases, indicating the potential for long-term repair and recovery capacity in microbes with sub-lethal damage (Han, et al., 2014; Mai-Prochnow, Murphy, McLean, Kong, & Ostrikov, 2014). The effect of post-treatment storage on ROS and RNS indicated that some reactive species had longer life time than 1 h, where their reaction with biological molecules continues after ACP treatment (Table 4, Figure 6). The effect of oxygen composition was further emphasised with extended post-treatment storage, which indicated the time ROS required for bactericidal action (>1 h).

4. Conclusion

Overall, the microbicidal effect of ACP is dependent on the oxygen content of applied gases interacting with treatment time and post-treatment storage time. While the capacity for ROS generation was mainly dependent on the oxygen content in applied gases, RNS formation was governed by both nitrogen content and presence of oxygen. Reactive species densities were different in relation to their anti-bacterial mode of actions and cell type. Most ROS react with both cell envelope and intracellular components, such as DNA. Their detected densities were dependent on the cell envelope structures, where the cell structure of Gram negative bacteria was more easily disrupted than Gram positive bacteria. This could have led to the formation of peroxides, which are more stable ROS. However, singlet oxygen and RNS are relatively short lived and highly convertible to other forms. Both ROS and RNS showed treatment time dependence, which could improve the microbicidal effect. Furthermore, reaction between reactive species and bacteria could be enhanced by extending post-treatment storage time.

This understanding of the roles and impact of reactive species generated with in-package high voltage ACP on microbiological targets can advance applications to foods, particularly where the microbiological target can be estimated, the gaseous environment controlled and the food matrix suitability for processing using High voltage ACP selected.

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Conflicts of Interest

There are no conflicts of interest.

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Tables

Table 1. *E. coli* NCTC 12900 inactivation efficacy with treatment and post-treatment storage time in different gases

Gases	Plasma treatment time (s)	Post-treatment storage time					
		0 h		1 h		24 h	
		Cell density (Log ₁₀ CFU/ml)	SD*	Cell density (Log ₁₀ CFU/ml)	SD*	Cell density (Log ₁₀ CFU/ml)	SD*
1	C	8.00 ^a	0.05	7.92 ^a	0.04	7.92 ^a	0.04
	15	7.47 ^b	0.11	7.45 ^b	0.03	6.79 ^b	0.18
	60	7.21 ^{bc}	0.36	7.43 ^b	0.14	7.00 ^b	0.06
	300	7.04 ^c	0.09	6.65 ^e	0.14	6.95 ^b	0.10
2	C	7.99 ^a	0.15	7.92 ^a	0.04	7.92 ^a	0.04
	15	7.80 ^{ad}	0.04	7.41 ^b	0.03	7.07 ^{bd}	0.08
	60	7.78 ^{ad}	0.15	7.11 ^d	0.02	6.70 ^c	0.08
	300	7.61 ^d	0.13	6.82 ^f	0.01	2.31 ^e	0.73
3	C	7.99 ^a	0.15	7.92 ^a	0.04	7.92 ^a	0.04
	15	8.00 ^a	0.05	7.61 ^c	0.06	7.15 ^d	0.18
	60	7.85 ^{ad}	0.04	7.08 ^d	0.05	6.90 ^{bc}	0.06
	300	7.66 ^d	0.11	6.26 ^g	0.06	4.23 ^f	0.13
4	C	7.91 ^a	0.02	7.92 ^a	0.04	7.92 ^a	0.04
	15	7.99 ^a	0.08	7.11 ^d	0.10	6.34 ^g	0.15
	60	7.95 ^a	0.07	5.17 ^h	0.48	4.20 ^h	0.16
	300	6.60 ^e	0.19	ND* ⁱ	-	ND* ⁱ	-

Different letters indicate a significant difference at the 0.05 level between different treatment times and gas mixtures

SD* : Standard deviation

ND* : Under detection limit, complete inactivation

Gas 1: 70% N₂+ 30% CO₂; Gas 2: 90% N₂+ 10% O₂; Gas 3: Air; Gas 4: 70% O₂+ 30% CO₂.

548 Table 2. *S. aureus* ATCC 25923 inactivation efficacy with treatment and post-treatment
549 storage time in different gases

Gases	Plasma treatment time (s)	Post-treatment storage time					
		0 h		1 h		24 h	
		Cell density (Log ₁₀ CFU/ml)	SD*	Cell density (Log ₁₀ CFU/ml)	SD*	Cell density (Log ₁₀ CFU/ml)	SD*
1	C	8.19 ^a	0.03	8.18 ^a	0.06	8.18 ^a	0.06
	15	7.52 ^b	0.11	7.09 ^b	0.05	6.73 ^b	0.17
	60	7.27 ^c	0.09	5.99 ^c	0.07	6.78 ^b	0.10
	300	7.37 ^{bc}	0.08	5.76 ^d	0.16	6.60 ^b	0.25
2	C	8.16 ^a	0.07	8.18 ^a	0.06	8.18 ^a	0.06
	15	7.19 ^{cd}	0.11	7.20 ^b	0.07	6.81 ^b	0.19
	60	7.37 ^c	0.08	6.93 ^b	0.25	6.44 ^c	0.08
	300	7.00 ^d	0.13	6.07 ^e	0.20	4.72 ^d	0.73
3	C	8.16 ^a	0.07	8.18 ^a	0.06	8.18 ^a	0.06
	15	7.87 ^f	0.10	7.14 ^b	0.11	6.97 ^b	0.22
	60	7.83 ^f	0.07	6.67 ^f	0.09	6.44 ^c	0.03
	300	7.75 ^f	0.04	6.59 ^f	0.05	3.54 ^e	0.51
4	C	7.88 ^g	0.05	8.18 ^a	0.06	8.18 ^a	0.06
	15	7.42 ^{bd}	0.15	6.66 ^g	0.05	6.18 ^f	0.13
	60	7.50 ^b	0.04	2.94 ^h	0.68	3.15 ^g	0.12
	300	6.68 ^d	0.26	ND* ⁱ	-	ND* ^h	-

550 Different letters indicate a significant difference at the 0.05 level between different treatment
551 times and gas mixtures

552 SD*: Standard deviation

553 ND*: Under detection limit, complete inactivation

554 Gas 1: 70% N₂+ 30% CO₂; Gas 2: 90% N₂+ 10% O₂; Gas 3: Air; Gas 4: 70% O₂+ 30% CO₂.

Table 3. *L. monocytogenes* NCTC 11994 inactivation efficacy with treatment and post-treatment storage time in different gases

Gases	Plasma treatment time (s)	Post-treatment storage time					
		0 h		1 h		24 h	
		Cell density (Log ₁₀ CFU/ml)	SD*	Cell density (Log ₁₀ CFU/ml)	SD*	Cell density (Log ₁₀ CFU/ml)	SD*
1	C	7.49 ^a	0.31	7.85 ^a	0.12	7.85 ^a	0.12
	15	6.84 ^b	0.14	6.43 ^b	0.07	6.88 ^b	0.07
	60	6.94 ^b	0.15	6.18 ^{bd}	0.18	6.62 ^b	0.34
	300	6.85 ^b	0.09	5.09 ^c	0.12	6.10 ^c	0.13
2	C	7.98 ^c	0.11	7.85 ^a	0.12	7.85 ^a	0.12
	15	7.21 ^d	0.13	6.58 ^b	0.16	5.55 ^d	0.13
	60	7.16 ^e	0.06	6.01 ^{bd}	0.10	4.68 ^e	0.19
	300	6.24 ^f	0.19	3.59 ^e	0.15	ND* ^f	-
3	C	7.98 ^c	0.11	7.85 ^a	0.12	7.85 ^a	0.12
	15	8.06 ^c	0.08	5.55 ^f	0.11	5.63 ^d	0.12
	60	7.81 ^c	0.07	5.12 ^c	0.09	1.83 ^g	0.67
	300	7.72 ^g	0.06	ND* ^g	-	ND* ^f	-
4	C	8.27 ^c	0.02	7.85 ^a	0.12	7.85 ^a	0.12
	15	7.02 ^{bd}	0.21	3.94 ^h	0.23	ND* ^f	-
	60	3.70 ^h	0.03	ND* ^g	-	ND* ^f	-
	300	ND* ⁱ	-	ND* ^g	-	ND* ^f	-

Different letters indicate a significant difference at the 0.05 level between different treatment times and gas mixtures

SD*: Standard deviation

ND*: Under detection limit, complete inactivation

Gas 1: 70% N₂+ 30% CO₂; Gas 2: 90% N₂+ 10% O₂; Gas 3: Air; Gas 4: 70% O₂+ 30% CO₂.

Table 4. In-package ozone concentration after different ACP treatment and post-treatment storage time

Post-treatment storage time (h)	Plasma treatment time (s)	Ozone concentration (ppm)							
		Gas 1	SD*	Gas 2	SD*	Gas 3	SD*	Gas 4	SD*
0 h	15	ND*	ND*	127	12	393	12	547	220
	60	ND*	ND*	380	35	1000	200	1533	462
	300	ND*	ND*	2067	115	2433	404	7067	611
1 h	15	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
	60	ND*	ND*	7	12	63	32	243	80
	300	ND*	ND*	253	99	633	153	1067	115
24 h	15	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
	60	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*
	300	ND*	ND*	ND*	ND*	ND*	ND*	ND*	ND*

SD* : Standard deviation

ND* : Under detection limit, non-detectable

Gas 1: 70% N₂+ 30% CO₂; Gas 2: 90% N₂+ 10% O₂; Gas 3: Air; Gas 4: 70% O₂+ 30% CO₂.

573

574 Figure 1. Emission intensities of dielectric barrier discharge atmospheric cold plasma

575 operating under atmospheric pressure

576 (●Gas 1: 70% N₂+ 30% CO₂; ■Gas 2: 90% N₂+ 10% O₂; ◆ Gas 3: Air; ▲ Gas 4: 70%

577 O₂+ 30% CO₂.)

578 (a) Emission intensities at 309.65 nm

579 (b) Emission intensities at 336.65 nm

580

Figure 2. General reactive oxygen species density detection by DCFH DA

15, 60, 300 s treatment at 70 kV_{RMS} without post-treatment storage

(a) PBS solution;

(b) *E. coli* suspension;

(c) *S. aureus* suspension;

(d) *L. monocytogenes* suspension.

■ Gas 1: 70% N₂+ 30% CO₂; ▨ Gas 2: 90% N₂+ 10% O₂; ▤ Gas 3: Air; ▧ Gas 4: 70% O₂+ 30% CO₂.

Figure 3. Singlet oxygen detection by SOSGR

15, 60, 300 s treatment at 70 kV_{RMS} without post-treatment storage

(a) PBS solution;

(b) *E. coli* suspension;

(c) *S. aureus* suspension;

(d) *L. monocytogenes* suspension.

■ Gas 1: 70% N₂+ 30% CO₂; ▨ Gas 2: 90% N₂+ 10% O₂; ▩ Gas 3: Air; ▪ Gas 4: 70% O₂+ 30% CO₂.

Figure 4. H₂O₂ concentration detection by Amplex Red

15, 60, 300 s treatment at 70 kV_{RMS} without post-treatment storage

(a) PBS solution;

(b) *E. coli* suspension;

(c) *S. aureus* suspension;

(d) *L. monocytogenes* suspension.

■ Gas 1: 70% N₂+ 30% CO₂; ▨ Gas 2: 90% N₂+ 10% O₂; ▩ Gas 3: Air; ▪ Gas 4: 70% O₂+ 30% CO₂.

Figure 5. General reactive nitrogen species density detection by DAF FM DA

15, 60, 300 s treatment at 70 kV_{RMS} without post-treatment storage

(a) PBS solution;

(b) *E. coli* suspension;

(c) *S. aureus* suspension;

(d) *L. monocytogenes* suspension.

■ Gas 1: 70% N₂+ 30% CO₂; ▨ Gas 2: 90% N₂+ 10% O₂; ▩ Gas 3: Air; ▪ Gas 4: 70% O₂+ 30% CO₂.

Figure 6. Nitrite/Nitrate concentration in PBS solution

15, 60, 300 s treatment at 70 kV_{RMS}

(a) Nitrite concentration without post-treatment storage;

625 (b) Total Nitrite/Nitrate concentration without post-treatment storage;
626 (c) Nitrite concentration with 1 h post-treatment storage;
627 (d) Total Nitrite/Nitrate concentration with 1 h post-treatment storage.
628 (e) Nitrite concentration with 24 h post-treatment storage;
629 (f) Total Nitrite/Nitrate concentration with 24 h post-treatment storage.
630 ● Gas 1: 70% N₂+ 30% CO₂; ■ Gas 2: 90% N₂+ 10% O₂; ♦ Gas 3: Air; ▲ Gas 4: 70% O₂+
631 30% CO₂.
632
633