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Atmospheric cold plasma interactions with modified atmosphere packaging inducer gases for safe food preservation

HanL.[a], BoehmD.[a], AmiasE.[a], MilosavljevićV.[a], CullenP.J.[a,b], BourkeP.[a,*]

[a] Plasma Research Group, School of Food Science and Environmental Health, Dublin Institute of Technology, Ireland
[b] School of Chemical Engineering, UNSW, Sydney, Australia

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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 15 s 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 et al., 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; O_3, O atom, singlet oxygen and nitric oxides are reported as the main reactive species, while H_2O_2, OH radicals and HNO_x ($x = 1,4$) are also generated with humid applied gases (Moiseev et al., 2014; Takamatsu et al., 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

* Corresponding author at: School Food Science and Environmental Health, Dublin Institute of Technology, Dublin 1, Ireland.
E-mail address: paula.bourke@dit.ie (P. Bourke).

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the red colour of meat products. However, because of the high dissociation energy, inert gas CO_2 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% O_2 + 30% CO_2 for MAP (Sørheim, Nissen, & Nesbakken, 1999) and cooked meats are stored in 70% N_2 + 30% CO_2 (Smiddy, Papkovsky, & Kerry, 2002). At the same time, low oxygen/high nitrogen (10% O_2 + 90% N_2) 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 optimized 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% N_2 + 30% CO_2 (Gas 1), 90% N_2 + 10% O_2 (Gas 2) and 70% O_2 + 30% CO_2 (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 Log_{10} 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 5 mm 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 5 mm 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 # 18 M, 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 Synergy™ 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 μM for 15 min at 37 °C immediately after ACP treatment. Samples of 200 μl 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