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Microbiological Control and Mechanisms of Action of High Voltage Atmospheric Cold Plasma

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Technological University Dublin, lu.han@tudublin.ie

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MICROBIOLOGICAL CONTROL AND MECHANISMS OF ACTION OF HIGH VOLTAGE ATMOSPHERIC COLD PLASMA

Lu Han

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MICROBIOLOGICAL CONTROL AND MECHANISMS OF ACTION OF HIGH VOLTAGE ATMOSPHERIC COLD PLASMA

Ms Lu Han

A thesis submitted to Dublin Institute of Technology in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

School of Food Science and Environmental Health

College of Sciences and Health

Dublin Institute of Technology

Supervisors:
Dr. Paula Bourke
Dr. PJ Cullen

June 2016
Abstract

Dielectric barrier discharge-atmospheric cold plasma (DBD-ACP) is a promising non-thermal technology effective against a wide range of microorganisms. These studies were performed using a custom built DBD-ACP system. The inactivation efficacy was found to be governed by a series of critical control parameters, including treatment time, mode of exposure (Direct/Indirect exposure), applied voltage, applied gas content but was also very dependent on the characteristics of treatment targets. In this study, these parameters were investigated using in-package design along with a post-treatment storage procedure to align with industrial processing times as well as mitigation of post-process contamination. A range of food borne pathogens were used as targets to optimize system and process parameters and further reveal the inactivation mechanisms.

The inactivation efficacy of ACP against all applied strains was dependent on treatment time, applied voltage and oxygen percentage. Reactive species, especially reactive oxygen species (ROS) are the main microbicidal agents of plasma. By increasing the value of the above processing parameters, the detected amount of ROS increased and enhanced the bactericidal effect. Due to the in-package design of this study, post-treatment storage time investigated (0, 1 and 24 h) also had positive effect on inactivation level, which provided a retention time for reaction and effect with longer live or recombined reactive species. Additionally, the mode of exposure resulted in different inactivation levels, where the chemistry associated with the mode of exposure to the discharge was altered with or without samples in discharging area. Inactivation mechanisms were different for the Gram negative and positive bacteria studied. The obvious loss of cell membrane integrity was shown for Gram negative bacteria, while the damage on Gram positive bacteria was more significant on
intracellular components (DNA in this study). This effect was indicated as a result of their different cell envelope structures, which dominated ROS action/inactivation patterns. Using five knockout mutants and parent strain of *E. coli* as target cells, cellular responses were observed as general regulation, short-term and long-term actions with regard to ROS scavenging and cellular repair.

Furthermore, the optimized ACP parameters were applied to meat product models to ascertain the impact of the technology to model food surface contamination and to challenge the processing parameters previously determined as critical in the preceding studies. Increasing the microbial challenge to ACP from liquid suspensions through to colonised surfaces did require greater treatment duration at a higher voltage level. Comprehensive antimicrobial actions of ACP generated reactive species were based on the food matrix complexity and the structure of the target cells exposed. Subsequently, the additional protection effect of nutritive composition against reactive species was observed. Nonetheless, the results indicate that ACP can be employed to address a range of microbiological safety challenges pertinent to the meat industry, particularly where the processing advantage of in package treatment is available. However, careful consideration of additional hurdles is required along the product chain.
Declaration

I certify that this thesis which I now submit for examination for the award of Doctor of Philosophy, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any other third level institution. The work reported on in this thesis conforms to the principles and requirements of the DIT's guidelines for ethics in research.

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_________________________________________________ Date ____________________

Signature


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### Abbreviations

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<tr>
<td>DBD</td>
<td>Dielectric barrier discharge</td>
</tr>
<tr>
<td>ACP</td>
<td>Atmospheric cold plasma</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>RF/RFP</td>
<td>Radio frequency/radio frequency plasma</td>
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<tr>
<td>ICP</td>
<td>Inductively coupled plasma</td>
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<tr>
<td>DNA/RNA</td>
<td>Deoxyribonucleic acid/ribonucleic acid</td>
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<tr>
<td>DSB/SSB</td>
<td>Double-strand break/single-strand break</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>MDA</td>
<td>Methyleneedianiline</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>IV</td>
<td>Intravenous</td>
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<tr>
<td>PP</td>
<td>Polypropylene</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
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<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
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<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
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<tr>
<td>AP sites</td>
<td>Apurinic/apyrimidinic (AP) sites</td>
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<tr>
<td>EPS</td>
<td>Extracellular polymeric substances</td>
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<tr>
<td>MAP</td>
<td>Modified atmosphere packaging</td>
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<tr>
<td>HOA</td>
<td>High oxygen atmosphere</td>
</tr>
<tr>
<td>EMA</td>
<td>Equilibrium modified atmosphere</td>
</tr>
<tr>
<td>CFA</td>
<td>Cyclopropane fatty acid</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>8-oxodeoxyguanosine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>TSB+G/-G</td>
<td>Tryptic soy broth with glucose/without glucose</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
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<tr>
<td>BE</td>
<td>Beef extract</td>
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<tr>
<td>MRD</td>
<td>Maximum recovery diluent</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>g (centrifuge)</td>
<td>gravitational force</td>
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<tr>
<td>g (mass)</td>
<td>Gram</td>
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<tr>
<td>L/ml</td>
<td>Litre/millilitre</td>
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<td>M</td>
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<td>minute</td>
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<td>ppm</td>
<td>parts per million</td>
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<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>RMS</td>
<td>root mean square</td>
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<tr>
<td>J</td>
<td>joule</td>
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<tr>
<td>OES</td>
<td>Optical emission spectroscopy</td>
</tr>
<tr>
<td>OAS</td>
<td>Optical absorption spectroscopy</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>DCFH-DA</td>
<td>2’,7’-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>SOSGR</td>
<td>Singlet oxygen sensor green reagent</td>
</tr>
<tr>
<td>DAF-FM DA</td>
<td>4-amino-5-methylamino-2’,7’-difluorofluorescein diacetate</td>
</tr>
<tr>
<td>AFU</td>
<td>Arbitrary fluorescence unit</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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Chapter 1. INTRODUCTION

1.1 Traditional Sterilization and Pasteurisation Technologies

Sterilization is a term referring to any process that eliminates (removes) or kills all forms of microbial life, including transmissible agents (such as fungi, bacteria, viruses, spore forms, etc.) present on a surface, contained in a fluid, in medication, or in a compound such as biological culture media (Block, 2001; Fraise et al., 2012). It is a very important process across the food industry, healthcare environments and for medical surgery.

Sterilization can be achieved by applying heat, chemicals, irradiation, high pressure, and filtration or combinations thereof. Heat has been used as the most efficient and widely used sterilization method before non-thermal sterilization technologies were developed. However, heat may induce changes in texture and flavour of food, or cause damage to heat sensitive target materials. Non-thermal sterilization technology, including chemical and physical sterilizations, are alternative choice to heat sterilization. There are many chemicals which could be applied in sterilization processing, such as chlorine, alcohol, formaldehyde, glutaradehyde, ozone, hydrogen peroxide, peracetic acid, iodophors, phenolics, quaternary ammonium compounds and metals. Chemical sterilization has drawbacks, such as unpleasant smell, hazardous residue, surface damage or incomplete inactivation sometimes. Similarly, physical sterilization methods such as filtration and gamma radiation are expensive and not applicable to a wide range of foods.

There is a need to develop effective alternatives to current sterilization methods like heat, gamma irradiation and ethylene oxide. The reason behind this is mainly due to disadvantages associated with these methods. Also, the development of novel polymeric biomaterials that are usually sensitive to these methods. Using these
methods, the different properties of polymeric materials like molecular weight, transparency etc. can be altered (Loo et al., 2005; Woo and Sandford, 2002). 

Heat treatment is one of the most widely used methods for food preservation. It is applied as low-heat and high-heat processing. Low-heat food processing has been used for decades and remains a cornerstone of food processing. Thermal sterilization always inactivates pathogens by damaging and degrading their DNA and protein. The inactivation effect mostly relies on the treatment temperature and processing time. (Juneja and Sofos, 2001) However, thermal sterilization can affect the texture, flavour and nutritive components, which make it unsuitable for some products, such as fresh cut fruits and vegetables. Cold temperature storage has disadvantages in application in that it can only slow or reduce the growth of food pathogens rather than inactivate them.

Non-thermal decontamination technology refers to those sterilization methods operating at ambient temperatures or below. Consequently, non-thermal sterilization technology may induce less damage to samples, can be safer and easier to manipulate and cheaper in cost than thermal technologies. They can be applied to samples sensitive to temperature and pressure, e.g. fresh produce, medicines, temperature sensitive material and clinical instruments.

Chemical sterilization includes chlorine and non-chlorine washes, since chlorine is the most important and widely used chemical sterilization reagent. These chemicals can inactivate microbes by denaturing proteins and attaching nucleic acids (Favero and Bond, 1991; Zhang and Zhou, 1996). Although they are usually effective and inexpensive, there are concerns about leaving harmful residues, nutrition and quality damages, which limit their applications on food processing.
The use of heavy metals as antiseptics or disinfectants has been explored as a novel chemical sterilization method (Brady et al., 2003). Metals such as silver, iron, and copper could be used for environmental control, disinfection of water, reusable medical devices or incorporated into medical devices. They are effective against a wide variety of microorganisms with limitation of high cost (Bright et al., 2002; Rusin et al., 2003).

As physical non-thermal sterilization technologies, filtration and ultrasound are promising for fluids that would be damaged by other sterilization methods (Levy, 2001). However, their effectiveness and applicable area are limited (Piyasena et al., 2003).

With the wavelength ranging from 200-300 nm, UV radiation provides a non-ionizing radiation sterilization method (Laroussi, 1996). Inactivation of microorganisms results from destruction of nucleic acid through induction of thymine dimers. Bacteria and viruses are more easily killed by UV light than bacterial spores (Russell, 1999). UV can be applied in health-care environment for airborne pathogens, but its efficacy is easily affected by distance and shields (Shechmeister, 1991).

Ionizing radiation sterilization technologies include electron beam, X-ray and gamma rays. During ionizing, the collisions of atoms result in damage of chemical bonds in cell components. These damages include breakage of DNA backbone, oxidation of liquid and radiolysis of water molecules, which lead to cell death. (Juneja and Sofos, 2001) Although sterilization efficacy of electron beam can be improved with higher dosing rate and less exposure time, it is less penetrating than gamma or X-ray. Compared with X-ray, the source of gamma radiation cannot be switched off, which increase its danger during application. These technologies are commonly used for sterilization of disposable medical equipment, such as syringes, needles, cannulas and
IV sets, and food, due to their high efficacy and low damage to treated samples. However, there are many disadvantages associated and limitation in terms of safety. These radiation requires proper shields to protect workers and the environment from radiation exposure.

As an alternative choice of radiation, atmospheric cold plasma is well documented as a novel ionizing sterilization method.

**1.2 Atmospheric cold plasma**

Plasma is often referred to as the fourth state of matter according to a scheme expressing an increase in the energy level from solid to liquid to gas and ultimately to plasma. Thus, any source of energy which can ionise a gas to plasma may be employed for generation of plasma. This energy can be thermal, or carried by either an electric current or electromagnetic radiations. In this study, the electric field transmits energy to the gas electrons, and is then transmitted to the neutral species by elastic collisions (where the energy can slightly raise energy of species) and inelastic collisions (with high energy and create excited species and ions). (T Endero et al., 2006)

After inelastic collisions, plasmas are conductive assemblies of charged particles, neutrals and fields that exhibit collective effects. When a gas is energised to such an extent that the molecules of the gas breakdown to give free electrons, radicals, positive and negative ions, quanta of electromagnetic radiation, while some molecules may still remain neutral (incomplete ionisation), then this state of the gas is known as plasma.

**1.2.1 Configurations**

Plasma is commonly formed in a neutral gas by providing sufficient energy, which is capable of causing ionisation of the gas. There are few well reported approaches for plasma generation at atmospheric pressure, including corona discharge, Radio-Frequency Plasma (RFP), gliding arc discharge, microwave and Dielectric Barrier
Discharge (DBD). The categories were defined by the characteristics of source power and ionizing patterns. However, some equipment design can combine the categories to increase the stability and output power (Moreau et al., 2008).

1.2.1.1 Corona discharge

Corona is referred to as a weak luminous discharge, which is generated at atmospheric pressure near sharp points, edges, or thin wires using very large electric fields. The electric field and the densities of charged particles are among the most important physical parameters that need to be determined to control the operation of a corona discharge. A corona discharge starts around electrodes with small radii of curvature, where the electric field intensity is very high. The ionization process is then restricted to a small region surrounding that electrode called the ionization region. The rest of the discharge volume is filled with ions drifting in a much lower electric field. The generation of charged particles and electric field of corona discharge have been well studied by Béquin et al. (2013) and Yanallah and Pontiga (2012).

![Diagram of point to plane corona discharge](image)

Figure 1.1 Schematic of point to plane corona discharge
Adapted from Béquin et al. (2013)
1.2.1.2 **Radio-Frequency Plasma**

RFP are formed in a flow of gas by an externally applied radio frequency field. It is recommended that the frequency at which the source operates is given and the gas type defined. These are capacitive discharges produced more often in noble gases such as helium or argon by the application of RF power.

1.2.1.3 **Gliding arc discharge**

The gliding arc is a well-known example of a gliding discharge, which was first used in chemical processes initially for gas conversions and decontamination applications. Gliding discharge is an auto-oscillating periodic phenomenon developing between at least two diverging electrodes submerged in a laminar or turbulent gas flow. Plasma generated by the gliding discharge has thermal or non-thermal properties depending on the system parameters such as power input and flow rate. The treatment efficacy has been studied with respect to the parameters of length of discharge, voltage, flow rate, and frequency of the power (Mutaf-Yardimci et al., 2000).

1.2.1.4 **Microwave Plasma**

These plasma systems are based on high frequency (GHz range) microwave discharges in the gas. Microwave plasma is widely reported on the synthesis of nanomaterials, especially the growth of diamond films (Bates, 2007; Bower et al., 2000; Hozumi and Takai, 1997; Kamo et al., 1983; Kobashi et al., 1988). Microwave discharge patterns are able to generate both thermal and non-thermal plasma. The frequency range of these sources is always limited by system size and frequency of ionization and ion transfer (Fridman, 2008a). The most commonly used frequencies are ranging from 300 MHz to 2450 MHz. The key advantage of high-frequency fields is a stabilising effect on the plasma and generation of streamerless plasmas in molecular gases (Bárdos and
Recently, there are studies on non-thermal plasma sterilization technologies with microwave sources (Deilmann et al., 2008; Lee et al., 2005).

### 1.2.1.5 Dielectric barrier discharge

Dielectric-barrier discharges (DBD) are characterised by the presence of one or more insulating layers in the current path between metal electrodes in addition to the discharge space. When the potential across the gap reaches the breakdown voltage, the dielectric barriers acts as a stabilizing material leading to the formation of a large number of micro-discharges and inhibits the glow-to-arc transition in a DBD design (Tendero et al., 2006). The classical DBD configurations utilize planar or cylindrical arrangements with at least one dielectric layer placed between the electrodes.

![Diagram of DBD configurations](image)

Figure 1.2 Common configurations of the dielectric-barrier discharges (DBDs) (a, b, c) Planar, (d) Cylindrical. Adapted from Kogelschatz et al. (1997).
This discharge was initiated in an annular gap between two coaxial glass tubes. By using coaxial external electrodes a radial electric field was applied by an alternating voltage of sufficiently high amplitude to cause electrical breakdown in the flowing gas. Since the electric current is forced to pass through the glass walls acting as dielectric barriers the discharge is commonly referred to as the dielectric-barrier discharge (Kogelschatz et al., 1999). DBD atmospheric cold plasma, which was investigated for ozone generation, may offer advantages over other plasma generation system in terms of cost, practicality and reliability (Kim et al., 2008; Morent and Leys, 2005).

Moreover, new properties and physics might be achieved when scaling down those described plasma sizes to submillimeters. These can be called as microdischarges. By reducing the size, the significant changes include increasing the power density, changes of plasma composition, significant sheath effect and relatively high electron energy (Fridman, 2008a).

The design of plasma generation equipment could be various based on described configurations, where those configurations can be combined or further modified. In some designs, there are possible limitations of the approach for scale-up, such as low efficacy with inert gases. DBD configuration offers an economic and reliable method for generation of plasma with an option for a broad range of applications and the potential to scale-up to larger industrial installations (Kogelschatz, 2003; Kostov et al., 2009). Hence, this study focused microbiological interactions with DBD-ACP.

1.2.2 Applications

The use of atmospheric cold plasma (ACP) has gained increased attention in recent years with a wide range of potential applications, including food and medical surface sterilization, clinical treatment of open wounds, removal of toxic compounds from air
and modification of surface properties (Desmet et al., 2009; Dirks et al., 2012; Dobrynin, 2007; Dobrynin et al., 2009; Eto et al., 2008; Müller and Zahn, 2007). ACP presents various advantages for microbial control over existing technologies including short processing times and operation at low temperature thus mitigating against changes in product quality indicators (Laroussi et al., 2002).

1.2.2.1 Sterilization and decontamination

A wide range of applications for atmospheric plasma have been described for inactivation of bacteria, spores, viruses, bacteriophage and fungi (Hati et al., 2012; Laroussi et al., 2002; Venezia et al., 2008). Some of the earlier applications of plasma in medicine relied mainly on the thermal effects of plasma. Recently, atmospheric cold plasma effects have been studied for various sub-lethal purposes such as genetic transfection, cell detachment, wound healing (treatment of chronic wounds), dermatology (treatment of skin irritations and diseases), dentistry (periodontitis prophylaxis), surgery (infection control), equipment decontamination and others (Fitzpatrick et al., 2008; Fridman, 2008b; Ginsberg et al., 2002).

The mechanism of microbial inactivation by plasma is complex, with the role of various charged particles and generated reactive species under current investigation. A number of anti-microbial agents including reactive oxygen species (ROS), reactive nitrogen species (RNS), ultraviolet (UV) radiation, energetic ions, and charged particles can be generated in a gas discharge. Depending on the type of gas utilised for generation of plasma, the concentration, type and range of reactive species may vary (Alkawareek et al., 2012).

Microorganisms can occur on raw and minimally processed produce at populations ranging from $10^3$ to $10^9$ CFU g$^{-1}$. Although washing produce in water may remove some soil and other debris, it cannot be relied upon to completely remove
microorganisms and may result in cross-contamination of food preparation surfaces (Nguyen - the and Carlin, 1994). ACP is an emerging non-thermal pasteurization method for the enhancement of food safety (Kim et al., 2011). ACP is very effective against a wide range of food borne pathogens in the treatment of nuts, liquid and fresh produce surfaces (Deng et al., 2007; Montenegro et al., 2002; Zhang, Oh, et al., 2013). Its strong inactivation efficacy with short treatment time make industrial scale-up promising (Niemira, 2012). Moreover, there are studies about effect of plasma treatment on plant germination. Under optimized critical control parameters, plasma treatment can increase the germination rate or delay germination (Sera et al., 2010; Volin et al., 2000; Živković et al., 2004).

**1.2.2.2 Other applications**

Atmospheric cold plasma (ACP) has been applied as an important tool for the abatement of pollutants and for promoting various chemical reactions in gas or in liquid. Indoor air cleaners have been proven to be effective for the removal of harmful gases, odour and allergens without producing harmful residues or increasing temperature (Hammer, 2002; Mizuno, 2007; Rong et al., 2006).

RF inductively coupled plasmas (ICPs), a type of ACP, have been described can be used as excitation sources for spectroscopic analysis. Microplasmas are particularly interesting as this technology has the potential of further integration with complementary devices onto a single “chip”, resulting in miniaturized total analytical systems. (Tendero et al., 2006)

Plasma has been widely documented for surface applications, including cleaning (described in sterilization applications), etching and activation. Surface etching means modifying surface by embedding elements using plasma discharge. Its etching rate is
governed by: plasma gas composition, substrate nature, working conditions (power, gas flow rate, substrate position). The etching efficiency can be enhanced by metastable energetic species, which play an essential part in excitation, ionization and dissociation phenomena (Tendero et al., 2006). Surface activation could be achieved by grafting chemical functions (plasma active species) on the material surface in order to give it specific properties by varying its surface energy. The plasma gas composition influences the treated materials superficial properties. In the polymer industry, air corona discharge-based plasma (similar to oxygen plasma) treatment is used to modify the surface properties of polymers. It has been shown that such air plasmas can dramatically improve the wettability and adhesion of polymer surface. Plasma modification has been widely reported on the surface of polypropylene (PP), polyethylene (PE), polyethylene terephthalate (PET) and polytetrafluoroethylene (PTFE) materials. (Cheng et al., 2006; Cui and Brown, 2002; Liu et al., 2004; Tendero et al., 2006)

ACP has also been recognized for the production of chemicals (e.g. methanol from methane and other selective-oxidation reactions, reforming reduction of supported metal catalysts and for the removal of hazardous compounds (e.g. nitrogen oxides and volatile organic compounds) from gas streams (Huang et al., 2011; Roland et al., 2005). It is expected that future efforts using optical diagnostics and modeling and simulation techniques will allow researchers to identify the critical species and kinetic routes responsible for the observed catalytic effects (Paulmier and Fulcheri, 2005).

ACP can also be used to ‘activate’ or ‘crack’ hydrocarbon fuels, which promotes the combustion of the fuels (reducing unburned hydrocarbons and allowing fuel burning in regimes where the emissions of CO and NO\textsubscript{x} are expected to be reduced; e.g., ultra-lean-burn conditions) (Rosocha and Kim, 2006).
1.2.3 Advantages of ACP for Microbial Control

Atmospheric cold plasma is an emerging technology for microbial control with advantages in terms of cost, safety and ease of manipulation. Atmospheric cold plasma generates a number of anti-microbial agents, including reactive oxygen species (ROS), reactive nitrogen species (RNS), ultraviolet (UV) radiation, energetic ions, and charged particles, during a gas discharge. A wide range of applications for DBD-ACP have been described for inactivation of bacteria, spores, viruses, bacteriophage and fungi (Hati et al., 2012; Laroussi et al., 2002; Venezia et al., 2008). The novel application of in-package plasma introduced in this study offers the potential of produce safety and shelf-life extension by reducing pathogenic and spoilage organisms, with the in-package processing preventing recontamination events.

A clear understanding of the critical control parameters governing any process technology is required for optimization and validation. In terms of achieving optimal anti-microbial efficacy, it is also important to understand how these process and system variables interact with the mechanism of inactivation. A greater understanding of the interaction between process parameters in tandem with the mode of action could lead to more successful applications, where for example, different target microbiological species may require different processing conditions.

Low cost: ACP is generated at room temperature with atmospheric pressure. Comparing with thermal technologies, no special temperature or pressure equipment is required. Power requirements are relatively low. The product about to be sterilized could be packed properly without any special after treatment procedure. ACP saves cost from equipment, input power and post processing.

Safe and environmental friendly: The reactive species generated depend on gases applied. Among all reactive species generated during discharge, ozone is the longest
living species. Ozone and other charged particles are easily exhausted without any toxic residues and unpleasant smell.

Highly effective: ACP is very effective on inactivation of microbes with short treatment time.

1.2.4 Critical control Parameters governing ACP efficacy

A clear understanding of the critical control parameters governing any process technology is required for optimization. In terms of achieving optimal anti-microbial efficacy, it is also important to understand how these process and system variables influence the mechanism of inactivation.

1.2.4.1 Output Power

Voltage/output power is one of the most important parameters governing ACP effectiveness, which has been widely reported. Deng et al. (2007) studied the ACP bactericidal effect on *Escherichia coli*. Their research showed the ACP inactivation effects varied with the power voltage (17-25 kV) and frequency (1-2.5 kHz) applied. Increased inactivation found with increasing voltage (2.56-3.8 kV) is mainly due to increased energy input and energy density, which may raise the generation of reactive species (Cheng et al., 2014). While higher inactivation rates could be achieved by raising voltage and/or frequency, technological challenges and limitation, cost, and negative impacts on quality must be considered in practice. Moreover, the effect of treatment time was also observed in mammalian cells by analysing cell viability and apoptosis, with plasma dose of 0.13-7.8 J/cm² (Kalghatgi et al., 2011).

In a study of pure oxygen plasmas, ACP effect has been enhanced at high power levels, where bacterial inactivation of *B. subtilis* spores was also influenced. D-values of survival were dependent on the discharge power supplied. At 300 W, a D-value of
approximately 8 minutes was found, while at higher discharge powers (350 and 400 W), the D-value decreased to approximately 3 minutes. (Boscariol et al., 2008)

1.2.4.2 Treatment time

Similar to the power applied, increasing the exposure time can lead to a decrease the number of surviving bacteria in the exposure spot.

In many research publications, the inactivation efficacy for different bacteria was found to be increased by treatment time (Ghomi et al., 2009; Rupf et al., 2010). Deng et al. (2007) increased \( E. \text{coli} \) reduction on almond surface from 1 to 5 \( \log_{10} \) CFU ml\(^{-1} \) by increasing treatment time from 10 sec to 30 sec. Kvam et al. (2012) demonstrated that plasma rapidly inactivates planktonic cultures with \( >5 \log_{10} \) CFU ml\(^{-1} \) kill in 30 sec. By increasing treatment time from 7 sec to 30 sec, the cell surface damage showed a time-dependent manner, and resulted in a loss of membrane integrity and leakage of intracellular components (nucleic acid, protein, ATP), and ultimately focal dissolution of the cell surface with longer exposure times. The increase of exposure time (5-60 sec) led to the generation of higher concentration of reactive species, which cause more severe damage and further resulted in cell death (Cheng et al., 2014).

Both input power and exposure time of plasma treatment can control applied plasma doses and further govern the microbicidal efficacy, where cell damage is a plasma dose dependent factor. It is reported that low dose ACP treatment cause repairable damage to genomic DNA, while higher dose will lead to greater amount of damage and result in cell apoptosis with 2-5 J/cm\(^2\) (Dobrynin et al., 2009).

1.2.4.3 Mode of Exposure

In many research publications, direct and indirect exposure of plasma shows different effects on inactivation. Mode of exposure is suspected to play a key role in microbial inactivation. When microbes are treated with plasma directly, they are exposed
simultaneously to charged particles, UV and all active plasma components such as ozone (O$_3$), hydroxyl radicals (OH$^-$) and other excited molecular and atomic species, and thus maximum inactivation effect is always obtained in this case.

Typically, indirect exposure indicates remote exposure, which means samples are located outside the discharge area, which employs mostly uncharged atoms and molecules that are generated in plasma, but involves small, if any, flux of charges to the surface. In indirect treatment, the active uncharged species are typically delivered to the surface via gas flow through a plasma region. Thus, the indirect sterilization is the result of relatively long-lived or recombined species. These species are products of secondary reactions, such as hydrogen peroxide and peroxynitrite, which is lower in energy but still have damaging effect on essential macromolecules. Their chemical reactions and antimicrobial effect will be further described in following sections.

Both indirect and direct non-thermal plasma treatments permit some degree of tuning of the plasma properties (Fridman et al., 2007). However, due to the absence of charged species, direct exposure of ACP has been reported to achieve higher efficacy than indirect exposure (Dobrynin et al., 2009; Fridman et al., 2007; Okubo et al., 2004).

Moreover, indirect exposure treatments had different design due to the experimental devices, where Fridman et al. (2007) used a grounded mesh instead of remote exposure. The in-package treatment in this study is different from previously reported direct and indirect treatment, which highlighted the long term effect of reactive species. Additionally, the characteristic of samples in DBD design may change the plasma properties by increasing barrier effect or in-package humidity.
1.2.4.4 Gases and humidity

Reactive species generated in the discharge are governed by voltage, treatment time and gases, in which gases play the most important role (Gallagher et al., 2004; Kirkpatrick et al., 2007). Due to the cost, the most popular gases applied in ACP sterilization are argon, helium and a mixture of nitrogen and oxygen (including atmospheric air).

Helium is a commonly preferred carrier gas for several reasons. Its high thermal conductivity assists in heat removal from the application site, minimizing substrate damage. The rich ultraviolet emission spectrum provides greater decontamination capability, and helium has a lower operating discharge voltage at atmospheric pressure even though it has the highest ionization potential (Konesky, 2009).

With the presence of oxygen, aggressive active chemical species, singlet oxygen ($^1O_2$), hydroxyl radicals (OH'), hydrogen peroxide (H$_2$O$_2$) and ozone (O$_3$) etc. are created. Ozone is a proven sterilizing agent, but it is known to have negative health effects and its longer lifetime can potentially lead to human exposure.

The reactions of ozone with water could decrease total oxidative stress, which was well explained from a series of reactions (Table 1.1).

<table>
<thead>
<tr>
<th>I</th>
<th>O$_3$ + HO$^-$ → HO$_2$$^\cdot$ + O$_2$$^\cdot$</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>HO$_2$$^\cdot$$\leftrightarrow$O$_2$$^\cdot$ + H$^\cdot$</td>
</tr>
<tr>
<td>III</td>
<td>O$_3$ + O$_2$$^\cdot$ → O$_3$$^\cdot$ + O$_2$</td>
</tr>
<tr>
<td>IV</td>
<td>O$_3$$^\cdot$ + H$^\cdot$ → HO$_3$$^\cdot$</td>
</tr>
<tr>
<td>V</td>
<td>HO$_3$$^\cdot$ → HO$^\cdot$ + O$_2$</td>
</tr>
<tr>
<td>VI</td>
<td>HO$^\cdot$ + O$_3$ → HO$_4$$^\cdot$</td>
</tr>
<tr>
<td>VII</td>
<td>HO$_4$$^\cdot$ → HO$_2$$^\cdot$ + O$_2$$^\cdot$</td>
</tr>
<tr>
<td>VIII</td>
<td>HO$_4$$^\cdot$ + HO$_4$$^\cdot$ → H$_2$O$_2$ + O$_3$</td>
</tr>
</tbody>
</table>
Ozone chemistry in the presence of water is different than dry air. Water in an ACP discharge, either as humidity in gas or as liquid in the system, will result in the abundance of hydroxyl radicals, hydrogen peroxide and hydronium ions (H$_3$O$^+$). Those species are highly effective at altering liquid pH, disrupting cell membranes and damage DNA of bacteria (Moreau et al., 2008). The presence of water can not only react with ozone, but also vary the generation of reactive species by quenching effect (Moiseev et al., 2014). Thus, the increasing of humidity in applied gases would affect the inactivation efficacy (Patil et al., 2014).

Besides applied gas type and humidity, gas flow rate has also been reported as an important parameter (Du et al., 2012). In the ACP configurations with gas flowing through discharging area, the flow rate of gases could be associated with their ionization extent. This could further change the gas-plasma chemistry and sterilization results (DiSanto et al., 2011; Du et al., 2012; Oda et al., 2004). However, in-package treatment design in this study will not be affected by this parameter, due to the retention of the applied gases in a sealed container.

The system and process parameters employed in DBD-ACP, including voltage level, induced gas composition, mode of exposure and treatment time, as well as environmental and product conditions, such as relative humidity, water content and product composition may all effect the reactive species generated, thereby influencing inactivation efficacy (Fridman et al., 2007; Ghomi et al., 2009; Kikuchi et al., 2011; Majumdar et al., 2009). A greater understanding of the interaction between process parameters in tandem with the mode of action could lead to more successful applications where for example different target microbiological species may require different processing conditions. This approach is also of critical importance where
retention of sensitive product quality characteristics is needed in addition to microbiological safety.

1.3 Plasma chemistry

As described above, reactive species generated during the discharges include reactive oxygen species (ROS), reactive nitrogen species (RNS), ultraviolet (UV) radiation, energetic ions and charged particles. ROS and RNS play the main role on inactivation because of high activity among themselves and with biomolecules. Some of their redox reactions are listed below.

Table 1.2 List of reactive species (ROS/RNS) and some redox reactions

<table>
<thead>
<tr>
<th>Reactive species</th>
<th>Formula</th>
<th>Reactions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Radicals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superoxide</td>
<td>O₂•</td>
<td>O₂ + H⁺ + e⁻ ⇌ H₂O</td>
<td>(Beckman et al., 1990)</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>•OH</td>
<td>•OH + H⁺ + e⁻ ⇌ H₂O</td>
<td>(Arjunan et al., 2015)</td>
</tr>
<tr>
<td>Hydroperoxyl (protonated superoxide)</td>
<td>HO₂•</td>
<td>HO₂• + H₂O⁺ ⇌ H₂O</td>
<td>(Baskin and Salem, 1997)</td>
</tr>
<tr>
<td>Carbonate</td>
<td>CO₃•</td>
<td>CO₃• + e⁻ ⇌ CO₃²⁻</td>
<td>(Arjunan et al., 2015)</td>
</tr>
<tr>
<td>Alkoxyl</td>
<td>RO•</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peroxyl</td>
<td>RO₂•</td>
<td></td>
<td>(Baskin and Salem, 1997)</td>
</tr>
<tr>
<td>Carbon dioxide radical</td>
<td>CO₂•</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Radicals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H₂O₂</td>
<td>H₂O₂ + 2 H⁺ + 2 e⁻ ⇌ 2 H₂O</td>
<td>(Baskin and Salem, 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H₂O₂ + 2 e⁻ ⇌ 2 OH</td>
<td>(Arjunan et al., 2015)</td>
</tr>
<tr>
<td>Ozone</td>
<td>O₃</td>
<td>O₃ + 2 H⁺ + 2 e⁻ ⇌ O₂ + H₂O</td>
<td>(Arjunan et al., 2015; Baskin and Salem, 1997)</td>
</tr>
</tbody>
</table>
Singlet Oxygen

\[ ^1\text{O}_2 + ^1\text{O}_2 + 4 \text{H}^+ + 4 \text{e}^- \rightleftharpoons 2 \text{H}_2\text{O} \]

Arjunan et al., 2015

Organic peroxides

\[ \text{ROOH} \]

Arjunan et al., 2015

Peroxynitrite

\[ \text{ONOO}^- \rightleftharpoons \text{ONOOO}^+ + \text{CO}_2 \]

Arjunan et al., 2015

Nitrosoperoxycarbonate

\[ \text{ONOOCO}_2^- \]

Arjunan et al., 2015

Dissolved Oxygen

\[ \text{O}_2 \]

Arjunan et al., 2015

<table>
<thead>
<tr>
<th>Reactive nitrogen species</th>
<th>Free Radicals</th>
<th>Non-Radicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide</td>
<td>( \bullet \text{NO} )</td>
<td>( \bullet \text{NO} + \text{O}_2\bullet \rightleftharpoons \text{ONOO}^- )</td>
</tr>
<tr>
<td>Nitrogen dioxide</td>
<td>( \bullet \text{NO}_2 )</td>
<td>( \bullet \text{NO}_2 + \text{e}^- \rightleftharpoons \text{NO}_2^- )</td>
</tr>
<tr>
<td>Nitrous acid</td>
<td>\text{HNO}_2</td>
<td>( \text{ONO}^- + \text{H}^+ \rightleftharpoons \text{ONO}_2^- )</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>\text{ONOO}^-</td>
<td>( \text{ONO}_2^- \rightarrow \text{ ONOO}^- \rightarrow \text{ ONOO}_2^- \rightarrow \text{ ONOO}_3^- + \text{H}^+ )</td>
</tr>
<tr>
<td>Peroxynitrous acid</td>
<td>\text{ONO}_2^-</td>
<td>( \text{ONO}_2^- \rightarrow \text{ONO}_3^- + \text{H}^+ )</td>
</tr>
<tr>
<td>Alkyl peroxynitrites</td>
<td>\text{ROONO}^-</td>
<td>( \text{ROONO}^- \rightarrow \text{ ROON}_2^- \rightarrow \text{ ROON}_3^- + \text{H}^+ )</td>
</tr>
<tr>
<td>Alkyl peroxynitrates</td>
<td>\text{RO}_2\text{ONO}^-</td>
<td>( \text{RO}_2\text{ONO}^- \rightarrow \text{ ROO}_2\text{ONO}^- \rightarrow \text{ ROO}_3\text{ONO}^- \rightarrow \text{ ROO}_4\text{ONO}^- + \text{H}^+ )</td>
</tr>
</tbody>
</table>

By discharging in oxygen containing gases, ozone is one of the main antimicrobial agents, especially with low humidity. Besides ozone, other reactive species have also been studied on their reaction with water. Yusupov et al. (2014) investigated the interactions of O, OH, HO\(_2\) radicals and H\(_2\)O\(_2\) with water molecules, and revealed the immediate reaction of O atoms with water molecules. This reaction results in the formation of two OH radicals, which further exchange a hydrogen atom and reform the reactants. Therefore, OH, HO\(_2\) and H\(_2\)O\(_2\) can travel through the water layer and
eventually reach the surface of biomolecules. Moreover, O$_2^-$ and H$_2$O$^+$ can be formed during diffusion of those radicals in water, which will further involved in cell interactions.

As a main composition of air, dissociation of N$_2$ molecules has been well studied, which requires much more energy than other compositions. N$_2$ molecules were dissociated through two main mechanisms and transformed into excited N$_2$ (Shintani et al., 2010). However, discharges in N$_2$-O$_2$ mixtures, which is commonly used as air or other gas mixtures, are considerably different from pure N$_2$ or O$_2$ dissociations. The generation of N atoms will decrease with the addition of O$_2$ above some critical percentage (8-10%), while NO density increases (Capitelli et al., 2013). Moreover, simultaneous production of O$_2^-$ and NO can lead to the generation of ONOO$^-$ (as in table 2), which may cause significant injury to different cellular structures (Novo and Parola, 2008).

Except N$_2$ and O$_2$ as usual air composition, CO$_2$ has also been widely used in industrial manufacturing processes. Plasma treatment can dissociate CO$_2$ and result in CO and O$_2$ (from generation of O atoms). In CO$_2$-O$_2$ mixture, the dissociation mainly results in the generation of O atoms, with CO and CO$_2^*$ intermediated. However, the presence of water molecules may help speed up conversion of CO to CO$_2$ by generating HO$_2$, OH and H radicals (Fridman, 2008a).

Besides reacting with reactive species, water molecules can be dissociated in gas phase as water vapour. However, the energy efficiency of H$_2$O dissociation is usually low, because of the reverse reactions (Fridman, 2008a).

ACP generated reactive species discussed above have strong antimicrobial effect by targeting cell components. There are many studies on microbial inactivation mechanisms of ACP treatment, which attribute inactivation to cell morphology
changes and intracellular damage. Electric field generated with input voltage has a polarization effect on cells, which is also a killing effect. Further inactivation effects are combined results of all the species, although the inactive interactions may vary because of the composition of the utilised gas (Alkawareek et al., 2012). Whereas, reactive species generated (ROS/RNS) during the discharges may play the main role on inactivation.

1.3.1 Cell morphology and surface damage

Basically, ACP treatment devices are electric field generators, which can ionize gases inside a field. The electric field has a polarization effect on cells within the discharge area, resulting in morphology changes and ultimately cause cell death.

Cell morphology and surface changes can be easily detected by microscopy (bright field microscopy, fluorescence microscopy, scanning electron microscopy and confocal microscopy). El-Hag et al. (2011) studied the electric field influence on microbe size and shape with confocal microscopy. Reduction of cell sized was observed through the decrease of cell radius, while greater reductions were observed for spherical cells than elliptical cells. Their study also showed that cells with smaller size and thicker membranes have lower cell number reductions by electric field treatment. Similarly, bacillary cells became coccoid and shrunken after ACP treatment, while irregular shapes were also observed by bright field microscopy and fluorescence microscopy (Joshi et al., 2011; Kvam et al., 2012). Moreover, electric field can cause the disruption of cell membrane, while Gram positive bacteria can support higher turgor pressure than Gram negative bacteria (Laroussi et al., 2003). A careful investigation of the target of these charges revealed that most processes occur on the cell membrane, including the phospholipid bilayer of mammalian cells or the polysaccharide membrane of bacterial cells, indicating the primary target of reactive
species generated by discharge systems is cell envelope (Dobrynin et al., 2009). On the other hand, electric field also has a killing effect on eukaryotic cells. Treating dinoflagellates with non-thermal plasma, Tang et al. (2008) found that plasma effects the surface of some species of dinoflagellates, with morphological changes and cell shrinkage observed by scanning electron microscopy.

Reactive species generated by ACP can directly react with cell envelope components, which will result in cell wall damage and cause subsequent effects leading to inactivation. The difference structure between Gram negative and positive bacteria results in different effects on to cell shape and surface damage by ACP treatment (Figure 1.3).

![Cell envelope structure of Gram negative and Gram positive bacteria](image)

**Figure 1.3** Cell envelope structure of Gram negative and Gram positive bacteria

Adapted from (Brown et al., 2015)

Gram negative bacteria, e.g. *E. coli*, have disrupted cell membranes, while Gram positive bacteria exhibit no obvious damage of cell surface. The rigidity of the cell membrane of *B. subtilis* could be the reason of different visible response reported by
(Laroussi et al., 2003). This could due to the tight structure and strength of Gram positive bacteria cell wall consisting of peptidoglycan, while Gram negative bacteria are covered by a thin layer of peptidoglycan and an outer membrane containing lipopolysaccharide.

During plasma treatment, generated ROS can react with both lipopolysaccharide and peptidoglycan thus breaking the molecule structure by damaging C-O, C-N and C=C bonds (Figure 1.4) (Chung et al., 2013; Yusupov et al., 2013; Yusupov et al., 2012). Furthermore, polysaccharides are easier to peroxidise than lipids (Dobrynin et al., 2009). Due to the outer membrane components, envelopes of Gram negative bacteria are possibly more vulnerable than Gram positive bacteria and mammalian cells.

![Figure 1.4 Breaking mechanisms of important bonds in biomolecules](image-url)
(a), (b), (c) Three steps of O atom interaction and formation of C=C bond; (d), (e), (f) Breaking mechanisms of three important ether C–O bonds by O atom; (g), (h), (i) Three steps of O atom breaking C-N bonds. Adapted from Yusupov et al. (2012)

1.3.2 Intracellular damage

Reactive species generated by ACP have a strong oxidative stress on cells. The small size of reactive molecules can easily cross cell membranes and cause intracellular damage. Lipid peroxidation, which leads to cell leakage, was detected by methylenedianiline (MDA) measurements (Joshi et al., 2011). The product of lipid peroxidation, MDA, also participates in DNA damage. Reactive species have strong oxidizing effects on the double bonds of unsaturated lipids and the sulfhydryl groups of enzymes, which are important for maintaining cell viability (Cabisco et al., 2000). UV has been reported to induce dimer and cross dimer of DNA thymine, which is also a lethal mechanism (Dobrynin et al., 2009; Joshi et al., 2011; Laroussi and Leipold, 2004). In former studies, thymine cross-linking, DNA double-strand breaks (DSBs) and DNA single-strand breaks (SSBs) were observed following various ACP exposure doses (Dobrynin et al., 2009). ROS and products from ROS attack may also react with DNA and proteins. DNA adducts generated by reactive aldehydes can cause mutagenesis of genomic DNA, which may lead to cell death. Comparing to mammalian cells, bacterial DNA is more exposed to reactive species because of frequency of replication, which renders the unfolded DNA more vulnerable.

With the presence of water molecules in the discharge area, OH• molecules are produced in DBD plasma. OH molecules can pass through cell membrane easily and cause lethal effects (Imlay et al., 1988). The OH• radicals can react with all the purine/pyrimidine bases as well as the deoxyribose backbone generating both base-
derived and sugar-derived products, by attacking C-H and C=C bonds. Some ROS, such as O$_2^{-}$ and H$_2$O$_2$ are likely to convert into OH’ radicals via the Fenton reaction and Haber-Weiss reaction, and cause cellular damage (Figure 1.5).

![Figure 1.5 Cellular oxidative damaging mechanism](image)

Reactions between O$_2^{-}$, H$_2$O$_2$ and OH’ radicals. Adapted from (Graves, 2012)

OH’ radicals can also react with proteins surrounding DNA (e.g., histone) and produce DNA-protein cross-links, as the most reactive oxidant (Figure 1.6 a). Moreover, these agents can cause SSBs or DSBs in the DNA, base modifications, helix-distorting bulky lesions or cross-links of DNA strands that are repaired by biochemically distinct DNA repair pathways (Figure 1.6 b) (Arjunan et al., 2015). It is also reported that ACP has inactivation effect on in vitro enzymes, indicating that ACP is able to change the complex three-dimensional structure, thus inactivation key enzymes (Dobrynin et al., 2009).
Figure 1.6 ROS induce strand breaks in DNA

(a) •OH radical-induced products of the deoxyribose sugar in DNA and (b) ROS-induced SSBs containing blocked termini such as 3′-phosphoglycolate, 3′-phosphate,
5′-OH and 5′-deoxyribosephosphate. Adapted from Arjunan et al. (2015).

Gaseous nitric oxide (NO) and nitrogen dioxide (NO₂) have been proven to induce DNA SSBs by (Görsdorf et al., 1990). RNS can be rapidly endogenously formed due to the reaction of nitric oxide and superoxide, can damage proteins, lipids and DNA as cited in Shigenaga et al. (1997). Nitric oxide could convert into other RNS such as ONOO⁻, HNO₂, and N₂O₃. N₂O₃ can directly react with DNA, causing nitrosation of the primary amines in DNA and lead to deamination. Nucleobases, especially guanine, adenine and cytosine are deaminated to xanthine, hypoxanthine and uracil, respectively. This could cause mispairing during replication and lead to mutation or SSBs. ONOO⁻ reacts only with guanine, and produce 8-oxo-guanine and 8-nitro-guanine by oxidizing or nitrosating respectively. This can also leave apurinic/apyrimidinic (AP) sites which can lead to the formation of an SSB. ONOO⁻ also directly attacks the sugar phosphate backbone of the DNA by abstracting an H atom from the deoxyribose, which then opens the deoxyribose sugar generating strand breaks (Arjunan et al., 2015). It is also a strong oxidant able to react directly with thiol groups, iron-sulphur centres and the active site -SH groups in tyrosine phosphatases. In physiological conditions, the production of ONOO- is quite low and oxidative injury is minimized by endogenous antioxidant defences. When increased in pathological conditions, ONOO- can act either as a direct oxidising species or indirectly by decomposing into highly reactive radicals and can react with proteins (tyrosine nitration or direct reactions with specific amino acids), lipids (lipid peroxidation) and nucleic acids (Capitelli et al., 2013). Moreover, the inactivation efficacy of RNS could be stimulated with the presence of ROS, and finally increase the products as nitrite and nitrate in solution (Boxhammer et al., 2012).
Overall, atmospheric cold plasma is a ‘chemical cocktail’ of cell lethal species, which act with both cell envelope and intracellular components. However, microbes have stress response systems to increase their resistance during ACP treatment.

1.4 Microbial response in industrial processing

1.4.1 Food contamination

The risk associated with the transmission of foodborne pathogenic infections has become a global issue that affects the entire food industry (Doyle and Erickson, 2006). In the European Union, in 2011, a total of 5,648 food-borne outbreaks were reported, resulting in 69,553 human cases, 7,125 hospitalizations and 93 deaths (EFSA and ECDC, 2013).

Meat safety remains a major industrial challenge presented by the emergence of pathogens with low infectious doses, increased virulence and resistance to antibiotics and cross-contamination or recontamination of foods, food contact surfaces and water within the food production chain (Sofos and Geornaras, 2010). The main pathogens associated with meat produce include Bacillus cereus, Campylobacter spp., Clostridium perfringens, Clostridium botulinum, Escherichia coli, Listeria monocytogenes, Salmonella spp., Staphylococcus aureus, Yersinia enterocolitica, Aeromonas, Brucella, Clostridium difficile, Enterobacter and Shigella (Stoica et al., 2014). In real food processing environments, most human pathogens grow predominantly as biofilms (Giaouris et al., 2014), which may form in all areas, including floors, walls, pipes and drains (Sofos and Geornaras, 2010). Biofilms are defined as complex microbial communities enclosed in hydrated extracellular polymeric substances (EPS), which comprise polysaccharides, proteins,
phospholipids, teichoic and nucleic acids (Shi and Zhu, 2009). Biofilms are characterized by an enhanced resistance as compared to their planktonic counterparts to most environmental stresses encountered in food production plants (Bridier et al., 2014; Giaouris et al., 2014). Therefore, in the light of the issues associated with biofilm resistance, diversity of produce and microorganisms implicated in food related health outbreaks and an increase in numbers of the reported human illnesses there is a need for the development of more efficient strategies to reduce the risks linked with microbiological safety of food produce.

*E. coli* and *S. aureus* are key food-contaminating pathogens, and have been reported to have strong multi-drug resistance (Braoudaki and Hilton, 2004; Brown, 2001). Their high rate of mutations can lead to cross protective effects against environmental stresses, including oxidative stress. It has been reported that *L. monocytogenes* has the capability to reproduce at low temperatures with the regulation of cold shock proteins (Bayles et al., 1996), making it a major pathogen of concern in the cold chain. Moreover, *L. monocytogenes* has been shown to be the most resistant on meat surfaces compared with the other main food pathogens (Chorianopoulos, 2012). Therefore, our study was focused on these three bacteria, which represent both Gram negative and positive pathogens.

### 1.4.2 Modified atmosphere packaging

Modified atmosphere packaging (MAP) is widely used in the food industry to avoid contamination and weight loss and also applied for extending shelf-life (Kerry et al., 2006; Sivertsvik et al., 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). Oxygen and CO₂ are also popular MAP gas contents, which were found to be effective in
inhibiting enzymatic browning and controlling microbial quality (Jacxsens et al., 2001).

In MAP of fruits and vegetables, both High Oxygen Atmospheres (HOA) (i.e. >70% O₂) and low O₂ Equilibrium Modified Atmosphere (EMA) packaging (3% O₂–5% CO₂–balance N₂) were used for packaging of ready-to-eat vegetables and respiring products, respectively (Jacxsens et al., 2001). However, relatively high O₂ atmospheres, more than 70%, were found to reduce the food quality in carrots (Amanatidou et al., 2000). Therefore, the percentage of oxygen content may vary in line with product characteristics and requirements.

In the meat industry, high oxygen levels (70–80%) are also used in MAP to reduce microbial growth within the package and preserve the bright red colour of fresh meat as well as tenderness and juiciness (Lund et al., 2007; Okayama et al., 1995). Carbon dioxide is also employed for inhibiting bacterial growth (Sivertsvik et al., 2002), protein oxidation and maintaining the red colour of meat products. Typically, fresh red meat MAP uses 70% O₂+30% CO₂ (Sørheim et al., 1999) and cooked meats are stored in 70% N₂+30% CO₂ (Smiddy et al., 2002).

1.4.3 Microbial resistance response in food processing environment

Most of the foodborne microorganisms are able to survive under diverse conditions by adaptive responses, including preservation technologies like high salts, high fat, high temperature and low pH. Among all microbes, E. coli is the most important and well known food pathogen. It has been well studied under multi environmental stress response.

RpoS is one of the most important transcriptional regulator in E. coli, not only for stationary phase growth but also different stress conditions, such as starvation, hyper-
osmolarity, pH downshift, or non-optimal high or low temperature (Hengge-Aronis, 2002).

During starvation, metabolic activity and growth were reduced and many degradative enzymes and substrate capturing enzymes were produced, such as proteases, lipases, glutamine synthetase and alkaline phosphatase (Chung et al., 2006; Kjelleberg et al., 1987; Matin et al., 1989; Siegele and Kolter, 1992). Moreover, glucose starvation can induce the increase of DnaK protein synthesis, thermotolerant, H$_2$O$_2$-resistant and changes of morphology (Rockabrand et al., 1995).

It is also noticed that starvation can improve the osmotic resistance in *E. coli* by inducing similar proteins, such as Pex starvation proteins and heat shock proteins (Jenkins et al., 1990). Two protective mechanisms were usually involved in surviving hyperosmotic stress, discharging the excess solutes outside the cells and accumulating compatible solutes or osmolytes (Griffiths, 2005).

In the food environment, the decrease of pH could be attributed to the results of fermentation or acid preservatives. Food pathogens can develop pH dependant or pH independent acid tolerance under low pH conditions, which enhanced microbial survival in fermentation food such as salami sausage or apple cider (Leyer et al., 1995).

The formation of cyclopropane fatty acid (CFA) content was reported as a major factor in the acid resistance of *E. coli*, which is regulated by RpoS (Chang and Cronan, 1999). As a main concern of food safety, acid resistance of *E. coli* O157 could not only increase the resistance to other environmental stress, but also enhance the virulence (Beales, 2004; Benito et al., 1999; Buchanan et al., 1999; Mazzotta, 2001).

The outcome of non-optimal temperature exposure includes heat shock and cold shock, which are widely used in food sterilization and preservation procedures. Many of the environmental stresses stated above can induce heat resistance in *E. coli*, where the
synthesis of heat shock proteins are observed. Over 30 proteins associated with the physiological response to heat have been identified, including GroES, GroEL, and DnaK chaperone proteins. They are involved in the folding, repair and degradation of proteins (Seyer et al., 2003). Moreover, DnaK interfaces extensively with the upstream chaperon trigger factor and the downstream chaperonin, which make it the central hub in E. coli network (Calloni et al., 2012).

Similarly, heat resistance can result in resistance to other environmental stress caused by food processing. The response to cold temperature is mainly due to synthesis of cold shock proteins. They bind RNA without apparent sequence specificity and with low binding affinity (Jiang et al., 1997). These cold shock proteins function as RNA chaperones at low temperatures (Graumann and Marahiel, 1998).

During respiration and other metabolic reactions, reactive oxygen species could be generated under aerobic conditions, including hydroxyl radical (OH\(^-\)), superoxide anion (O\(_2\)\(^-\)), peroxyl radical (ROO\(^-\)) and hydrogen peroxide (H\(_2\)O\(_2\)) (González-Flecha and Demple, 1995; Imlay, 2003). As described before, reactive oxygen species have a damaging effect on biological macromolecules, such as breaking membrane functions, affecting enzyme activities, and causing DNA damage, which lead to cell death (Imlay, 2003). Virtually all aerobic organisms have evolved complex antioxidant defence systems, comprising low molecular weight antioxidants, antioxidant enzymes for scavenging ROS, and reparation enzymes for removing oxidative damage of macromolecules (Pomposiello et al., 2001). The oxidative stress response in E. coli also overlaps with other stress, such as heat shock, carbon starvation or DNA damage, where the SOS response involved (Farr and Kogoma, 1991). Low molecular weight antioxidants comprise endogenous antioxidants (uric acid, coenzyme Q, lipoic acid etc.) and nutritional antioxidants (vitamin C, vitamin E, carotenoids and phenolic
compounds) (Grune et al. 2004). There are several genes reported to be important under oxidative stress.

SoxRS and OxyR are two crucial redox-responsive transcription regulators in *E. coli* and serve as paradigms of redox-operated genetic switches (Figure 1.7).

![Figure 1.7 Activation of OxyR (a) and SoxRS (b) regulons in *E. coli*. Adapted from Imlay (2013).](image-url)
Their activation is associated with the transcription of sets of genes (regulons), whose products relieve the stress by eliminating oxidants and preventing or repairing oxidative damage (Pomposiello et al., 2001).

Oxidative species, such as hydrogen peroxide, can oxidize the thiol groups and form disulfide bonds (Figure 1.7 a). This reaction can activate OxyR regulon, which further acts as a transcription activator and induces a series of antioxidative enzymes (Cabisco et al., 2000; Christman et al., 1989; Mukhopadhyay and Schellhorn, 1997). In OxyR regulon, YaaA protein has been found to be a key element for hydrogen peroxide response by suppress intracellular iron levels (Liu et al., 2011). Additionally, several operons, including catalase-peroxidase (katG) and alkyl hydroperoxide reductase (ahpCF) can also be inactivated by OxyR, depending on the exogenous and endogenous H₂O₂ concentration (Mai-Prochnow et al., 2014).

The oxidation of [2Fe-2S] cluster of the subunit can activate SoxR, which can be shut down when reduced (Figure 1.7 b). Oxidation of SoxR induces the transcription of the soxS gene, which encoding the protein binds promoter regions of the target genes to recruit RNA polymerase (Pomposiello et al., 2001). These inducible genes associate the antioxidative and repairing activities, including manganese superoxide dismutase (sodA), the DNA repair enzyme endonuclease IV (nfo), and superoxide resistant isozymes of fumarase (fumC) and aconitase (acnA).

Listeria monocytogenes also has capability of developing adaption to environmental stress, such as acid, osmotic, heat and cold stress. The adaptation of microorganisms to low temperature represents a food safety risk since refrigeration is one of the most common methods used for preserving foods, where L. monocytogenes is the main concern in the cold chain. (Garcia et al., 2001; Russell, 2002). The resistance against environmental stress has been reported to afford cross protection to other stress, and
able to enhance the virulence of infection on target cells (Faleiro et al., 2003; Gahan and Hill, 1999). The σ^B factor is the main resistance regulator under environmental stress by upregulating associated genes, while PrfA also play an important role (Kazmierczak et al., 2003; Kazmierczak et al., 2006).

The σ^B factor also associated with multi resistance response in *S. aureus*, except starvation. It could be involved in recovery from heat shock and in acid and hydrogen peroxide resistance but as yet not observed in resistance to ethanol or osmotic shock (Chan et al., 1998). A recognition site for σ^B was located in the promoter region of *katA*, the gene encoding the sole catalase (Horsburgh et al., 2002). Under oxidative stress, ClpP protease plays an important role in stress response of *S. aureus* by regulating associated genes, which involved in oxidative stress response, autolysis and SOS DNA repair (Michel et al., 2006). *S. aureus* is well known for its strong antimicrobial resistance profile. β-lactam antibiotics exert bactericidal effect by inhibiting the synthesis of cell wall. However, the overexpression of a number of genes induced by these inhibitors led to antibiotic resistance. *PbpB* is an essential gene for cell wall synthesis, which is associated with *vraSR* and some additional genes that belong to the VraSR regulon which led to β-lactam resistance, such as stress responses genes *msrA*, *htrA*, *psrA* and *hslO* (Michel et al., 2006; Utaiida et al., 2003).

### 1.4.4 Cell response and regulation against ACP treatment

ACP has adverse effects on cell surface with strong effects on intracellular components. Bacteria have different repair systems for stresses including oxidizing stress. Repairing systems are also reported as further research on ACP inactivation in former studies. Those researches focused on specific genes transcription and expression levels. It is observed that there are oxidized bases like 8-oxodeoxyguanosine (8-OHdG), which have a repairing effect on damaged DNA
strands in mammalian cells (Figure 1.8). Accumulation of 8-OHdG was detected in ACP treated cells, which revealed the recovery system started by the oxidative damage of DNA. (Brun et al., 2012)

Figure 1.8 Reaction of 2’-deoxyguanosine with hydroxyl radicals
Radical adducts followed by reduction to 7-hydro-8-hydroxy-2’-deoxyguanosine, and by oxidation to 8-hydroxy-2’-deoxyguanosine (8-OHdG) or its tautomer 8-oxo-7-hydro-2’-deoxyguanosine (8-oxodG). Adapted from Brun et al. (2012)
Similarly, the effect of ACP on mammalian cells have been shown to lead to formation of DNA strand breaks, which can arrest replication. Histone are chief protein in formation of chromatin in eukaryotic cells. The phosphorylation on the H2AX histone at serine 139 occurs following ionizing or other DNA damage treatment and generates γ-H2AX as product, which is used as an novel indicator for DNA DSBs (Kuo and Yang, 2008; Rogakou et al., 1998). Kalghatgi et al. (2011) studied DBD-ACP induced DNA damage in mammalian cells by testing γ-H2AX. The amount of detected DSBs varied with the plasma dose. With cell response to ACP stress, the SOS and other repair system made the DNA damage of DSBs reversible for lower plasma doses (shorter treatment time/ lower voltage or input power), which induce repairable damages on cell components. This repair effect mostly occurs in mammalian cells rather than bacteria cells, while bacteria have a strong repair system for thymine dimers. Minimal effects (repairable damages, comparing with other treated results) on DNA were observed at plasma doses below 1 J cm$^{-2}$, while DBSs were observed at higher doses of 2 to 6 J cm$^{-2}$ (Kalghatgi, 2010). However, there are repairing effects of DBSs within 24 h. Cell apoptosis occurred with ACP doses above 7 J cm$^{-2}$, which caused sufficient DNA damage. Intracellular DSBs are related to ROS generated during ACP discharges and can be blocked by intracellular and extracellular anti-oxidants. (Kalghatgi, 2010)

Additionally, positive ions generated during a plasma discharge can be involved in chain oxidation processes and result in oxidation or peroxidation of cell components. They also have an effect on ion-channel activities in cells, which can change the concentrations of ions such as calcium within cells (Dobrynin et al., 2009). Correlated changes of genes expression reveals both damage and repair mechanisms. Sharma et al. (2009) investigated the differential gene expression on a genome-wide
scale of *E. coli* after ACP exposure. With micro-array analysis, it showed higher expression of SOS regulon, oxidative correlated genes and DNA repair genes with minimized housekeeping genes. The microarray analysis was based on results of *E. coli* K12 MG1655 after 120 sec exposure to ACP. A gene involved in the removal of superoxide radicals, *katG* was noted to have a 10-fold increase in expression. *RecA*, a gene involved in DNA recombination and repair, was also greatly up-regulated. At the same time, most housekeeping genes, including genes involved in metabolism and transportation, were observed to be down-regulated. In *Deinococcus radiodurans*, a highly resistant bacteria to radiation, Roth et al. (2010) reported an increase of expression of genes involved in DNA repair, oxidative stress response and cell wall synthesis. Thus, the repair systems are targeted on the damaged caused by UV and oxygen radicals resulting from ACP exposure.

As a result of DNA damage, there are chances of mistakes in repairing response. This could cause mutagenesis in target microbes and induce the microbial resistance to ACP treatment. Boxhammer et al. (2013) studied the mutagenic effect of ACP with bactericidal dosages. The results interestingly showed the UV radiation dominates the mutation rather than other reactive species. This could due to the dimer and cross dimer formation caused by UV, instead of strand breakage by ROS or RNS. Whereas, reactive species has strong damaging effect on cell components, which could led to cell death and minimize the survival of UV mutations.

### 1.5 Research questions

The aim of the current study is optimisation of ACP process parameters and to assess mechanism of actions. The microbial inactivation efficacy of ACP treatment is governed by system, process and target product parameters. The mechanism of
inactivation relies on the generation and interaction of reactive species. The corresponding cell damage and response can be attributed to these reactive species with target strains. Hence, both Gram negative and positive bacteria were involved in this study.

To apply a comprehensive investigation on this mechanism, the study design is outlined below:

- In order to set up the primary experimental study conditions, the effect of incubation media, treatment time, post-treatment storage time and mode of exposure were evaluated on standard strains of both Gram negative and positive bacteria, and is described in Chapter 3. The entire study employs high voltage levels, but here a relatively low voltage was applied at 40 kV against both *E. coli* and *L. monocytogenes*. The effect of those parameters on inactivation efficacy and cell membrane was obtained, which provided us with a primary understanding of the ACP inactivation mechanism with the DBD-ACP.

- To further optimize the ACP treatment, further process and system parameters were involved (Chapter 4). In this section, a range of higher voltages and two different gas mixtures, in addition to air, were applied, while *E. coli* NCTC 12900 strain was included in studies of the cell damaging mechanism. Cell membrane integrity and DNA damage were assessed to reveal the different inactivation mechanisms in Gram negative and positive bacteria chosen.

- In order to further investigate the reactive species generation and interaction with microbes during ACP treatment, a complex reactive species study was designed. The working gas mixtures were retained for evaluation with regards to the generation of different types of ROS/RNS. The inactivation of the 3
microbial contaminants *E. coli*, *L. monocytogenes*, *S. aureus* and their interaction with those reactive species further elucidated the plasma microbicidal action.

- To further understand the mode of action of ACP on microbes, the generation of reactive oxygen species and their cell penetration effect were investigated on both Gram negative and positive bacteria and is described in Chapter 6. The results provided a clearer understanding of the correlation of process and system parameters and target bacteria with inactivation efficacy.

- To investigate the regulatory response of microbes in tandem with critical processing parameters, the inactivation efficacy, intracellular ROS levels and cell membrane permeability were evaluated in several knock-out mutants of *E. coli* deficient in key components of the oxidative or general stress response after air ACP treatment. The understanding of cell surviving mechanism is another potential factor to be considered for the setup of processing parameters with respect to microbial resistance.

- The ultimate aim for expanding understanding of DBD-ACP with regard to interactions with microbiological targets is to further develop appropriate application to the food industry and among others with the understanding of the mechanism of actions. In order to assess the suitability of this technology for decontamination of meat products a number of key parameters were systematically investigated in Chapter 8. Key product parameters included the nature of the microbial contamination (A. microbial species) as well as product characteristics (B. product/medium composition) whereas process parameters comprised 1. voltage levels, 2. storage temperature and 3. gas composition. Initially, an optimal voltage level was determined for inactivation of the 3
microbial contaminants *E. coli, L. monocytogenes, S. aureus* (grown in the more realistic and resistant form of biofilms). This setting was then used for further investigations of the influence of medium composition in connection with storage temperature and with gas composition. The generation of intracellular ROS was compared to microbial inactivation levels to determine the influence these control parameters have on decontamination efficacy.
Chapter 2. MATERIALS AND METHODS

In this chapter, experimental materials and methods are described. Due to the ongoing contribution of this work towards ACP system design and optimisation, modifications to materials and methods were applied as appropriate throughout the study.

2.1 Experimental Design

In order to setup experimental conditions, a preliminary investigation was performed to assess effect of overnight culture, and some process and system parameters. Further studies on optimization of ACP system expanded to include more system process variables and post-treatment analysis. Hence, plasma generation devices and the packaging method were thus improved to facilitate either higher power or enhanced sealing to promote contact time and retention of species. This allowed a range of post-treatment analysis, in order to address the key research questions. Detailed experimental conditions and analysis are listed in Table 2.1.
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<td>8 Investigation of ACP application on meat model</td>
<td><em>E. coli</em> NCTC 12900, <em>L. monocytogenes</em> NCTC 11994, <em>S. aureus</em> NCTC 1803</td>
<td>Biofilm, $10^7$ CFU ml&lt;sup&gt;-1&lt;/sup&gt;, 12% BE, 3% BE, 12% BE</td>
<td>TSB, PBS</td>
<td>DIT 120 plus</td>
<td>60, 70, 80</td>
<td>Air, MP1, MP2</td>
<td>15, 30, 60, 120, 300</td>
<td>24</td>
<td>Direct</td>
<td>Microbiology analysis Ozone measurements ROS measurements Modelling microbial recovery Meat studies</td>
<td></td>
</tr>
</tbody>
</table>
2.2 Microbial strains, growth conditions and preparation of samples

The microorganisms, treatment media and ACP technologies used in this study are listed in Table 2.1. *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* strains were selected as the pertinent challenge microorganisms for studying the efficacy of the applied DBD-ACP treatments on both Gram positive and negative bacteria.

The specific microbial strains, growth conditions and preparation of cell suspensions are described below in Table 2.2.

Table 2.2 List of microorganisms, source information

<table>
<thead>
<tr>
<th>Gram negative pathogens</th>
<th>Microorganisms</th>
<th>Storage/Source information</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> ATCC 25922 (generic strain)</td>
<td>School of Food Science and Environmental Health, Dublin Institute of Technology</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> NCTC 12900 (non-toxigenic O157:H7)</td>
<td>National Collection of Type Cultures of the Health Protection Agency (HPA, UK)</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> BW 25113 (parent strain of knockout mutants)</td>
<td>All from National BioResource Project, Japan (NIG, Japan) (Baba et al., 2006)</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> JW 4024 (<em>ΔsoxR</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> JW 4023 (<em>ΔsoxS</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> JW 3933 (<em>ΔoxyR</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> JW 5437 (<em>ΔrpoS</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> JW 0013 (<em>ΔdnaK</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em> NCTC 11994</td>
<td>All from School of Food Science and Environmental Health, Dublin Institute of Technology</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 25923</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> NCTC 1803</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In cell regulatory response study, five mutants with knockout genes associated with stress regulation and responses were selected to investigate their role in providing protection against ACP or whether absence confirms enhanced sensitivity towards ACP (Table 2.3).

**Table 2.3 List of knock-out mutants and their characteristics**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Description</th>
<th>Gene information</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> BW 25113</td>
<td>Parent strain</td>
<td>A derivative of the F-, λ-, <em>E. coli</em> K-12 strain (Datsenko and Wanner, 2000)</td>
</tr>
<tr>
<td><em>E. coli</em> JW 5437</td>
<td>ΔrpoS</td>
<td>Regulatory factor, influence on expression of genes for stationary phase or stress response (Cheville et al., 1996; Matsuoka and Shimizu, 2011)</td>
</tr>
<tr>
<td><em>E. coli</em> JW 4024</td>
<td>ΔsoxR</td>
<td>Correlated with oxidative damage repairing and antioxidative actions (Nunoshiba et al., 1992), can be induced by both ROS and RNS (Ding and Demple, 2000; Greenberg et al., 1990)</td>
</tr>
<tr>
<td><em>E. coli</em> JW 4023</td>
<td>ΔsoxS</td>
<td>Triggered by soxR under oxidative stress (Pomposiello et al., 2001), and stimulates genes for oxidative and antibiotic resistance (Demple, 1996).</td>
</tr>
<tr>
<td><em>E. coli</em> JW 3933</td>
<td>ΔoxyR</td>
<td>Required for the induction of a regulon of hydrogen peroxide-inducible genes (Christman et al., 1989)</td>
</tr>
<tr>
<td><em>E. coli</em> JW 0013</td>
<td>ΔdnaK</td>
<td>Participating in initiation of DNA replication, which plays an important role in DNA repair, and is the central hub of chaperone network for regulation and stress response (Calloni et al., 2012; Skowyra et al., 1990)</td>
</tr>
</tbody>
</table>

### 2.2.1 Storage and Growth conditions

Bacterial strains were maintained as frozen stocks at -70 °C in the form of protective beads (Technical Services Consultants Ltd, UK), which were plated onto Tryptic Soy Agar (TSA, Scharlau Chemie, Barcelona, Spain) and incubated at 37 °C to obtain single colonies before storage at 4 °C.
A single colony was inoculated into Tryptic Soya Broth with Glucose (TSB+G 0.25%, Scharlau Chemie, Barcelona, Spain), Tryptic Soya Broth without Glucose (TSB-G) or into 12% beef extract (BE, Scharlau Chemie, Barcelona, Spain) and incubated overnight (18 h) at 37 °C.

2.2.2 Preparation of samples

Both planktonic cells and biofilms were used in our studies. Procedures for preparation are described below.

For planktonic samples, cells were harvested by centrifugation at 8,720 g for 10 min after overnight incubation. The cell pellet was washed twice with sterile phosphate buffered saline (PBS, Oxoid LTD, UK). The bacterial cell density of overnight culture was determined by measuring absorbance at 550 nm using McFarland standard (BioMérieux, Marcy -l'Etoile, France) and a working inoculum corresponding to required 1.0 ×10⁸ CFU ml⁻¹ was prepared. Cells were then adjusted to the required density of each experiment with different treatment media (as described in Table 3).

For planktonic samples, three types of sample holders were used, including 92×16 mm petridish, 6 well plate or 96 well plate (all from Sarstedt, Nümbrecht, Germany). Prior to ACP treatment, aliquots of 10 or 3 ml of cell suspensions were dispensed into petridish or 6 well plate, respectively. For experiments with a smaller liquid volume, 100 µl/well cell suspensions in PBS, 3% or 12% BE were dispensed into the wells of 96 well microtiter plate.

Bacterial biofilms were grown by adding 200 µl of 12% BE bacterial suspension with a cell density of 1.0 × 10⁷ CFU ml⁻¹ into the wells of 96 well microtiter plates (Sarstedt). The plates were incubated at 37 °C for 48 h. After 24 h of incubation the supernatant from each well was replaced with fresh BE, with further incubation for 24 h. After incubation, the BE containing suspended bacterial cells was removed and
wells were rinsed three times with sterile deionized water and dried, leaving only attached bacterial biofilms for further investigation. Negative controls were obtained by using BE without inoculation. Prior to each experiment, those microtiter plates containing biofilms were air dried for 60 min.

Biofilm and planktonic samples of 100 µl/well in 96 well plates were used in the meat model study, while 3 ml cell suspensions in 6 well plates were used for studies of ACP characterisation. All other experiments used 10 ml cell suspensions in petridishes.

2.3 **DBD-ACP system & extrinsic parameters**

The schematic diagrams of the Dielectric-Barrier Discharge (DBD) atmospheric cold plasma (ACP) systems used in this study are shown in Figure 2.1 and 2.2.

Steady state of plasma generation was achieved prior to each experiment by predischarging for 5 min using separate empty plastic containers to ensure consistency.

2.3.1 **DIT 60**

DBD-ACP plasma generation device DIT 60 was used for studies on effect of glucose, treatment and post-treatment time effect, mode of exposure and SEM sample preparation.

DIT 60 consisted of a high voltage transformer, a voltage variac (0 – 100%, output voltage could be controlled within 0~60 kV), and two aluminium electrodes for generation of plasma. The system was operated at 40 kV at atmospheric pressure. Voltage and input current characteristics of the system were monitored using an InfiniVision 2000 X-Series Oscilloscope (Agilent Technologies Inc., USA). The two electrodes were separated by a dielectric barrier i.e. the polypropylene container, which acted as a sample holder. The distance between the two electrodes was kept identical (2.2 cm, which is the thickness of the polypropylene box) for all experiments.
2.3.2 DIT 120

DIT 120 consists of a high voltage transformer (with input voltage 230 V at 50 Hz), a voltage variac (0 – 100%, output voltage could be controlled within 0~120 kV), and two 15-cm diameter aluminium electrodes for generation of plasma. The system was operated at voltage levels of either 56 kV\text{RMS} or 70 kV\text{RMS} at atmospheric pressure. Voltage and input current characteristics of the system were monitored using an
InfiniVision 2000 X-Series Oscilloscope (Agilent Technologies Inc., USA). The two electrodes were separated by two dielectric barrier, 1mm thick polypropylene boards. A sealed container, which acted as a sample holder and another dielectric barrier, was located between the polypropylene boards. The distance between the two electrodes can be calculated from the thickness of the container.

Figure 2.2 A schematic diagram of the experimental plasma device DIT 120
(a) The schematic diagram of DIT 120; (b) The image of DIT 120 device.
2.3.3 DIT 120 plus

DIT 120 plus was a modified design of DIT 120, with a perspex dielectric layer instead of the polypropylene board between polypropylene box and high voltage electrode. The perspex dielectric layer, with a thickness of 10 mm, provided a more stable discharging environment by preventing unexpected sparking. DIT 120 plus system was used in the studies of microbial regulatory response and for the technology application study using the meat model (Table 2.1).

2.3.4 Plasma treatment

Prepared samples were then placed in a dielectric container and sealed before plasma treatment. Two types of container were used.

2.3.4.1 Packaging and gas mixtures

For all experiments, two different types of containers were used depending on experimental optimization and the particular study, rigid polypropylene plastic box (2.2 cm thick) and PET+PE tray (196*154 mm and 45 mm depth, Holfield Plastics Limited, Ireland). The containers were considered as a dielectric barrier in DBD-ACP treatment.

Packaging procedure was aligned with the type of sample container (polypropylene box or plastic tray) and working gas. Four gases were assessed as plasma working gases in addition to air, which was dependent on the specific application aims. The Safebag project investigating fresh produce application, required gas mixtures SB1: 90% N₂ + 10% O₂ and SB2: 65% O₂ + 30% CO₂ +5% N₂, while Meat Pack project provided MP1: 70% N₂+ 30% CO₂ and MP2: 70% O₂ + 30% CO₂.

For experiments performed in polypropylene boxes (bacterial samples in petri dishes or 96 well plates), each container was sealed inside a polypropylene bag. There was no additional process for samples treated with air, while for other working gas
experiments, a vacuum was applied and the sample was then filled with the required working gas at a controlled flow rate of 0.5 l min\(^{-1}\) for 1 min using a flow regulator. For experiments performed in plastic trays (bacterial samples in 6 well plates or sliced meat), samples were placed inside a PET+PE tray, prior to sealing using a vacuum packager (Lavezzini VG600, UK). The packaging machine was operated at optimised packaging conditions (30 sec 99% vacuum phase; 30 s gas flushing phase; 1.5 sec at 112 ºC sealing time and temperature) according to the selected packaging material, Polyester/BLL/LDPE+anti-fog coating film (thickness 0.06 mm, STEPHENS, Ireland).

2.3.4.2 Voltage

DIT 60 system voltage could be controlled within 0~60 kV. Output power was monitored by peak-to-peak value with oscilloscope. Applied voltage in DIT 60 studies was adjusted to 40 kV. DIT 120 and DIT 120 plus system provided higher voltages, which could be controlled within 0~120 kV. Output power was monitored by peak-to-peak value with oscilloscope and RMS on controller screen. Various output power was investigated through the studies described below and listed in Table 2.1.

2.3.4.3 Mode of exposure

For direct plasma treatment, petri dishes or 96 well plates containing bacterial samples were placed in the centre of the rigid polypropylene plastic container directly between the electrodes within the plasma discharge. In case of indirect plasma treatment, a separate container was used and samples were placed so as to achieve treatment outside the plasma discharge. Each container was sealed within the high barrier polypropylene bag (B2630; Cryovac Sealed Air Ltd, Dunkan, SC, USA) to retain
reactive species inside the package during ACP treatment and post-treatment storage times. Mode of exposure design is shown in Figure 2.3.

(a) Electrodes
Discharging area
Sealed PP container
Direct exposed sample

(b) Electrodes
Discharging area
Sealed PP container
Indirect exposed sample

Figure 2.3 Schematic diagrams of mode of exposure design
(a) Direct exposure; (b) Indirect exposure.

Samples in plastic trays were only treated with direct exposure, because of the similar size of container and electrode.

2.3.4.4 Treatment time

Treatment times ranged from 5 sec to 5 min. The effect of treatment time was investigated in association with inactivation efficacy and pattern and reactive species generation. In each study, both short and long treatment times were applied, in order to observe non-obvious and obvious inactivation and cell damage. Treatment times applied for each experiment were listed in Table 2.1.
2.3.4.5 Post-treatment storage

After treatment, samples were kept sealed and stored for either 0, 1 or 24 h at room temperature prior to unsealing and analysis. Refrigerated storage was used for food related studies in compliance with processing requirements in the food industry. Otherwise, all samples were stored at room temperature.

2.3.4.6 Meat challenge studies

The meat challenge study was performed on 3 types of meat products in conjunction with MAP packaging. Cooked thin-sliced turkey, raw pork loin and raw lamb chop were purchased from a local butcher shop and stored at 4 °C until use. *E. coli* was used for the raw meat samples and *S. aureus* was selected as the challenge microorganism for the cooked sliced turkey. Cell suspensions in PBS with concentration 8-9 Log_{10} CFU ml^{-1} were used as the working inoculum. For inoculation, the samples were visually divided into two parts. Each part was spot-inoculated with 100 µl of bacterial culture. Inoculated samples were dried for 1 h in a laminar flow cabinet to allow the attachment of bacteria to the surface. Prior to the treatment the inoculated samples were placed inside a PET+PE tray and sealed with 30% CO_{2}+70% N_{2} and 30% CO_{2}+70% O_{2} gas using a vacuum packager (as mentioned before).

Based on earlier results, samples were treated with 80 kV_{RMS} ACP for 60 and 300 s. Lamb chop was stored for up to 14 days post treatment with sampling on days 0, 1, 4, 7, 11 and 14. Pork loin was stored for up to 14 days with sampling on days 0, 1, 4, 7, 11 and 14. Cooked turkey slices were stored for up to 35 days with sampling on days 0, 1, 7, 14, 21, 28 and 35. Control samples were untreated. The storage temperature for all samples was 4 °C.
2.4 Post-treatment measurements and analysis

2.4.1 Microbiological analysis

To assess remaining cells of planktonic bacterial populations, serial dilutions were prepared using maximum recovery diluent (MRD, Scharlau Chemie, Barcelona, Spain), which were further plated on TSA with 100 µL on each plate. In order to obtain low microbial detection limits, 1 ml of the treated sample was dispensed and spread onto 3 TSA plates as described by ISO (1996), incubated at 37 ºC for 24 h and counted as total colonies from all 3 plates. Any 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\textsubscript{10} CFU ml\textsuperscript{-1} units. All experiments were carried out in duplicate and replicated at least twice.

To quantify the effects of ACP treatment on biofilm, PBS (100 µL) was added into the wells containing biofilms. The 96 well plate was then sonicated for 5 min to detach the bacterial cells into the solution using a water table sonicator (Branson EMT, USA, Mexico). PBS cell suspensions from each well of corresponding sample were mixed to obtain an average surviving bacterial population, serially diluted in MRD and surface plated on TSA.

For food samples, untreated control and ACP treated samples were analysed. The samples were aseptically cut into two pieces, transferred into separate sterile stomacher bags (BA6041, Seward LTD, UK) with 10 ml of sterile MRD and stomached for 2 min. The resulting suspension was serially diluted in MRD. Aliquots of appropriate dilutions were plated on Baird Parker agar (Biokar, Ireland) supplemented with egg yolk (Sigma Aldrich, Ireland) for *S. aureus* and McConkey agar for *E. coli* and incubated for 48 h at 37 ºC. All treatments were performed in duplicate (2 trays) and
replicate samples were obtained from each tray to ensure reproducibility of the experimental data. Results are reported in $\log_{10}$ CFU g$^{-1}$ units.

2.4.2 Plasma diagnostic and gas analysis

Gas composition were analysed using optical emission spectroscopy (OES) as real time monitoring or gas tube detectors after treatment and storage.

2.4.2.1 Optical emission spectroscopy

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. UV Enhanced Smart CCD Spectrometers have been optimized for maximum performance in the ultraviolet, and for multichannel operation with ultra-low trigger delay, a gate jitter and the spectral resolution of 0.6 nm.

The fibre optic from the spectrometer was placed directly towards a package that allows the light to cross the centre of the side wall of the polypropylene container. The fibre had a numerical aperture of 0.22 mm and was optimized for use in the ultraviolet and visible portion of the spectrum with a wavelength range of 200 – 920 nm. A 5 mm diameter lens collected light from a column across the diameter of the package and focused onto a 200 $\mu$m multi-mode fibre. The other end of the 2 m long fibre was connected to the spectrometer. Applied gas mixtures were flowing through the container at 0.5 bar, and out from the lens window during discharging. Data was collected 20 sec/5 min with duplicate.

2.4.2.2 Ozone measurements

Ozone concentrations were measured using GASTEC gas tube detectors (Product # 18M, Gastec Corporation, Kanagawa, Japan) after treatment and individual storage time.
2.4.3 Reactive oxygen species measurements

2.4.3.1 DCFH-DA general reactive oxygen species

The oxidant-sensing fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), is a nonpolar dye, which is converted into the nonfluorescent polar derivative DCFH by cellular esterases and switched to highly fluorescent DCF when oxidized by intracellular ROS and other peroxides (Gomes et al., 2005). DCFH-DA was purchased from Sigma-Aldrich (USA) as dry powder. It was first prepared as 50 mM in DMSO as stock solution and further diluted with PBS before use. After ACP treatment and storage, bacterial cells were incubated with DCFH-DA at a final concentration of 5 μM in PBS for 15 min at 37 °C in dark. Incubated aliquots of 200 μL were transferred into each well of 96 well fluorescence microplate wells (Fisher Scientific, UK) and measured by Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments Inc.) at excitation and emission wave lengths of 485 and 525 nm (Joshi et al., 2011). Untreated control of each strain was used to determine the original intracellular ROS before ACP treatment. Each sample was read in 3 wells as triplicate and replicated at least twice. Data shown in figures represent the intracellular ROS concentrations generated after ACP treatment expressed as arbitrary fluorescence units (AFU).

2.4.3.2 Other reactive oxygen species measurements

To evaluate different type of reactive oxygen species generated, 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 in dark after ACP treatment. Incubated aliquots of 200 μL were transferred
into each well of 96 well fluorescence microplate wells and measured by Synergy™ HT Multi-Mode Microplate Reader at excitation and emission wave lengths of 485 and 528 nm. (Joshi et al., 2011) Untreated controls of each strain were used to determine the blank level before ACP treatment. Each sample was read in 3 wells as triplicate and replicated at least twice. Data shown in figures represent the intracellular ROS concentrations generated after ACP treatment expressed as arbitrary fluorescence units (AFU).

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. Bacterial cell suspensions were incubated with 50 µM of Amplex Red reagent and 0.1 U ml\(^{-1}\) HRP provided by kit at 37 °C for 10 min in dark after ACP treatment. Incubated aliquots of 100 µl were transferred into each well of 96 well fluorescence microplate wells and measured by Synergy™ HT Multi-Mode Microplate Reader at excitation and emission wave lengths of 530 and 590 nm. (Boxhammer et al., 2012; Joshi et al., 2011) Standard curve was obtained with serial H\(_2\)O\(_2\) solutions after incubating with 50 µM of Amplex Red reagent and 0.1 U ml\(^{-1}\) HRP and measured in same way. Each sample was read in 2 wells as duplicate and replicated at least twice. Fluorescence signals were recorded as arbitrary fluorescence units (AFU) and then converted to concentration of µM using the H\(_2\)O\(_2\) standard curve.

2.4.4 Reactive nitrogen species measurements

DAF-FM DA, 4-amino-5-methylamino- 2',7'-difluorofluorescein diacetate, is a cell-permeable fluorescent probe for the detection of nitric oxide (NO). It permeates into living cells and is rapidly transformed into water-soluble DAF-FM by cytosolic esterases and ideal for detection of intracellular NO.
DAF-FM DA was purchased from Sigma-Aldrich (USA) as 5 mM stock solution in DMSO. It was further diluted with PBS before use. Cells were incubated with DAF-FM DA at a final concentration of 1 μM in PBS for 15 min at 37 °C in dark. Incubated aliquots of 200 μL was transferred into each well of 96 well fluorescence microplate wells and measured by Synergy™ HT Multi-Mode Microplate Reader at excitation and emission wave lengths of 485 and 528 nm. Untreated controls of each strains were used to determine the intracellular RNS before ACP treatment. Each sample was read in 3 wells as triplicate and replicated at least twice. Data shown in figures represent the intracellular ROS concentrations generated after ACP treatment expressed as arbitrary fluorescence units (AFU).

Since NO and other reactive nitrogen species have a very short life, nitrates and nitrites can be measured as evidence for emerging RNS in liquids. The commercial 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 with all storage times. Hundred μl of samples were incubated with 50 μl of Griess Reagent R1 and 50 μl of Griess Reagent R2 provided by kit at room temperature for 10 min to develop colour change. The absorbance was measured using Synergy™ HT Multi-Mode Microplate Reader at wave length of 550 nm. A standard curve (0 – 35 μM) was prepared using the standard nitrite and nitrate chemicals provided in the kit, and used to convert absorbance results to concentration of nitrite/nitrate in μM. (Boxhammer et al., 2012)

2.4.5 Cell morphology

To assess the impact of plasma treatment on cell surface and morphology changes, treated cells were collected for scanning electron microscopy (SEM) analysis. This was based on a noticeable difference in plasma inactivation efficacy with respect to
post-treatment storage time. Bacterial cells were prepared as described by Thanomsub et al. (2002) with minor modifications (Patil et al., 2011).

In brief, the samples were concentrated by centrifugation and the cell pellet was fixed in ice-cold 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) (SCB) for 2 h. The pellet was washed with the same buffer three times and fixed in 1% osmium tetroxide for 2 h at 4 °C. After 2 h of fixation, bacterial cells were washed with SCB followed by three washes with distilled water. The samples were dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 90%, 95%, and 99.5%) and freeze dried (Labconco, FreeZone 6; Mason Technology, Dublin, Ireland). Samples were sputter-coated with gold particles using Emitech K575X Sputter Coating Unit resulting in a coating of 10 nm after 30 s. Further, the samples were examined visually by using a FEI Quanta 3D FEG Dual Beam SEM (FEI Ltd, Hillsboro, USA) at 5 kV.

2.4.6 Cell integrity

2.4.6.1 Leakage

Membrane integrity was examined by determination of the release of material absorbing at 260 nm and 280 nm (Virto et al., 2005). The UV absorbance at 260 nm and 280 nm (A260 and A280) were used to indicate the effect on cell membrane integrity. Untreated (bacterial cells in PBS) and ACP-treated samples were centrifuged at 13,200 g for 10 min. Untreated controls determined the release of any intracellular material before ACP treatment. Supernatant of 200 μl from each sample was transferred into UV transparent microtitre plate wells (Corning, U.S.A.) and measured by Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments Inc.) at 260 nm and 280 nm.
2.4.6.2 Fluorescence probe

After ACP treatment, cells were incubated with propidium Iodide (PI, Sigma Aldrich Ltd, Dublin, Ireland) at a final concentration of 3 μM in PBS for 15 min at 37 °C, then washed once with PBS and resuspended in PBS for flow cytometry (CyFlow Partec Int.) analysis with excitation and emission wave lengths of 485 and 590 nm, respectively. For each analysis 100,000 events were recorded.

2.4.7 DNA damage

2.4.7.1 Genomic DNA extraction

Due to the cell leakage induced by ACP treatment, treated aliquots were first precipitated by ethanol with a final concentration of 70% for 5 min. Samples were then centrifuged at 13,200 g for 10 min to obtain the pellet. Genomic DNA was then extracted from the pellet by Wizard Genomic DNA Purification Kit (Promega, USA) as per manufacturer’s instructions. The amount of DNA was quantified by measuring the absorbance at 260 nm.

2.4.7.2 Genomic DNA extraction and PCR

The genomic DNA amplification of conserved bacterial regions i.e. 16S rRNA was performed using the primers listed in Table 2.3. The multi-copy gene, 16S rRNA was chosen because of its widely existence in all bacteria (Glazer and Nikaido, 2007). Moreover, the multi copies of 16S rRNA were spread on genomic DNA, which can reveal the possible preference of sites of ACP induced DNA damage. All reactions were performed with GoTaq Colorless Mastermix (Promega, USA). A 25 μl PCR reaction system was used which contained, 12.5 μl Mastermix, 2 μl of each primer (0.2 nM), 2 μl of genomic DNA as template (0.2 ng) and sterilized water to make the final volume up to 25 μl. The amplification programme employed was: 95 °C for 5 min as
first denaturation step, 25 cycles of 95 ºC for 45 sec for denaturation, 51 ºC for 30 sec annealing and 72 ºC for 90 sec extension, and 72 ºC for 10 min for final extension.

Table 2.4 Primers in DNA damage study

<table>
<thead>
<tr>
<th>Organism</th>
<th>5’-3’ Sequence</th>
<th>PCR product length</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>Forward: CAG GCC TAA CAC ATG CAA GT</td>
<td>1410 bp</td>
</tr>
<tr>
<td><em>E. coli</em> NCTC 12900</td>
<td>Reverse: CGA AGG TTA AGC TAC CTA CTT</td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em> NCTC 11994</td>
<td>Forward: TAAAGAGAGT TTGATCCTGG C</td>
<td>1418 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCT ACC GAC TTC GGG TGT T</td>
<td></td>
</tr>
</tbody>
</table>

Electrophoresis was performed in 0.8% agarose gel, with Ethidium Bromide (Sigma Aldrich Ltd, Dublin, Ireland) staining at 140 V. Genomic DNA samples (20 ng) were loaded for each well, with exACTGene 1 kb plus marker (Fisher BioReagents, UK). 16S rRNA PCR products were loaded with digested BenchTop pGEM DNA marker (Promega, USA). Digested BenchTop pGEM DNA marker was used for providing more comparable bands around target length (1.4 kb).

2.4.7.3 Fluorescence probe

DNA damage analysis was carried out on samples with the DNA quantification probe, SYBR Green I (Zipper et al., 2004). *E. coli* samples after ACP treatment were incubated with 100 µg ml⁻¹ lysozyme at 37 ºC for 4 h to break the cell envelope and release the intracellular DNA. Because of the different cellular structures in Gram positive bacteria, *S. aureus* samples were incubated with 100 µg ml⁻¹ lysozyme and 10 µg ml⁻¹ lysostaphin at 37 ºC for 4 h. Cell digestion effects were verified by colony counts on TSA plates. Cells without ACP treatment were digested and used as positive control group, while untreated cells without digestion were used as negative controls.
After the cell digestion, the solution was incubated with SYBR Green I (1:10,000, Sigma Aldrich Ltd, Dublin, Ireland) at working concentration (1:1) for 15 min at 37 °C. Aliquots of 200 µL from each sample was transferred into wells of 96 well fluorescence microplate and measured by Synergy™ HT Multi-Mode Microplate Reader at excitation and emission wave lengths of 485 and 525 nm.

2.4.8 Modelling microbial recovery

Samples in 96 well plates were ACP treated for 60, 120 and 300 s and stored at 4 ºC for 24 h post treatment. Aliquots of 20 µl from each well were analysed using plate count. The microtiter plates were then placed in a microplate spectrophotometer (ELx808, BioTek Instruments Inc., U.S.A.) set at 37ºC. Absorbance readings were taken at 600 nm every 30 min for 48 h. The OD values were used to quantify the growth rate, $\mu_{\text{max}}$ and the lag phase, $\lambda$, of all the bacteria studies using the following equation (Biesta-Peters et al., 2010; Cuppers and Smelt, 1993):

$$TTD_i = \lambda + \frac{1}{\mu_{\text{max}}} \cdot \log \left( \frac{N_{\text{turb}}}{N_i} \right)$$

(Equation 1)

where $TTD_i$, is time to detection (h) of the inoculum level $i$, chosen as the time at which the sample in the well reaches an OD$_{600}$ of 0.2 (Biesta-Peters et al., 2010), $\lambda$ is the duration of the lag phase (h), $N_{\text{turb}}$ is the number of bacteria per ml (CFU ml$^{-1}$) at which an OD$_{600}$ of 0.2 is observed, $N_i$ is the number of organisms per ml of the inoculum at time zero, and $\mu_{\text{max}}$ is the maximum specific growth rate (h$^{-1}$). $N_{\text{turb}}$ was determined through separate experiments. The goodness of fit of these curves was validated experimentally. The kinetic parameters ($\mu_{\text{max}}$ and $\lambda$) were calculated by performing a regression analysis on the data correlating $TTD$ with $\log (N_{\text{turb}}/N_0)$ using equation 1 for each replicate separately (Millan Sango et al., 2015).
2.4.9 Statistical analysis

Surviving bacterial populations either in planktonic or biofilm form following treatment were subjected to statistical analysis, which was performed using SPSS 22.0 (SPSS Inc., Chicago, U.S.A.). Means were compared using analysis of variance (ANOVA) using Fisher’s Least Significant Difference-LSD at the 0.05 level.
Chapter 3. PRIMARY EXPERIMENTAL CONDITION SETUP

This study was performed using an in-package treatment as a novel way to prevent post processing contamination. Process variables were examined in terms of their antimicrobial effects and effects on cell morphology towards increasing understanding of how plasma processes may be optimised. Therefore, the present study aimed to relate the microbial inactivation efficacy associated with defined process treatment parameters of treatment time, post-treatment storage time and mode of exposure to ozone measurements and observations of morphological effects using scanning electron microscopy for both gram positive and gram negative cells. Meanwhile, the effect of potential acid stress was also investigated, which might be caused by the absence or presence of glucose in media.

3.1 Effect of Presence or Absence of Glucose on Plasma Inactivation Efficacy

The effect of presence (TSB+G) or absence of glucose (TSB-G) on ACP inactivation of bacteria in PBS is shown in Table 3.1. In both cases, *E. coli* populations were completely inactivated after 5 min of plasma treatment at 40 kV followed by 1 h post-treatment storage time. However, for *L. monocytogenes* NCTC 11994, inactivation efficacy was dependant on the presence or absence of glucose in the growth medium in combination with the mode of plasma exposure. Direct plasma exposure reduced *L. monocytogenes* by 8 \( \log_{10} \) CFU ml\(^{-1} \) regardless of glucose condition. However, indirect plasma exposure reduced *L. monocytogenes* populations by 4.4 and 2.5 \( \log_{10} \) CFU ml\(^{-1} \) within TSB+G or TSB-G, respectively (p<0.05). Based on these results, all further studies on the effects of plasma treatment parameters on inactivation efficacy included growing cells in TSB-G to ensure the greatest challenge to the system.
Table 3.1 Cell number reduction with regards to glucose absence and presence in overnight culture

<table>
<thead>
<tr>
<th>Organism</th>
<th>Absence(-) or presence(+) of glucose</th>
<th>Mode of Plasma Exposure</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Direct</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Initial cell density (Log₁₀ CFU ml⁻¹)</td>
<td>Reduction (Log₁₀ CFU ml⁻¹)</td>
<td>SD*</td>
<td>Initial cell density (Log₁₀ CFU ml⁻¹)</td>
<td>Reduction (Log₁₀ CFU ml⁻¹)</td>
<td>SD*</td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>-</td>
<td>7.8</td>
<td>7.8ᵃ</td>
<td>0.1</td>
<td>7.8</td>
<td>7.8ᵃ</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.7</td>
<td>7.7ᵃ</td>
<td>0.0</td>
<td>7.8</td>
<td>7.8ᵃ</td>
</tr>
<tr>
<td>L. monocytogenes NCTC 11994</td>
<td>-</td>
<td>8.5</td>
<td>8.5ᵃ</td>
<td>0.0</td>
<td>8.6</td>
<td>2.5ᵇ</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8.2</td>
<td>8.2ᵃ</td>
<td>0.0</td>
<td>8.2</td>
<td>4.4ᵇ</td>
</tr>
</tbody>
</table>

*SD: Standard deviation. All experiments were carried out in duplicate and replicated at least twice.

Experimental conditions: 5 min treatment at 40 kV; 1 h post-treatment storage; treated in PBS

3.2 Effects of ACP-DBD Process Parameters on Bacteria Inactivation

Efficacy

Table 3.2 shows the inactivation efficacy of ACP against E. coli ATCC 25922 and L. monocytogenes NCTC 11994 in PBS. ACP inactivation is related to a range of extrinsic control parameters. In this study we examined the effects of direct versus indirect exposure, treatment time and post-treatment storage time towards optimizing the ACP process design.
Table 3.2 Cell number reduction with regards to process parameters

<table>
<thead>
<tr>
<th>Organism</th>
<th>Treatment Time (min)</th>
<th>Post-treatment Storage Time (h)</th>
<th>Mode of Plasma Exposure</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Direct</td>
<td>Indirect</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial cell density</td>
<td>Reduction (Log_{10} CFU ml^{-1})</td>
<td>SD*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Log_{10} CFU ml^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>1</td>
<td>1</td>
<td>7.8</td>
<td>5.0 (^{a})</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>7.8</td>
<td>7.8 (^{b})</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>7.9</td>
<td>7.9 (^{a})</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>8.3</td>
<td>8.3 (^{a})</td>
<td>0.4</td>
</tr>
<tr>
<td>L. monocytogenes NCTC 11994</td>
<td>1</td>
<td>1</td>
<td>8.3</td>
<td>1.4 (^{a})</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>8.3</td>
<td>8.3 (^{b})</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>8.5</td>
<td>8.5 (^{a})</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>7.9</td>
<td>7.9 (^{a})</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^{*}\)SD: Standard deviation. All experiments were carried out in duplicate and replicated at least twice.

Experimental conditions: 1 or 5 min treatment at 40 kV following 1 or 24 h post-treatment storage; treated in PBS

3.2.1 Direct and Indirect Exposure

Exposure of E. coli to direct plasma for 1 min with 1 h of post-treatment storage, decreased the cell population by 5.0 Log_{10} CFU ml^{-1}, while indirect exposure reduced the population by only 2.6 Log_{10} CFU ml^{-1}. For L. monocytogenes, direct and indirect plasma exposure for 1 min with 1 h post-treatment storage time, reduced the population by only 1.4 and 1.1 Log_{10} CFU ml^{-1} respectively.

3.2.2 Treatment Time

Increasing treatment time from 1 min to 5 min, while retaining a 1 h post-treatment storage, resulted in total inactivation of E. coli. Similar results were obtained for L. monocytogenes (Table 3.2). Increasing treatment time to 5 min yielded complete
inactivation with direct exposure and up to 2.5 $\log_{10}$ CFU ml$^{-1}$ reduction of *L. monocytogenes* with indirect exposure.

### 3.2.3 Post-treatment Storage Time

Two post-treatment storage times, 1 or 24 h, were assessed ($p<0.05$). Using a 24 h post-treatment storage time increased inactivation efficacy of ACP for *E. coli* with the shorter treatment time of 1 min yielding no survivors ($p<0.05$). Similarly, in case of *L. monocytogenes* greater inactivation rates were attained with 24 h post-treatment storage compared with only 1 h of post-treatment storage time ($p<0.05$) (Table 3.2).

### 3.2.4 Ozone measurements

The effect of process parameters on ozone measurements is shown in Figure 3.1. Increasing the treatment time to 5 min gave in-package ozone concentrations ranging from 2600 ppm to 3000 ppm or 2600 ppm to 3800 ppm by direct and indirect exposure respectively (0 h). Following a 1 h post-treatment storage time, the ozone concentration ranges decreased to 180 ppm to 300 ppm and 160 ppm to 500 ppm with direct and indirect exposure, respectively. With a shorter treatment time of 1 min, the ozone concentration ranged from 1800 ppm to 2200 ppm and 2600 ppm to 3200 ppm immediately after treatment. A similar trend as for 5 min treatment was observed after a 1 h post-treatment storage time, where ozone concentration ranges decreased to between 140 ppm to 160 ppm and 260 ppm to 320 ppm following direct and indirect exposure. No ozone was detected in either treatment condition after the 24 h post-treatment storage time.
Figure 3.1 In-package ozone concentration after ACP treatment with respect to process parameters. Experimental conditions: 1 or 5 min treatment at 40 kV measurements immediately after treatment or after 1 or 24 h post-treatment storage.

3.3 SEM Observation of Plasma Treated Bacteria

Post-treatment storage time was observed to be an important factor for the efficacy of ACP inactivation and further appraisal of the effects of the ACP in conjunction with post-treatment storage time were warranted. Bacterial populations that had been exposed to ACP for 1 min with a post-treatment storage of both 1 and 24 h were chosen for SEM analysis. Compared to untreated *E. coli* cells, ACP treated cells with 1 h post treatment storage showed slightly different cell morphology. The surface of plasma exposed cells was visibly rougher than untreated cells (Figure 3.2 a, b, c). Untreated *L. monocytogenes* exhibited a smooth cell surface structure, whereas those exposed to ACP had a rough and shrunken appearance (Figure 3.3 a, b, c). Significant damage including rupture in the cell membrane to the *E. coli* cell structure was observed with
the increased post-treatment storage time of 24 h (Figure 3.2 a, d, e). \textit{L. monocytogenes} cells showed noticeable alteration in the cell surface with roughness and wrinkles appearing on the surface which was more significant with longer storage time (Figure 3.3 a, d, e). There were no obvious effects noted of the mode of plasma exposure on the visual appearance of the cell surface structure. (Figure 3.2 and 3.3). Overall, cell shrinkage and dehydrated appearance of cells after plasma exposure were observed.

(a) Untreated control

(b) 1 h post-treatment storage of direct exposure

(c) 1 h post-treatment storage of indirect exposure
Figure 3.2 Scanning electron microscopy image of *E. coli* ATCC 25922

Experimental conditions: untreated and plasma exposed (1 min) following 1 or 24 h post-treatment samples in PBS. Circles indicate cell envelope breakage.

(a) Untreated control
Experimental conditions: untreated and plasma exposed (1 min) following 1 or 24 h post-treatment samples in PBS. Arrows indicate cell shrinkage.

Figure 3.3 Scanning electron microscopy image of *L. monocytogenes* NCTC 11994

3.4 Discussion

In this study a significant influence of process parameters on inactivation efficacy within PBS was observed. Direct plasma exposure to both types of bacteria always resulted in efficient inactivation with the shorter treatment time of 1 min. However,
for total inactivation of *L. monocytogenes*, a 5 min treatment time in conjunction with 24 h post-treatment storage time was required. Previous studies also reported that direct exposure to plasma discharge produces much faster bacterial inactivation than indirect plasma discharge. Fridman *et al.* concluded that charged particles produced from direct plasma discharge plays an important role for inactivation of microorganisms, with synergistic action of long living atoms as well as UV radiation generated by plasma (Fridman et al., 2007). Similarly, better inactivation of *E. coli* was reported after direct plasma exposure on the surface of agar (Dobrynin et al., 2009). Dobrynin *et al.* observed that direct exposure of bacteria to plasma generated reactive species such as ozone (O₃), hydroxyl radicals (OH•), excited molecular, atomic species, UV and charged particles produced a maximum inactivation effect by comparison with indirect exposure (Dobrynin et al., 2009). However, in the current study, the longer post-treatment storage time of 24 h had an overarching effect where enhanced inactivation of both bacteria was achieved by incorporating this storage time, and the effect of mode of plasma exposure was nullified to a certain extent. This effect could be due to the diffusion of plasma generated reactive species into the liquids during the period of post-treatment storage. Oehmigen *et al.* suggested the diffusion of reactive species into liquid as one possible means of antimicrobial plasma effect (Oehmigen et al., 2010). Ikawa *et al.* suggested that for indirect plasma exposure to a liquid, that the bactericidal effects were due to the highly reactive species generated in the solution by plasma-liquid interaction (Ikawa et al., 2010).

The addition of glucose to the media was investigated to determine the effect of presence or absence of glucose in the growth medium on plasma inactivation efficacy. Presence of glucose in TSB causes media pH reduction from 7.0 to 5.0. This can cause mild acid stress to microorganisms resulting in cross-protective effects against applied
stresses (Kroll and Patchett, 1992; Samelis et al., 2003). In our research, indirect ACP exposure of *L. monocytogenes* population grown in TSB+G showed greater sensitivity compared to that grown in TSB-G (p≤0.05). Based on these results, all further studies on the effects of plasma treatment parameters on inactivation efficacy included growing cells in TSB-G to ensure the greatest challenge to the system. Plasma-liquid interactions were reported in number of studies (Ikawa et al., 2010; Julák et al., 2012; Tang et al., 2008), where inactivation was found to be dependent on acidification of media following plasma exposure due to formation of nitrous acid (HNO₂) and nitric acid (HNO₃). Where buffered solutions were exposed to plasma and a decrease in pH was not evident, a reduced antimicrobial activity was noted (Oehmigen et al., 2010). Julák et al. studied persistent microbicidal effect in solutions exposed to plasma produced by positive and negative DC discharges in air, where it was noted that the microbicidal effects were dependent on the volume of treated solution (Julák et al., 2012). Complete inactivation was obtained in only 1 ml of PBS solution after plasma exposure by positive streamers, stored for 4 weeks. However, no inactivation was attained when the volume was increased to either 5 or 10 ml. Similarly, Oehmigen et al. reported plasma inactivation efficacy was dependent on sample volume (Oehmigen et al., 2010). The smallest sample volume of 1.5 ml resulted in either 3 or 1.5 Log₁₀ CFU ml⁻¹ reductions of number of viable *E. coli* and *Staphylococcus aureus* suspended in PBS, after 15 min of indirect plasma treatment, respectively (Oehmigen et al., 2010). It was concluded that the plasma discharge in buffered solution avoided the pH change (no acidification of medium), thus resulting in a reduced antimicrobial plasma activity. Another possibility suggested was incomplete diffusion of reactive species generated by plasma or generated by plasma-liquid based interactions within non-stirred large volumes of liquid. Our results demonstrate a much more effective treatment. A 10 ml
volume of *Escherichia coli* ATCC 25922 and *Listeria monocytogenes* NCTC11994 exposed to 1 min ACP treatment and 24 h storage achieved greater than a $5 \log_{10}$ CFU ml$^{-1}$ reduction. This increased bactericidal effect is likely the result of the greater applied voltage (40 kV compared to 10 kV) and the containment of reactive species inside the package leading to retention of effect in the liquid.

In the present study using the larger volume, increasing ozone concentrations were measured after longer ACP treatment time, with indirect exposure resulting in slightly higher O$_3$ concentrations than direct exposure (Figure 3.1). A strong influence of post-treatment storage time was also noted, thus suggesting penetration of plasma generated reactive species with enhanced antimicrobial activity even in large volume buffered liquid samples. The diffusion of generated reactive species would be dependent on their half-life. Species with very short half-lives will not have sufficient time for diffusion. The inactivation observed in this study, might be due to the diffusion of long-lived species such as ozone during storage. The diffusion of reactive chemical species into the liquid samples during the surface-DBD plasma treatment was recently demonstrated by Oehmigen *et al.* (Oehmigen *et al.*, 2010). Ozone is one of the stable reactive species responsible for inactivation of microorganisms. From the ozone measurement results, it was noted that detectable ozone concentration decreased during storage. After 1 h post-treatment storage, ozone was 90% depleted, and 100% depleted after 24 h storage. Arjunan *et al.* (2012) and Vandamme *et al.* (2011) reported that intracellular ROS keep working after plasma treatment up to 3 h or even 24 h.

From the SEM results, more visible damage was evident on *E. coli* surfaces than *L. monocytogenes*, indicating cell breakage effects for *E. coli* inactivation, while that for *L. monocytogenes* can be attributed to damage inside the cell envelope. The results of cell morphology were also correlated to the storage effect. The visual appearance and
changes of bacterial cell surface structures were similar to the patterns observed in bacterial survival rates after 1 min of ACP followed by either 1 or 24 h post-treatment storage time. *E. coli* cells were significantly damaged with the increased post-treatment storage time of 24 h. Similarly, ACP exposure of *L. monocytogenes* exhibited noticeable alteration in the cell surface with roughness and wrinkles appearing on the surface which was more prominent with longer storage times. Recently, some studies indicated no significant differences in the effect of plasma treatment on inactivation efficacy between Gram positive and negative bacteria (Kayes et al., 2007; Venezia et al., 2008), whilst other researchers found that gram-positive bacteria were more resistant than Gram-negative bacteria (Ermolaeva et al., 2011; Lee et al., 2006), depending on the plasma generation devices and species characterised.

In the current study, ACP-DBD plasma was more effective against *E. coli* compared to *L. monocytogenes*. ACP generation in air results in generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) including ozone. Oxidative stress exerted by ROS and RNS on cellular components (i.e. by attacking macromolecules of membrane component, such as lipopolysaccharide), makes *E. coli* vulnerable to plasma treatment (Dobrynin et al., 2009).

The use of ACP-DBD treatment was effective for inactivation of both challenge microorganisms. There were significant interactive effects of process parameters such as mode of exposure and treatment time, while post-treatment storage time emerged as an important factor to ensure complete or high inactivation assurance with a shorter treatment time. Additionally, the ACP applied yielded useful reductions in buffered liquid volumes of 10 ml, which is significant for industrial processing applications.

The comparison of the SEM images of ACP-DBD effects with population inactivation
confirmed the substantial effect of post-treatment storage time as a critical processing associated parameter that may be harnessed to facilitate shorter ACP treatment times. The process parameters evaluated in this study showed a significant effect on inactivation efficiency of the DIT 60 ACP system used. Significant interactive effects of process parameters such as treatment time, post-treatment storage time and mode of plasma exposure were observed. Additionally, the inactivation efficacy may be correlated to the target micro-organism characteristics. A greater inactivation efficacy of plasma was achieved with a 24 h post-treatment storage time for both challenge microorganisms, irrespective of plasma treatment time. However, the mode of plasma exposure also had a significant impact, for shorter treatment and storage times, where direct exposure to plasma had greater inactivation efficacy. These patterns are reflected within Scanning Electronic Microscopy (SEM) images, where *E. coli* cells formed irregular shapes with structural disintegration evident, but *L. monocytogenes* cells displayed shrinkage and wrinkling. Overall, the novel in-package, DBD-ACP technology showed an efficient inactivation of high concentrations of *E. coli* and *L. monocytogenes* in liquids under both direct and indirect treatment modes, correlated with treatment time and post-treatment storage time. However, there are other important process parameters such as voltage level and applied gas mixture need to be optimized, which also have great effect on inactivation efficacy based on literature.

Furthermore, the SEM results provide a glance of ACP inactivation mechanism against bacteria, which need to be further investigated. Consideration of the target characteristics in addition to processing or quality retention considerations are critical to develop this technology for industrial application.
Chapter 4. OPTIMIZATION OF ATMOSPHERIC COLD PLASMA TREATMENT SYSTEM

This study investigated the inactivation effect of DBD-ACP with different inducer gas types. Based on the primary study, the interactive effects with two high voltage levels, mode of exposure and treatment time were examined. The relationship between inactivation patterns and the intracellular damage patterns were further explored. Cell membrane integrity and genomic DNA damage were selected as indicators. This study evaluated the effects described above on two target organisms *E. coli* and *L. monocytogenes* to compare the different damage mechanisms for Gram negative and positive bacteria. Furthermore, two strains of *E. coli* with different resistance characteristics were compared. *E. coli* ATCC 25922 is a standard strain, while *E. coli* NCTC 12900 is non-toxigenic O157:H7 with higher virulence and resistance.

4.1 The effect of voltage level on DBD-ACP inactivation efficiency

The effect of voltage levels on ACP inactivation efficacy was investigated for *E. coli* strain 25922. ACP treatment of *E. coli* in PBS at 56 kV$_{RMS}$, using air as a working gas type, decreased the cell population by 1.8 and 1.6 Log$_{10}$ CFU ml$^{-1}$ after direct and indirect exposure, respectively. Similar effects were noted using 10% O$_2$+ 90% N$_2$ (SB1), where the reductions noted were 1.0 and 1.2 Log$_{10}$ CFU ml$^{-1}$ following direct and indirect ACP exposure, respectively (Table 4.1, p>0.05).

Increased voltage level of 70 kV$_{RMS}$ achieved significantly greater inactivation effects compared to 56 kV$_{RMS}$ (p<0.05). The indirect plasma exposure at 70 kV$_{RMS}$, for 30 s either in air or SB1, decreased the population by 7.9 and 3.2 Log$_{10}$ CFU ml$^{-1}$, respectively. Similarly, direct ACP exposure at higher voltage level using either gas
type resulted in better inactivation effects compared to lower voltage level tested (Table 4.1, p<0.05).

Table 4.1 Cell number reduction of E. coli ATCC 25922 with different applied voltage level of ACP treatment

<table>
<thead>
<tr>
<th>Voltage (kV&lt;sub&gt;RMS&lt;/sub&gt;)</th>
<th>Gas</th>
<th>Mode of Plasma Exposure</th>
<th>Initial cell density (Log&lt;sub&gt;10&lt;/sub&gt; CFU ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Reduction (Log&lt;sub&gt;10&lt;/sub&gt; CFU ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>SD*</th>
<th>Initial cell density (Log&lt;sub&gt;10&lt;/sub&gt; CFU ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Reduction (Log&lt;sub&gt;10&lt;/sub&gt; CFU ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>Air</td>
<td>Direct</td>
<td>8.0</td>
<td>1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.6</td>
<td>8.0</td>
<td>1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>SB1</td>
<td>Indirect</td>
<td>7.7</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2</td>
<td>7.7</td>
<td>1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4</td>
</tr>
<tr>
<td>70</td>
<td>Air</td>
<td>Direct</td>
<td>7.9</td>
<td>3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4</td>
<td>7.9</td>
<td>ND&lt;sup&gt;**c&lt;/sup&gt;</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SB1</td>
<td>Indirect</td>
<td>7.7</td>
<td>2.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2</td>
<td>7.7</td>
<td>3.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Different letters indicate a significant difference at the 0.05 level between voltage level and between gas types.

*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log<sub>10</sub> CFU ml<sup>-1</sup>)

Experimental conditions: 30 s treatment, 24 h post-treatment storage, treated in PBS

4.2 The effect of gas type on DBD-ACP inactivation efficiency

To assess the effect of gas type on ACP inactivation efficacy, the higher voltage level only was used. With direct exposure of ACP generated in either in air, 10% O<sub>2</sub>+ 90% N<sub>2</sub> (SB1) or 65% O<sub>2</sub>+ 30% CO<sub>2</sub>+ 5% N<sub>2</sub> (SB2), populations of E. coli ATCC 25922 were reduced by 3.4, 2.8 and 6.6 Log<sub>10</sub> CFU ml<sup>-1</sup>, respectively (p<0.05). For the same strain, indirect exposure in air and SB2 resulted in greater inactivation rates (under detection limit, <1.0 Log<sub>10</sub> CFU ml<sup>-1</sup>), but only 3.2 Log<sub>10</sub> CFU ml<sup>-1</sup> reduction was recorded when SB1 was utilised (Table 4.2). In the case of non-toxigenic strain E. coli
NCTC 12900, inactivation under detection limit (<1.0 Log$_{10}$ CFU ml$^{-1}$) was achieved only after indirect exposure in SB2 (p<0.05). The other gas mixtures were less effective. *L. monocytogenes* populations were inactivated under the detection limit (<1.0 Log$_{10}$ CFU ml$^{-1}$) after indirect exposure to ACP generated with all three gas types tested. Direct ACP exposure in air and SB2 was more effective than SB1 for inactivation of *L. monocytogenes* (Table 4.2, p<0.05).

Table 4.2 Cell number reduction of three strains with different applied gas mixtures of ACP treatment

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gas</th>
<th>Mode of Plasma Exposure</th>
<th>Direct</th>
<th>Indirect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial cell density (Log$_{10}$ CFU ml$^{-1}$)</td>
<td>Reduction (Log$_{10}$ CFU ml$^{-1}$)</td>
<td>SD*</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>Air</td>
<td>7.9</td>
<td>3.4 $^a$</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>SB1</td>
<td>7.7</td>
<td>2.8 $^b$</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>SB2</td>
<td>8.0</td>
<td>6.6 $^c$</td>
<td>0.1</td>
</tr>
<tr>
<td><em>E. coli</em> NCTC 12900</td>
<td>Air</td>
<td>7.9</td>
<td>1.8 $^{a,b}$</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>SB1</td>
<td>8.0</td>
<td>1.4 $^a$</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>SB2</td>
<td>8.0</td>
<td>2.1 $^b$</td>
<td>0.5</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> NCTC 11994</td>
<td>Air</td>
<td>8.3</td>
<td>ND* $^a$</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>SB1</td>
<td>8.2</td>
<td>4.1 $^b$</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SB2</td>
<td>8.2</td>
<td>ND* $^a$</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Different letters indicate a significant difference at the 0.05 level between gas types and between strains.

*SD: Standard Deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log$_{10}$ CFU ml$^{-1}$)

Experimental conditions: 70 kV$_{RMS}$ 30 s treatment, 24 h post-treatment storage, treated in PBS.
4.3 **Ozone measurements**

O$_3$ concentrations were measured immediately after plasma exposure with GASTEC gas tubes and are shown in Figure 4.1. Ozone concentrations of 1280 and 1000 ppm were noted immediately after direct ACP treatment in air or SB1, respectively. Indirect ACP treatment recorded slightly increased concentrations of 1440 ppm and 1367 ppm, respectively. Higher concentrations of 2000 and 4000 ppm were noted after direct or indirect ACP treatment in SB2, respectively. No ozone concentrations were recorded for stored samples after 24 h of post-treatment storage.

![Figure 4.1 In-package ozone concentration with regards to applied gas mixtures and mode of exposure](image)

Experimental conditions: measurements conducted immediately after 30 s treatment at 70 kV$_{RMS}$, treated in PBS
4.4 Effect on cell membrane integrity

Figure 4.2 represents the inactivation curve of bacterial strains following ACP exposure. It was observed that all bacterial strains studied were completely inactivated with 60 s of ACP treatment in air irrespective of the mode of exposure (Figure 4.2 a). *E. coli* ATCC 25922 populations were undetectable after 30 s of indirect ACP treatment, however, direct ACP treated populations were reduced by 3.4 log_{10} CFU ml^{-1}. *E. coli* NCTC 12900 was more resistant, with reductions of around 2 log_{10} CFU ml^{-1} recorded after both direct and indirect exposure. With a 5 s exposure, both *E. coli* ATCC 25922 and *E. coli* NCTC 12900 had less than 1 log_{10} CFU ml^{-1} reduction. However, *L. monocytogenes* NCTC 11994 was very sensitive, where even 5 s of treatment achieved reductions of 3.1 and 1.8 log_{10} CFU ml^{-1} with direct and indirect exposure, respectively. Meanwhile, 30 s of treatment decreased populations by about 6 log_{10} CFU ml^{-1}, regardless of mode of exposure.

Figure 4.2 b presents the release of intracellular components (nucleic acid) absorbing at 260 nm following plasma treatment. The results obtained at 280 nm (protein) were similar (Figure 4.2 c). For both *E. coli* strains, the absorption curves showed similar trends. A sharp increase in absorbance followed by a steady stage was recorded, indicating that cell integrity was compromised within 5 s of ACP treatment. While a similar trend was observed for the absorbance of *L. monocytogenes* NCTC 11994, the leakage recorded even after 120 s treatment was significantly less than that for *E. coli* strains within 5 s treatment.
Figure 4.2 Bacterial inactivation and cell leakage
Experimental conditions: 70 kV_{RMS} 0~120 s treatment in air following 24 h post-treatment storage. All experiments were carried out in duplicate and replicated at least twice.

4.5 DNA damage

Bacterial cells were treated with plasma and harvested cells were tested for DNA amplification by performing PCR as described in section 2.4.7.

Figure 4.3 represents extracted genomic DNA and PCR amplified products of untreated and ACP treated samples run on agarose gel electrophoresis. DNA samples were quantified by absorbance at 260 nm and adjusted to the same loading amount in each lane. ACP treated genomic DNA samples showed weaker band intensity than control i.e. untreated cells, thus indicating ACP treatment resulted in damage of DNA. With longer treatment time of 30 s, more damage was observed than with 5 s treatment (Figure 4.3 a, b, c). This pattern was noted for all strains studied, which correlated with microbial inactivation assessed by colony count method.

PCR results which are more sensitive for detection of small amount of DNA showed no noticeable difference between treated and untreated samples of *E. coli* (Figure 4.3 d and e). However, in case of *L. monocytogenes*, a 30 s of ACP treatment resulted in more DNA damage, with a weaker band intensity than 5 s ACP treated and control untreated samples (Figure 4.3 f). *L. monocytogenes* was more sensitive than other two strains of *E. coli* studied. These observations were correlated with the low survival rate of *L. monocytogenes* after ACP treatment.
Figure 4.3 Electrophoresis results of genomic DNA and 16s rRNA amplification

Experimental conditions: 70 kV<sub>RMS</sub> 0~30 s treatment in air following 24 h post-treatment storage

(a), (b), (c) Genomic DNA results. Lane: 1. 1 kb plus Marker; 2. control; 3. 5 s treated Direct; 4. 5 s treated Indirect; 5. 30 s treated Direct; 6. 30 s treated Indirect

(d), (e), (f) 16s rRNA amplification results. Lane: 1. control; 2. 5 s treated Direct; 3. 5 s treated Indirect; 4. 30 s treated Direct; 5. 30 s treated Indirect; 6. BenchTop pGEM (digested) Marker

4.6 Discussion

The influence of system and process parameters on inactivation were investigated including voltage, working gas and treatment time, with effects on cell membrane integrity and DNA damage assessed to complement inactivation efficacy data. In order to ensure system efficacy, ACP was tested against different types of bacteria which
can show different responses against plasma stress as noted previously (Hury et al., 1998; Kvam et al., 2012; Laroussi et al., 2003).

Applied voltage level had an impact on ACP antimicrobial efficacy. At higher voltage level greater microbial inactivation was achieved. The energy of ACP discharge is decided by applied voltage and frequency, thus generating different amount of reactive species which influence inactivation (Deng et al., 2007; Liu et al., 2013). Liu et al. (2013) studied the relationship between reactive species generation using helium as inducer gas and atmospheric non thermal plasma jet voltage level over time and found that four kinds of active species, N$_2^+$, OH, He and O, increased gradually with increasing applied voltage, which was attributed to higher inactivation efficacy (Liu et al., 2013). In the preliminary stages of our study, higher operating voltage resulted in higher inactivation efficacy, which could be ascribed to concentration of generated reactive species influencing the inactivation rate.

Besides voltage level, the working gas utilised for ACP discharge had a major effect on inactivation. The gas mixture with higher oxygen content (SB2: 65% O$_2$+30% CO$_2$+5% N$_2$) was the most effective for bacterial inactivation. Similar effects have been noted previously where oxygen gas plasma were very effective for microbial reduction (Hong et al., 2009; Hury et al., 1998; Laroussi and Leipold, 2004). ACP discharge in gas mixture containing oxygen generates highly reactive chemical species such as hydroxyl radicals (OH$^-$) and ozone (O$_3$). Presence of water either as humidity in gas or as liquid in a system during the plasma discharge results in the abundance of OH$^-$ radicals, H$_2$O$_2$ and hydronium ions H$_3$O$^+$ (Dobrynin et al., 2011; Pârvulescu et al., 2012). Thus, production of highly oxidizing species previously reported as having strong bactericidal effects resulted in significant inactivation effects. ACP inactivation efficacy in air was influenced by treatment time (Figure 4.2) and type of target cell.
ACP discharge in air also recorded better inactivation effects that can be assigned to generated ROS and RNS such as NO, NO$_2$.

The mode of ACP exposure showed some interesting inactivation effects which was related to the type of bacteria and working gas used. Direct plasma exposure was reported to have greater bactericidal effects than indirect exposure due to role of charged particles in synergy with generated reactive species (Dobrynin et al., 2009; Fridman et al., 2007). In our study, interestingly, in some cases, indirect mode of exposure was more effective than direct exposure. The possible explanation for this could be due to the prior recombining of reactive radicals generating reactive species with strong bactericidal effects, in addition to ozone, that might occur more predominantly in air and SB2.

The effects of ACP inactivation were also dependent on bacterial strains studied. Literature reports differential bacterial sensitivity towards plasma, based on their cell wall structures (Ermolaeva et al., 2011; Liang et al., 2012; Ma et al., 2008), while some suggested no clear differences in inactivation by cold atmospheric plasma treatment (Klämpfl et al., 2012). In our study, Gram positive L. monocytogenes populations were more sensitive than gram negative E. coli cells. Similar ACP sensitivity of bacterial species was reported previously where the lipopolysaccharide outer membrane of Gram negative bacteria was suggested to provide an enhanced protective barrier than the peptidoglycan cell wall structure of Gram positive bacteria against ACP generated reactive species (Tipa et al., 2011).

Bacterial inactivation by non-thermal plasma is a complex process and its mechanism of action is a subject of interest which is still not completely understood. Related to the system in use, it is warranted to evaluate specific system and process parameters in conjunction against potential target as one way of understanding mechanism of
action. In order to get some insights into the mechanism of ACP action, we investigated some biological consequences after ACP exposure. The effects of ACP process parameters on bacterial cell membrane integrity including effects on DNA damage were assessed. In common to other studies, there was a clear link to plasma treatment time and its inactivation efficacy (Deng et al., 2007; Ghomi et al., 2009; Joshi et al., 2011).

Joshi et al. (2011) attributed cell death to oxidation of DNA, protein and lipid during ACP treatment. However, Dobrynin et al. (2009) ascribed the primary target as cell membrane. The cell wall and vital intracellular macromolecules are also reported as main targets of reactive species (Dobrynin et al., 2009; Machala et al., 2009; Roth et al., 2010). In our study, the results of cell integrity measured by absorbance ratio 260/280, following ACP exposure showed different responses by Gram negative and Gram positive bacteria. The cell leakage (reflecting release of intracellular material such as proteins, DNA, RNA) results in our studies suggested the compromised cell membrane integrity of Gram negative bacteria, due to ACP exposure. Consisting of an outer membrane of lipopolysaccharide and thin layer of peptidoglycan, the cell wall of Gram negative bacteria is more vulnerable (Laroussi et al., 2003). However, a thick peptidoglycan layer is a much more stable structure of cell envelope for Gram positive bacteria, which resulted in different performance of cell membrane integrity during ACP treatment.

Comparing cell leakage and inactivation results with 5 s ACP exposure, a high leakage rate was detected for all strains whereas there were minor effects on cell viability noted (Figure 4.2). The possible explanation could be that short ACP exposure of 5 s results in reversible damage with the likelihood of activation of cell response system for repairing the damage (Dobrynin et al., 2009). Nevertheless, with increasing treatment
time, bacterial populations were reduced to undetectable levels. The plasma diffusion of generated reactive species into the cell results in damage of cell membrane and major cell constituents. The impact of ROS on bacterial cell components like nucleic acids, denaturation of proteins and peroxidation of lipids has been reported (Joshi et al., 2011).

The inactivation efficacy was also dependent on anti-oxidative activities of target bacteria.

Compared with *E. coli* ATCC 25922, non-toxigenic *E. coli* NCTC 12900 has been reported to have stronger resistance to acid stress, multi-drug resistance and higher rate of mutations, which has cross protective effect against wide range of environmental stresses including oxidative stress (Braoudaki and Hilton, 2004; Hosein, 2010; Maurer et al., 2005). These characteristics could impact the resistance of *E. coli* NCTC 12900 resulting in a different response to ACP stress comparing with *E. coli* ATCC 25922. Comparing the results of inactivation, *L. monocytogenes* was more sensitive to ACP treatment than two *E. coli* strains studied. In case of *L. monocytogenes*, the diffusion of ROS and RNS across the membrane would cause a severe irreversible damage of macromolecules including DNA, making the bacterial cells susceptible to ACP treatment. Therefore, we performed further investigations to assess ACP effects on genomic DNA damage and amplified DNA products.

In our study the extent of genomic DNA damage was dependent on type of bacteria and treatment time. Thus indicating that the concentration of ACP generated reactive species increased with time resulting in time dependent genomic DNA damage (Figure 4.3 a, b, c) with increased sensitivity towards plasma generated oxidative stress.

To further assess DNA fragmentation by plasma, amplification of DNA by PCR was performed that also revealed the DNA damage extent was dependent not only on the
type of bacteria but on ACP treatment time (Figure 4.3 d, e, f). Extensive DNA damage has been related to bacterial type and system parameters (Cooper et al., 2010; Joshi et al., 2011). Joshi et al. (2011) demonstrated the fragmentation of E. coli genomic DNA depending on length of plasma exposure (treatment time) and energy dose (J/cm²) using floating electrode DBD plasma application at 15 kV. Cooper et al. (2010) investigated DBD plasma treatment of Bacillus stratosphericus under dry environment at 30 kV for 120 s, and suggested direct interaction of charged particles or photons within plasma with bacterial cell envelope thus directly exposing internal components to plasma with extensive DNA damage.

In our study, 5 s of ACP treatment showed significant effects on membrane integrity but no significant impact on DNA damage was noted, thus suggesting repair is possible when the microbiological target is subject to short treatment time even at high voltage. After 30 s of treatment, population density was reduced in tandem with membrane leakage, while significant DNA damage was only evident for L. monocytogenes. Little DNA damage was noted for E. coli strains which could be due to the more layered Gram negative cell structure involving inner membrane, outer membrane and cell envelope that provides greater protection against plasma generated species. Further increasing treatment time could cause more adverse effects on nucleic acids resulting in irreversible DNA damage with loss of cell viability. The PCR results reveal the breakage of DNA strand on multi-sites. Cell viability can be maintained with little DNA damage observed with shorter plasma treatment time (Figure 4.3); specifically activities related to multi-copy genes may be unaffected. Using short treatment time up to 30 s at high voltage, the predominant effect of our system is related to the cell characteristics; where membrane damage may be the primary effect; while for L. monocytogenes intracellular components seemed to be the major target effected.
Recent studies highlighted activation of repair system of plasma treated bacteria in addition to up or down regulation of specific genes under ACP stress (Roth et al., 2010; Sharma et al., 2009).

Further research will involve plasma diagnostics to correlate both bacterial inactivation and the mechanism involved with reactive species generated during the process, thus enabling optimum process design appropriate to target. To understand in detail the ACP effects on intracellular targets, investigations on regulatory factors of ACP treated bacteria could elucidate the interaction between reactive species and cell response.

The process and system parameters investigated in the current study had a strong impact on bacterial inactivation. The higher voltage level of 70 kVRMS resulted in increased ACP inactivation efficacy. The working gas type significantly influenced inactivation process with greater efficacy recorded for gas mixture with higher oxygen content. The ACP inactivation efficacy was also dependent on target cell type, with greater sensitivity noted for Gram positive *L. monocytogenes*.

The studies assessing effects of ACP on cell membrane integrity and intracellular components also showed the damage pattern dependent on target cell type. The cell membrane integrity was compromised following shorter ACP treatment time but with little reduction in surviving population. Increasing treatment time had a major influence on bacterial reduction in addition to noticeable DNA damage observed for *L. monocytogenes*. Overall, the bacterial inactivation was well correlated with DNA damage effects following ACP exposure.

This study pointed to the need for a plasma diagnostic approach to correlate bacterial inactivation mechanism with generated reactive radicals in tandem with investigation
of cell regulatory responses by utilising knock-out strains to provide insight on bacterial protection or enhanced sensitivity towards ACP.
Chapter 5. EFFECT OF WORKING GAS ON ATMOSPHERIC COLD PLASMA EFFICACY AND MECHANISM OF ACTION

The influence that gas composition may have on inactivation efficacy in conjunction with other parameters was observed in Chapter 4. Further investigation of how any biocidal effect may be enhanced through working gas composition was warranted. Hence, the pattern of reactive species generated in association with the working gas composition and their interaction with microbes were evaluated. Pathogens with strong virulence and resistance characteristics were used, representing both Gram negative and positive bacteria. A gas mixture of MP1: 70% N₂ + 30% CO₂ was used and compared with a gas mixture of MP2: 70% O₂ + 30% CO₂. These gases and air are commonly employed in MAP by fresh and cooked meat processors.

5.1 Inactivation efficacy associated process and system parameters

Inactivation levels in Tables 5.1-5.3 were related to process and system parameters, including treatment time, post-treatment storage time and applied gas mixtures. There was no obvious difference in survival level of *E. coli* after treatment with 15 and 60 s in MP1 (70% N₂ + 30% CO₂) (between 7.47±0.11 to 6.79±0.10 Log₁₀ CFU ml⁻¹, p>0.05), while the numbers after 300 s treatment were slightly decreased. In SB1 (90% N₂ + 10% O₂) and air much lower survival levels were observed with 300 s treatment after 24 h storage, at 2.31±0.73 and 4.23±0.13 Log₁₀ CFU ml⁻¹ respectively. However, using MP2 (70% O₂ + 30% CO₂), populations were undetectable after 300 s treatment in conjunction with post-treatment storage time (1 and 24 h) (p<0.05). (Table 5.1)
Table 5.1 Surviving cell number of *E. coli* NCTC 12900 with respect to treatment and post-treatment storage time in different gases

<table>
<thead>
<tr>
<th>Gases</th>
<th>Plasma treatment time (s)</th>
<th>0 h</th>
<th>1 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cell density (Log$_{10}$ CFU ml$^{-1}$)</td>
<td>SD*</td>
<td>Cell density (Log$_{10}$ CFU ml$^{-1}$)</td>
</tr>
<tr>
<td>MP1 70% N$_2$+</td>
<td>0</td>
<td>8.00 $^a$</td>
<td>0.05</td>
<td>7.92 $^a$</td>
</tr>
<tr>
<td>30% CO$_2$</td>
<td>15</td>
<td>7.47 $^b$</td>
<td>0.11</td>
<td>7.45 $^b$</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.21 $^{bc}$</td>
<td>0.36</td>
<td>7.43 $^b$</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>7.04 $^c$</td>
<td>0.09</td>
<td>6.65 $^e$</td>
</tr>
<tr>
<td>SB1 90% N$_2$+</td>
<td>0</td>
<td>7.99 $^a$</td>
<td>0.15</td>
<td>7.92 $^a$</td>
</tr>
<tr>
<td>10% O$_2$</td>
<td>15</td>
<td>7.80 $^{ad}$</td>
<td>0.04</td>
<td>7.41 $^b$</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.78 $^{ad}$</td>
<td>0.15</td>
<td>7.11 $^d$</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>7.61 $^d$</td>
<td>0.13</td>
<td>6.82 $^f$</td>
</tr>
<tr>
<td>Air</td>
<td>0</td>
<td>7.99 $^a$</td>
<td>0.15</td>
<td>7.92 $^a$</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>8.00 $^a$</td>
<td>0.05</td>
<td>7.61 $^c$</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.85 $^{ad}$</td>
<td>0.04</td>
<td>7.08 $^d$</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>7.66 $^d$</td>
<td>0.11</td>
<td>6.26 $^g$</td>
</tr>
<tr>
<td>MP2 70% O$_2$+</td>
<td>0</td>
<td>7.91 $^a$</td>
<td>0.02</td>
<td>7.92 $^a$</td>
</tr>
<tr>
<td>30% CO$_2$</td>
<td>15</td>
<td>7.99 $^a$</td>
<td>0.08</td>
<td>7.11 $^d$</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.95 $^a$</td>
<td>0.07</td>
<td>5.17 $^h$</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>6.60 $^e$</td>
<td>0.19</td>
<td>ND* $^i$</td>
</tr>
</tbody>
</table>

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

*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log$_{10}$ CFU ml$^{-1}$)

Experimental conditions: 0~300 s treatment with indirect exposure at 70 kVRMS following 0, 1 and 24 h post-treatment storage.

Similar results were obtained from the inactivation of *S. aureus* represented in Table 5.2. The effect of increasing oxygen composition was clearly observed with the
decrease of surviving cell numbers (p<0.05), where 300 s treatment followed by 24 h post-treatment storage reduced populations to 6.60±0.25, 4.72±0.73, 3.54±0.51 Log_{10} CFU ml^{-1} or undetectable using the respective applied working gases.

Table 5.2 Surviving cell number of *S. aureus* ATCC 25923 with respect to treatment and post-treatment storage time in different gases

<table>
<thead>
<tr>
<th>Gases</th>
<th>Plasma treatment time (s)</th>
<th>Post-treatment storage time</th>
<th>Cell density (Log_{10} CFU ml^{-1})</th>
<th>SD*</th>
<th>Cell density (Log_{10} CFU ml^{-1})</th>
<th>SD*</th>
<th>Cell density (Log_{10} CFU ml^{-1})</th>
<th>SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP1 70% N₂+ 30% CO₂</td>
<td>C</td>
<td>0 h</td>
<td>8.19 ±0.03</td>
<td>0.06</td>
<td>8.18 ±0.06</td>
<td>0.06</td>
<td>8.18 ±0.06</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1 h</td>
<td>7.52 ±0.11</td>
<td>0.05</td>
<td>7.09 ±0.05</td>
<td>0.17</td>
<td>6.73 ±0.05</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1 h</td>
<td>7.27 ±0.09</td>
<td>0.07</td>
<td>5.99 ±0.07</td>
<td>0.14</td>
<td>6.60 ±0.07</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>1 h</td>
<td>7.37 ±0.08</td>
<td>0.16</td>
<td>5.76 ±0.10</td>
<td>0.05</td>
<td>6.60 ±0.07</td>
<td>0.25</td>
</tr>
<tr>
<td>SB1 90% N₂+ 10% O₂</td>
<td>C</td>
<td>0 h</td>
<td>8.16 ±0.07</td>
<td>0.06</td>
<td>8.18 ±0.06</td>
<td>0.06</td>
<td>8.18 ±0.06</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1 h</td>
<td>7.19 ±0.11</td>
<td>0.07</td>
<td>7.20 ±0.07</td>
<td>0.19</td>
<td>6.81 ±0.07</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1 h</td>
<td>7.37 ±0.08</td>
<td>0.25</td>
<td>6.93 ±0.07</td>
<td>0.08</td>
<td>6.44 ±0.07</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>1 h</td>
<td>7.00 ±0.13</td>
<td>0.20</td>
<td>6.07 ±0.10</td>
<td>0.73</td>
<td>6.44 ±0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>Air</td>
<td>C</td>
<td>0 h</td>
<td>8.16 ±0.07</td>
<td>0.06</td>
<td>8.18 ±0.06</td>
<td>0.06</td>
<td>8.18 ±0.06</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1 h</td>
<td>7.87 ±0.10</td>
<td>0.11</td>
<td>7.14 ±0.07</td>
<td>0.22</td>
<td>6.97 ±0.07</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1 h</td>
<td>7.83 ±0.07</td>
<td>0.09</td>
<td>6.67 ±0.07</td>
<td>0.03</td>
<td>6.44 ±0.07</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>1 h</td>
<td>7.75 ±0.04</td>
<td>0.05</td>
<td>6.59 ±0.07</td>
<td>0.51</td>
<td>3.54 ±0.05</td>
<td>0.51</td>
</tr>
<tr>
<td>MP2 70% O₂+ 30% CO₂</td>
<td>C</td>
<td>0 h</td>
<td>7.88 ±0.05</td>
<td>0.06</td>
<td>8.18 ±0.06</td>
<td>0.06</td>
<td>8.18 ±0.06</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1 h</td>
<td>7.42 ±0.15</td>
<td>0.05</td>
<td>6.66 ±0.05</td>
<td>0.13</td>
<td>6.18 ±0.05</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1 h</td>
<td>7.50 ±0.04</td>
<td>0.68</td>
<td>2.94 ±0.07</td>
<td>0.12</td>
<td>3.15 ±0.07</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>1 h</td>
<td>6.68 ±0.26</td>
<td>ND*</td>
<td>-</td>
<td>ND*</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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

*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log_{10} CFU ml^{-1})
Experimental conditions: 0–300 s treatment with indirect exposure at 70 kV$_{RMS}$ following 0, 1 and 24 h post-treatment storage

Table 5.3 Surviving cell number of *L. monocytogenes* NCTC 11994 with respect to treatment and post-treatment storage time in different gases

<table>
<thead>
<tr>
<th>Gases</th>
<th>Plasma treatment time (s)</th>
<th>Post-treatment storage time</th>
<th>0 h</th>
<th>1 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cell density (Log$_{10}$ CFU ml$^{-1}$)</td>
<td>Cell density (Log$_{10}$ CFU ml$^{-1}$)</td>
<td>Cell density (Log$_{10}$ CFU ml$^{-1}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD*</td>
<td>SD*</td>
<td>SD*</td>
</tr>
<tr>
<td>MP1 70% N$_2$ + 30% CO$_2$</td>
<td>C</td>
<td>7.49 a</td>
<td>0.31</td>
<td>7.85 a</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>6.84 b</td>
<td>0.14</td>
<td>6.43 b</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>6.94 b</td>
<td>0.15</td>
<td>6.18 bd</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>6.85 b</td>
<td>0.09</td>
<td>5.09 c</td>
<td>0.12</td>
</tr>
<tr>
<td>SB1 90% N$_2$ + 10% O$_2$</td>
<td>C</td>
<td>7.98 c</td>
<td>0.11</td>
<td>7.85 a</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>7.21 d</td>
<td>0.13</td>
<td>6.58 b</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
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<td>7.16 e</td>
<td>0.06</td>
<td>6.01 bd</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>6.24 f</td>
<td>0.19</td>
<td>3.59 e</td>
<td>0.15</td>
</tr>
<tr>
<td>Air</td>
<td>C</td>
<td>7.98 c</td>
<td>0.11</td>
<td>7.85 a</td>
<td>0.12</td>
</tr>
<tr>
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<td>15</td>
<td>8.06 c</td>
<td>0.08</td>
<td>5.55 f</td>
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</tr>
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<td>5.12 c</td>
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<td>7.72 g</td>
<td>0.06</td>
<td>ND* g</td>
<td>-</td>
</tr>
<tr>
<td>MP2 70% O$_2$ + 30% CO$_2$</td>
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<td>8.27 c</td>
<td>0.02</td>
<td>7.85 a</td>
<td>0.12</td>
</tr>
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<td>3.94 h</td>
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<tr>
<td></td>
<td>60</td>
<td>3.70 h</td>
<td>0.03</td>
<td>ND* g</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>ND* i</td>
<td>-</td>
<td>ND* g</td>
<td>-</td>
</tr>
</tbody>
</table>

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

*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log$_{10}$ CFU ml$^{-1}$)

Experimental conditions: 0–300 s treatment with indirect exposure at 70 kV$_{RMS}$ following 0, 1 and 24 h post-treatment storage
Table 5.3 represents the inactivation results of *L. monocytogenes*. As another Gram positive pathogen, much lower survival levels were observed by comparison with *S. aureus*. Extending treatment time from 15 s to 60 s or 300 s enhanced inactivation, where levels were undetectable using MP2 immediately after 300 s treatment, even without a post-treatment storage time. By extending post-treatment storage time to 24 h, no culturable cells were detected from this high oxygen mix even with only 15 s treatment. While SB1 and air also achieved inactivation under the detection limit (<1.0 $\log_{10}$ CFU ml$^{-1}$) with the longest treatment time employed of 300 s in conjunction with 24 h post-treatment storage; the MP1 gas mix had 6.10±0.13 $\log_{10}$ CFU ml$^{-1}$ surviving cells (Table 5.3).

### 5.2 Reactive species generation in gas phase

The in-package ozone concentration was measured using colorimetric tubes, which revealed its evolution in relation to the applied gas mixture, treatment and post-treatment storage time (Table 5.4).

<table>
<thead>
<tr>
<th>Post-treatment storage time (h)</th>
<th>Plasma treatment time (s)</th>
<th>Ozone concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MP1</td>
<td>SD*</td>
</tr>
<tr>
<td>0 h</td>
<td>15</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>ND*</td>
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<tr>
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<td>ND*</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td>24 h</td>
<td>15</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>ND*</td>
</tr>
</tbody>
</table>

ND*: Under detection limit (<1 ppm)
In-package ozone densities were similar for each bacterial or PBS sample. For MP1 samples, ozone measurements were all under the detection limit (<1 ppm). No ozone was detected in any treatment condition after the 24 h post-treatment storage time. The results correlated with the increasing inactivation in association with extending the post-treatment storage time, while the exhaustion time of in-package ozone is longer than 1 h.

Spectroscopic analysis of the discharge was carried out in the 4 gases at 70 kV_{RMS} over the range of 200–920 nm (Figure 5.1). Distinct peaks obtained in the near UV region corresponded to strong emissions from N\textsubscript{2} and N\textsuperscript{2+} excited species. The emission intensities during discharge in different gas mixtures were plotted in Figure 5.1. The emission of OH\textsuperscript{•} radicals at 309.65 nm (Figure 5.1 a) from air had higher intensities than other gases after 60 s discharge, while MP1 and SB1 had similar intensities during 300 s treatment which were slightly higher than those from MP2. The emission intensities of N\textsubscript{2} species at 336.65 nm (Figure 5.1 b) were influenced by the N\textsubscript{2} percentage in the applied gas mixtures. Air showed higher emission than MP2, although they had a similar nitrogen percentage. Other peaks of nitrogen species repeated similar patterns (data not shown).
Figure 5.1 Emission spectrum of dielectric barrier discharge atmospheric cold plasma operating in air under atmospheric pressure

(a) Emission intensity at 309.65 nm; (b) Emission intensity at 336.65 nm. All experiments were carried out in duplicate and replicated at least twice.

5.3 Reactive species generation in liquid phase

Figure 5.2-5.5 shows the results of reactive species analysis in PBS and cell suspensions of *E. coli*, *L. monocytogenes* and *S. aureus* in PBS immediately after ACP treatment.
General ROS densities are represented in Figure 5.2 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 5.2 a, while similar trends were also noticed from cell suspensions in Figure 5.2 b, c and d. The general ROS density assay results showed that fluorescence densities increased with the oxygen concentration in applied gas mixtures, where MP1 had the highest readings for both pure PBS or bacteria suspension samples (p≤0.05).

Figure 5.2 General reactive oxygen species density detection by DCFH DA
Experimental conditions: 15, 60, 300 s treatment at 70 kV RMS without post-treatment storage. All experiments were carried out in duplicate and replicated at least twice.
The generation of singlet oxygen and hydrogen peroxide repeated the correlation with oxygen content recorded in Figure 5.3 and 5.4, while the effect of treatment time was observed for singlet oxygen in PBS only, but in both PBS and bacterial samples for hydrogen peroxide.

Figure 5.3 Singlet oxygen detection by SOSGR

Experimental conditions: 15, 60, 300 s treatment at 70 kV_RMS without post-treatment storage. All experiments were carried out in duplicate and replicated at least twice.
Experimental conditions: 15, 60, 300 s treatment at 70 kV\textsubscript{RMS} without post-treatment storage. All experiments were carried out in duplicate and replicated at least twice.

RNS detection showed the same trend for both Gram positive and negative strains (Figure 5.5). Bacterial samples in SB1, which had the highest N\textsubscript{2} percentage, showed the highest fluorescence signal (p≤0.05). However, results from PBS samples differed, where those treated in air had the highest signals (p<0.05), followed by SB1 and MP1.
Figure 5.5 General reactive nitrogen species density detection by DAF FM DA

Experimental conditions: 15, 60, 300 s treatment at 70 kV\textsubscript{RMS} without post-treatment storage. All experiments were carried out in duplicate and replicated at least twice.

Nitrite and nitrate concentrations with various treatment and post-treatment storage time in different gas mixtures are presented in Figure 5.6 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<0.05). By extending post-treatment storage time, the nitrite concentration slightly increased in MP1 samples (from 12.16±0.18 µM to 15.49±0.03 µM with 300 s treatment, p<0.05), while treatment time also had an obvious positive
effect (p<0.05). However, nitrite/nitrate concentrations in SB1 and air increased, by extending post-treatment storage time from 0 h to 24 h, from similar levels (12.27±0.13 µM and 11.96±0.37 µM after 300 s treatment) to a much higher level (28.43±0.45 µM and 22.86 ±0.63 µM after 300s treatment) by comparison with MP1.

MP2 samples maintained very low nitrite/nitrate concentration (<1 µM) after 300 s treatment, which increased to 2.97±0.13 µM after storage for 24 h.
Figure 5.6 Nitrite/Nitrate concentration in PBS solution

Experimental conditions: 15, 60, 300 s treatment at 70 kV\textsubscript{RMS} following 0, 1 and 24 post-treatment storage. All experiments were carried out in duplicate and replicated at least twice.

(a) Nitrite concentration without post-treatment storage;
(b) Total Nitrite/Nitrate concentration without post-treatment storage;
(c) Nitrite concentration with 1 h post-treatment storage;
(d) Total Nitrite/Nitrate concentration with 1 h post-treatment storage.
(e) Nitrite concentration with 24 h post-treatment storage;
(f) Total Nitrite/Nitrate concentration with 24 h post-treatment storage.

5.4 Discussion

The effect of gas composition on ACP inactivation efficacy and mechanism with respect to reactive species was elucidated. The increasing oxygen content led to higher ROS generation, and correlated with increasing inactivation efficacy, which was also observed from sterilization of bacteria and endospores by Shintani et al. (2010). The results of ROS density in liquid assays showed that general ROS, singlet oxygen and
hydrogen peroxide increased with the oxygen concentration in applied gas mixtures, where MP2 had the highest readings for both PBS or bacteria suspension samples (Figure 5.2, 5.3 and 5.4, p≤0.05), confirming the inactivation mechanism with respect to processing variables of treatment time and oxygen percentage.

In PBS solution, DCFH DA could be altered by reactive species and transferred to DCFH form before entering the cells, which is further oxidized and detected as fluorescence signal (Gomes et al., 2005). The results detected represent total ROS post-discharge, while the detected levels from bacteria suspensions were significantly reduced by their interactions with cell components. At the same time, intracellular ROS detected from MP2 were much higher from S. aureus and L. monocytogenes than E. coli (Figure 5.2, p<0.05), although similar inactivation levels in all applied gases were achieved in E. coli and S. aureus (Table 5.1 and 5.2). This effect has been observed before due to the detected ROS densities and cell damage after plasma exposure (Gaunt et al., 2006; Han et al., 2014; Ziuzina et al., 2015). It indicated the different mechanism of action with Gram positive and negative bacteria, due to the different chemical structure of their cell wall (Laroussi et al., 2003; Shintani, 2015). The mechanism of action of reactive species was determined by their different key targets, which is the cell envelope for Gram negative bacteria and intracellular components for Gram positive microbes (Han et al., 2014). L. monocytogenes was the most sensitive target bacteria in our study, where no detectable survival was observed from Gas SB1, air and MP2 with extended treatment time and post-treatment storage time (Table 5.3). Similar reactive species patterns were observed from S. aureus and L. monocytogenes, which indicated a similar mode of action regardless of sensitivity (Figure 5.2, 5.3 and 5.4). 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 high frequency.

In contrast to total ROS results, the hydrogen peroxide concentrations in *E. coli* suspensions were much higher than those from PBS samples (Figure 5.4, p≤0.05), while Gram positive samples had much lower H$_2$O$_2$ concentrations (Figure 5.4, p>0.05). The cellular reactions with reactive species might lead to the generation of more H$_2$O$_2$ in Gram negative bacteria, while it was consumed by those in Gram positive bacteria. The generation of hydrogen peroxide in PBS also increased with oxygen content of applied gas mixtures, but samples from air were only slightly lower than those from MP2 (p<0.05). The decelerated increase of hydrogen peroxide concentration might be associated with the generation of other ROS, such as singlet oxygen. Moreover, air used was compressed atmospheric air with high humidity, while Gas SB1, MP1 and MP2 were obtained from dried gas cylinders. Moiseev et al. (2014) indicated that the humidity inside a package had a quenching effect on ozone generation, resulting in higher hydroxyl radical density, with a similar effect observed from Figure 5.1 a, where air showed a higher emission intensity of OH radical during discharging than the other gases. This was also observed from our OES results at 309.65 nm. Hydroxyl radicals have a short life and high reactivity, which can cause breakage of DNA strands (Shintani, 2015; Shintani et al., 2010), and might lead to the generation of hydrogen peroxide with better stability. However, it was reported that hydrogen peroxide can generate hydroxyl radicals through intracellular redox, such as Fenton reaction, but not in reverse (Graves, 2012; Kellogg and Fridovich, 1975).

Moreover, great differences in hydrogen peroxide concentrations were detected from cell suspensions (Figure 5.4). After exposure longer than 15 s, the readings from *E. coli* solutions were much higher than PBS samples for all gases, while those from *S.
*aureus* and *L. monocytogenes* suspensions were lower than PBS. The cell envelope of *E. coli* consists of lipopolysaccharides, which can be oxidized easily and reduce the strong oxidative species to less active species by reaction with O-H bond (such as lipid A) (Arjunan et al., 2015; Laroussi et al., 2003; Yusupov et al., 2013). These reactions might lead to the lesion of cell membrane and production of more peroxides, which is a possible reason why higher concentrations were detected from PBS than from *E. coli* samples. Our previous study showed obvious cell leakage after ACP treatment of Gram negative bacteria, but not Gram positive samples (Han et al., 2014).

Moreover, DCFH DA was reported as unsuitable for hydrogen peroxide detection without the presence of sufficient oxidases or peroxidases (Gomes et al., 2005). Much lower fluorescence signals were obtained in H$_2$O$_2$ solutions (up to 30%) with or without cells than ACP treated samples (data not shown). Therefore, hydrogen peroxide concentrations detected in this study were not included in general ROS results, which could explain their different patterns and the conversion between short-lived and long-lived ROS species.

In contrast to general ROS and hydrogen peroxide results, similar signal levels from Gram negative and positive bacteria samples were found with singlet oxygen (Figure 5.3). The possible reason might be that the singlet oxygen is a highly reactive species targeting various biological molecules, with strong penetrating effect and a relatively short life time (Joshi et al., 2011; Sousa et al., 2011). The singlet oxygen and other reactive oxygen species (superoxide, hydroxyl radical, hydrogen peroxide etc.) are cytotoxic species, which could cause damage to bacterial cells through several mechanisms. These include oxidation of membrane lipids and amino acids in proteins, cross-linking of proteins and oxidation of nucleic acid. It is reported the presence of hydrogen peroxide and nitric oxide could lead to the generation of singlet oxygen and
hydroxyl radical and result in more cell damage (Beckman et al., 1990; Noronha-Dutra et al., 1993). However, the intracellular signal was not achievable by our assay method. Different from the strong sterilization effect of ROS, the cellular damage of RNS was reported mostly 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), peroxynitrite (ONOO⁻), peroxynitrous acid (OONOH), and alkylperoxynitrite (ROONO) (Arjunan et al., 2015; Graves, 2012). The main reactive nitrogen species involved in bactericidal action are NO and NO₂ and their final format of nitrogen species after reaction are nitrite and nitrate. RNS detection showed the same trend for both Gram positive and negative strains (Figure 5.5 and 5.6).

The difference of intracellular ROS between Gram negative and positive species was not observed for RNS. Similar to singlet oxygen, RNS are short-lived species, but its half-life can be prolonged by converting to peroxynitrites which react widely with biomolecules (Graves, 2012; Sakudo et al., 2012). With 90% N₂ content, SB1 (90% N₂+ 10% O₂) gas showed highest RNS concentration in cell suspensions, while air had highest signal in PBS solution. In treated PBS solutions, DAF FM DA was the primary target for reactive species, due to absence of microbes or interfering organic compounds inside the solution. Similar to DCFH DA, the diacetate group of DAF FM DA can be removed by redox reaction before reacting with RNS. However, the amount of free ROS was decreased with the presence of bacterial cells. The amount of detected RNS could be clearly observed from cell suspensions results. Moreover, ROS have not only been reported to influence the fluorescence of DAF FM (Balcerczyk et al., 2005), but also have reversible or irreversible conversion with RNS (Balcerczyk et al., 2005;
Laroussi and Leipold, 2004). This could explain the increase of fluorescence signal from treated samples in MP2 and higher detected RNS levels of PBS samples in air than SB1 (Figure 5.5, p<0.05).

The generation of nitrogen species in liquid is the result of gas discharging in gas phase, which is shown in OES (Figure 5.1). The results showed the real time reactive nitrogen species emission, where SB1 had highest intensity. The amount of excited nitrogen was primarily dominated by the nitrogen content. Although a similar percentage of nitrogen was contained in MP1 and air, air had higher emission intensity at 336.65 nm and detected RNS densities. N₂ molecules have been reported to be resistant to ionization, with high dissociation energy (Shintani et al., 2010). In our study, the ionizing of N₂ level was much higher when mixing with O₂ than CO₂, while the dissociation energy of them are similar (Shintani et al., 2010).

Reactive species in this study were only reported immediately after ACP treatment, due to their short life time. However, their reactions with cell components were taking place during the storage procedure and led to further inactivation. After 1 h post-treatment storage time, inactivation levels were improved compared to all samples with no storage (Table 5.1, 5.2 and 5.3, p≤0.05). During storage, long-term effects on bacterial cells following treatment could be attributed to long-lived reactive species, which might result from the conversion of short-lived species. With in-package processing, post-treatment storage procedure could be an economic and effective way of enhancing the sterilization effect (Ziuzina et al., 2013). However, survival levels after 24 h post-treatment storage were slightly higher than 1 h stored samples in some cases, which may indicate long-term repairing and recovery effects in microbes with sub-lethal damage (Han et al., 2014; Mai-Prochnow et al., 2014). This was confirmed by the ozone and nitrite/nitrate assay results.
The effect of oxygen composition was revealed with extended post-treatment storage, which indicated the time that ROS required for bactericidal action (>1 h). The detected concentration of Nitrite/Nitrate increased with extended post-treatment storage time (Figure 5.6), which implied some RNS transferred to longer lived species in gas phase before diffusion into liquid solution. With similar levels detected immediately after ACP exposure, much higher nitrite/nitrate concentrations were obtained from SB1 and air samples than MP1 after 24 h post-treatment storage. This confirmed enhanced N₂ dissociation and generation of RNS when in the presence of oxygen in applied gas mixtures and indicated the RNS action as a long term reaction (>1 h).

The effect of post-treatment storage time on both 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. A retention of effect could lead to higher sterilization efficacy with reduced energy consumption.

Overall, the microbicidal effect of ACP is dependent on the oxygen content of applied gases, as well as treatment time and post-treatment storage time. While the capability of generating ROS was mainly dependent on the oxygen content in applied gases, RNS formation was governed by both nitrogen content and presence of oxygen. Furthermore, the intracellular reactive species densities were different in relation to their anti-bacterial mode of actions and cell type. Most ROS can 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, other reactive species, such as singlet oxygen and RNS, are relatively short lived species and highly convertible to other forms. Their densities were detected at similar levels in Gram
negative and positive bacteria samples. Moreover, both ROS and RNS showed a treatment time dependent manner, which could improve the microbicidal effect. The reaction between reactive species and bacteria could be enhanced by extending post-treatment storage time (retention). The optimization of ACP treatment can be determined with regards to operating process parameters, including treatment time, post-treatment storage time and applied gas mixture contents.

Future studies were warranted on the reactive species damaging patterns with regards to target characteristics.
Chapter 6. MODE OF MICROBICIDAL ACTION OF ATMOSPHERIC COLD PLASMA

Microbial inactivation efficacy by ACP has been comprehensively investigated in Chapters 3-5 in relation to a range of system and processing parameters. Interestingly, the microbial damaging patterns of ACP were observed as cell membrane integrity and genomic DNA damage in Chapter 4, which revealed the different damage mechanisms for Gram negative and positive bacteria. It was warranted to further expand understanding of the different patterns of damage against Gram positive and Gram negative bacteria. The interactive effects of intracellular ROS generation and DNA damage with treatment time were examined in conjunction with spectral diagnostics of the in package process to further elucidate the mechanism. Moreover, an optimized design of ACP system was used in this section named DIT 120-plus with a different dielectric barrier to prevent sparking during discharging.

6.1 Effect of treatment time and post-treatment storage time on plasma inactivation efficacy

The inactivation efficacy of ACP against *E. coli* NCTC 12900 and *S. aureus* ATCC 25923 is shown in Tables 6.1 and 6.2. Inactivation was related to both treatment time and post-treatment storage time.

After 1 min exposure of ACP, *E. coli* populations decreased by around $2 \log_{10} \text{CFU ml}^{-1}$ in conjunction with 24 h post treatment storage. When treatment time was increased to 3 min, bacterial populations were undetectable after either 1 or 24 h storage times. Without post-treatment storage, approximately 3.6 and 2.3 $\log_{10} \text{CFU ml}^{-1}$ reductions were detected with direct and indirect treatment after 3 min, but further
extending treatment time to 5 min resulted in $6 \log_{10} \text{CFU ml}^{-1}$ and at least $8 \log_{10} \text{CFU ml}^{-1}$ reductions for direct and indirect exposure respectively (Table 6.1, $p \leq 0.05$).

Table 6.1 Surviving cell numbers of *E. coli* NCTC 12900 with respect to treatment and post-treatment storage time

<table>
<thead>
<tr>
<th>Post-treatment storage time (h)</th>
<th>Plasma treatment time (min)</th>
<th>Mode of Plasma Exposure</th>
<th>Direct</th>
<th>Indirect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cell density (Log$_{10}$ CFU ml$^{-1}$)</td>
<td>SD*</td>
<td>Cell density (Log$_{10}$ CFU ml$^{-1}$)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<td>0.0</td>
<td>8.0$^a$</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7.6$^a$</td>
<td>0.1</td>
<td>7.3$^b$</td>
</tr>
<tr>
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</tr>
<tr>
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<td>5</td>
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<td>0.7</td>
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</tr>
<tr>
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<td>0.0</td>
<td>8.0$^a$</td>
</tr>
<tr>
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<td>7.2$^d$</td>
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<td>7.1$^b$</td>
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<td>ND$^e$</td>
<td>0.0</td>
<td>ND$^d$</td>
</tr>
</tbody>
</table>

Different letters indicate a significant difference at the 0.05 level between different treatment times and post-treatment storage times.

Experimental conditions: 80 kV$_{\text{RMS}}$ 1~5 min treatment in air following 0, 1 or 24 h post-treatment storage. Critical controls were provided as 0 min treated samples with 0, 1 and 24 h post-treatment storage.

*SD*: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND*: under detection limit ($<1.0 \log_{10} \text{CFU ml}^{-1}$)

A similar trend of ACP inactivation was recorded for *S. aureus*. With 24 h post-treatment storage, all treatment times used led to undetectable levels of bacteria,
irrespective of the mode of exposure. Increasing treatment time, from 1 min to either 3 or 5 min, yielded undetectable levels, with direct and indirect exposure, respectively, after 1 h storage. With no post treatment storage time, populations declined by approximately 1.8 and 6.1 Log$_{10}$ CFU ml$^{-1}$ by increasing treatment time from 1 min to 5 min with direct exposure (Table 6.2, p≤0.05). Similar effects were achieved with indirect exposure.

Table 6.2 Surviving cell numbers of *S. aureus* ATCC 25923 with respect to treatment and post-treatment storage time

<table>
<thead>
<tr>
<th>Post-treatment storage time (h)</th>
<th>Plasma treatment time (min)</th>
<th>Mode of Plasma Exposure</th>
<th>Cell density (Log$_{10}$ CFU ml$^{-1}$)</th>
<th>SD*</th>
<th>Cell density (Log$_{10}$ CFU ml$^{-1}$)</th>
<th>SD*</th>
</tr>
</thead>
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<td>0.2</td>
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<tr>
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<td>1</td>
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<tr>
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<td>3</td>
<td>Indirect</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Indirect</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>Indirect</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different letters indicate a significant difference at the 0.05 level between different treatment times and post-treatment storage times

Experimental conditions: 80 kV$_{RMS}$ 1~5 min treatment in air following 0, 1 or 24 h post-treatment storage. Critical controls were provided as 0 min treated samples with 0, 1 and 24 h post-treatment storage.
*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log \textsubscript{10} CFU ml\textsuperscript{-1})

6.2 Effect on cell membrane integrity

The absorbance of 260 and 280 nm which is commonly used for quantification of DNA and protein concentration, can also indicate the release of intracellular DNA and protein and loss of cell integrity (Virto et al., 2005). Different trends between \textit{E. coli} and \textit{S. aureus} were observed from their absorbance measured at 260 and 280 nm following plasma treatment (Figure 6.1).

For \textit{E. coli}, all absorption curves showed similar trends (Figure 6.1 a). With 24 h post-treatment storage, a sharp immediate increase in absorbance was followed by a steady stage, indicating that the cell integrity was compromised within 1 min of ACP treatment. In the case of 0 and 1 h post treatment storage samples, a sharp increase at 1 min of treatment was followed by a gradual increase in the absorbance as a function of treatment time (p\textless 0.05). In contrast, no leakage was recorded for \textit{S. aureus}, even after 5 min treatment (Figure 6.1 c, p>0.05). However, a small increase of absorbance was observed for the 24 h post-treatment storage sample group for both control and treated samples. Similar trends were observed at 280 nm (Figure 6.1 b, d).
Figure 6.1 Absorbance of ACP treated bacterial suspension in PBS at 260 and 280 nm with different post-treatment storage times

Experimental conditions: 1, 3, 5 min treatment at 80 kV$_{\text{RMS}}$ following 0, 1, 24 h post-treatment storage. Data points at 0 min treatment time refer to untreated control stored with 0, 1, 24 h in PBS. All experiments were carried out in duplicate and replicated at least twice.

6.3 Reactive oxygen and nitrogen species

The emission spectrum of ACP discharging is presented in Figure 6.2 a. Analysis of the discharge was carried out in air at 80 kV$_{\text{RMS}}$ over the range of 200–920 nm. Distinct
peaks obtained in the near UV and visible regions corresponded to strong emissions from N\textsubscript{2} and N\textsubscript{2}\textsuperscript{+} excited species. In Figure 6.2 b, one of the major emission intensities was one of second positive N\textsubscript{2} system from empty box and sample packages, where other major peaks had similar results (data not shown).

Figure 6.2 Emission spectrum of dielectric barrier discharge atmospheric cold plasma operating at 80 kV\textsubscript{RMS} in air under atmospheric pressure

(a) Emission spectrum of empty box as control; (b) Emission intensity at 336.65 nm.

All experiments were carried out in duplicate and replicated at least twice.
The in-package ozone concentration was investigated as described in 2.4.2.2, which revealed its correlation with treatment and post-treatment storage time (Table 6.3).

Table 6.3 In-package ozone concentration after different ACP treatment and post-treatment storage time with both *E. coli* and *S. aureus* samples

<table>
<thead>
<tr>
<th>Post-treatment storage time (h)</th>
<th>Plasma treatment time (min)</th>
<th>Ozone concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Direct</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>1600</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2400</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4200</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>330</td>
</tr>
<tr>
<td>24</td>
<td>1</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>ND*</td>
</tr>
</tbody>
</table>

ND*: Under detection limit (<1 ppm).

The in-package ozone densities were similar for each bacterial sample. Treatment time and post-treatment storage time had positive and negative effects respectively on the ozone concentration detected. Detected ozone concentrations were not significantly different from containers of *E. coli* or *S. aureus* samples with the same treatment parameters. No ozone was detected in either treatment condition after the 24 h post-treatment storage time. In air DBD-ACPs, the well-known generation–depletion cycle of ozone is interlinked to that of nitrogen oxides through several gas-phase reactions that generate N₂O, NO and O atoms starting from O₂ and N₂ (Moiseev et al., 2014).

The concentrations of ozone and nitrogen oxides (O₃, NO₂, NO₃, N₂O₄) for this DIT-120 set-up were quantified using absorption spectroscopy (OAS) and reported elsewhere (Moiseev et al., 2014). The measurements of ozone using the gas detectors
compare with those reported using OAS.

Figure 6.3 shows the detected ROS density results of *E. coli* and *S. aureus* suspensions in PBS, where a similar trend of ROS generation in response to ACP is demonstrated for both bacteria. With regard to the effect of mode of exposure, with indirect treatment the ROS density increased gradually as a function of treatment time from 1 min to 5 min, by comparison with direct treatment where ROS density was lower with prolonged treatment.

![Figure 6.3](image)

Figure 6.3 *E. coli* NCTC 12900 and *S. aureus* ATCC 25923 intracellular ROS density assay by DCFH DA

Experimental conditions: 1, 3, 5 min treatment at 80 kV\textsubscript{RMS} with 0 h post-treatment storage. All experiments were carried out in duplicate and replicated at least twice.

* indicate a significant difference at the 0.05 level between *E. coli* and *S. aureus*

### 6.4 DNA damage

Figure 6.4 presents the dsDNA quantity of *E. coli* and *S. aureus* before and after ACP treatment. The control group from both bacteria showed a similar signal strength,
which proved a similar initial DNA amount from samples. However, different signal levels were observed from the two treated strains. *E. coli* samples showed a gradual reduction of fluorescence signal in correlation with treatment time. However, there was only a trace of fluorescence signal from *S. aureus* samples after treatment (p≤0.05).

![Graph](image)

**Figure 6.4** *E. coli* NCTC 12900 and *S. aureus* ATCC 25923 DNA quantification assay by SYBR Green 1

Experimental conditions: 1, 3, 5 min treatment at 80 kV$_{	ext{RMS}}$ with 24 h post-treatment storage. All experiments were carried out in duplicate and replicated at least twice.

* indicate a significant difference at the 0.05 level between *E. coli* and *S. aureus*

### 6.5 Scanning Electron Microscopy

From the SEM results (Figure 6.5), more visible damage was evident on *E. coli* surfaces than *S. aureus*, indicating cell breakage/lysis effects for *E. coli* inactivation, while ACP treatment caused irregular shape and cell shrinkage in *S. aureus.*
Figure 6.5 SEM images of *E. coli* NCTC 12900 and *S. aureus* ATCC 25923

Experimental conditions: 1 min treatment with indirect ACP exposure at 80 kV<sub>RMS</sub> following 24 h post-treatment storage. Red arrows indicate obvious changes of cell morphology comparing with untreated control.

(a) Untreated *S. aureus* ATCC 25923

(b) Treated *S. aureus* ATCC 25923

(c) Untreated *E. coli* NCTC 12900

(d) Treated *E. coli* NCTC 12900
6.6 Proposed Inactivation Mechanism

Figure 6.6 illustrates the proposed mechanism of action of ACP with Gram negative and Gram positive bacteria based on the results described here for *E. coli* and *S. aureus*.

![Diagram of ACP action on Gram negative bacteria](image)

- **a** Gram negative bacteria
- **b**
- **c** cell leakage main effect

![Diagram of ACP action on Gram positive bacteria](image)

- **d** Gram positive bacteria
- **e**
- **f** severe damage to intracellular components

Figure 6.6 Proposed mechanism of action of ACP with Gram negative and positive bacteria

(a), (b), (c) the proposed inactivation mechanism of Gram negative bacteria: (a), structure of Gram negative bacteria before treatment, cell envelope consists of thin layer of peptidoglycan and lipopolysaccharide; (b), ACP generated ROS attacking both cell envelope and intracellular components, where cell envelope is the major target; (c), inactivation mainly caused by cell leakage, with some DNA damage possible.

(d), (e), (f) the proposed inactivation mechanism of Gram positive bacteria: (d), structure of Gram positive bacteria before treatment, cell envelope consist a thick rigid layer of peptidoglycan; (e), ACP generated ROS attacking both cell envelope and
intracellular components, where intracellular materials are the major targets; (f), inactivation mainly caused by intracellular damage (eg. DNA breakage), but not leakage.

After ACP treatment, generated reactive oxygen species, associated with process and system parameters, attack both cell envelope and intracellular components. For Gram negative cells the cell envelope is the major target of ROS. Reactions of ROS with cell components cause disruption of the cell envelope and result in leakage, with some possible damage of intracellular components (eg. DNA). For Gram positive cells the intracellular components are the major target of ROS. Reactions of ROS will cause severe damage of intracellular components (eg. DNA), but not cell leakage. Lower intracellular ROS in Gram negative bacteria can be result of both ROS depletion by cell envelope components and the cell leakage.

6.7 Discussion

From the results of inactivation efficacy, there is clearly a strong effect of increasing treatment time, even without any post treatment storage time. However, a surviving population could be below the detection limit (<1.0 Log \text{_{10}} CFU ml^{-1}) with recovery possible during storage under some treatment and storage conditions. No further enrichment procedures were employed in this study. Incorporating a post-treatment storage time increased the inactivation efficacy significantly, especially with 24 h post-treatment storage time, which could be attributed to the amount of reactive species generated and their extended reaction time with bacteria due to the containment within a sealed environment and also exposure to recombined longer lived species again facilitated by containment (Table 6.1 and 6.2). Similar results have been observed in
our former studies (Han et al., 2014). A post treatment storage time with retained antimicrobial efficacy has two-fold potential advantage, whereby the initial exposure could be minimal with enhanced efficacy during storage which is compatible with treatment of sensitive samples. Additionally a post treatment storage stage is compatible with many industrial processes. However, with applications to the food, beverage and pharmaceutical industries in mind, the strong oxidative effect with long ACP exposure time could adversely affect some ingredients by inducing surface oxidation, which has been observed from ozone food sterilization technologies (Kim et al., 1999). A challenge for developing ACP applications in the food industry is to optimize the dose or gas mixtures applied to ensure control of microbiological risks whilst maintaining food quality characteristics.

A hypothetical mechanism of action of ACP against *E. coli* and *S. aureus* was proposed and is shown in Figure 6.6. Different reaction mechanism with ROS and cell components are discussed below from reactive species and cell damage results. The leakage studies recorded pointed to different modes of action. High leakage levels were observed with all treatment and post-treatment storage steps for *E. coli* (p≤0.05), but not in *S. aureus* (p>0.05) (Figure 6.1). The cell wall of Gram positive bacteria consists of peptidoglycan with tight structure and strength, while Gram negative bacteria are covered by a thin layer of peptidoglycan and an outer membrane of lipopolysaccharide. During plasma treatment, generated ROS can react with both lipopolysaccharide and peptidoglycan thus breaking the molecule structure by damaging C-O, C-N and C-C bonds. (Chung et al., 2013; Yusupov et al., 2013; Yusupov et al., 2012) However, an obvious leakage was only observed from *E. coli*. With the higher lipid content, lipid peroxidation may have taken place on lipopolysaccharides and resulted in the breakage of the cell envelope. (Laroussi et al.,
This could suggest that reactive species reacted with the cell wall in different patterns. Reactions with other cell wall components, such as peptidoglycan, could be also involved. Furthermore, Figure 6.5 visually illustrates the difference between *E. coli* and *S. aureus* after ACP treatment and further supports our hypothesis on the pattern of damage. The effect of shrinkage but not breakage has also been reported on another Gram positive bacteria, *L. monocytogenes* (Cullen et al., 2014).

As a main inactivation species, the ozone level inside the package showed strong correlation to treatment time and post-treatment storage time, but not to the type of bacteria in the sample (Table 6.3). However, the fluorescent signal recorded for *S. aureus* was three times that of *E. coli*, thus indicating a much higher intracellular ROS density in *S. aureus* than for *E. coli* (Figure 6.3, p≤0.05). A similar time correlated ROS generation was reported by other researchers using a plasma jet treatment. Intracellular ROS increased over 5 min of treatment by air plasma from a jet (Ali et al., 2014), with a similar trend reported on generation of RNS (Cheng et al., 2014). Plasma treatment time determines the input energy during discharging. As the key reactive species for oxygen containing working gases, the generation of ROS consumes most of the energy in air plasma. It has been suggested that in-package ROS can penetrate cell membranes by active transport across the lipid bilayer or transient opening of pores in the membrane (Sensenig et al., 2011). This could explain the correlation between treatment time and ozone/intracellular ROS. The mode of exposure also adds complexity, where an obvious difference in reactive species was observed from OES and DCFH DA assay according to mode of exposure (Figure 6.2 and 6.3). Lower reactive species levels were detected from samples exposed to direct plasma than the indirectly exposed samples. This could be due to the quenching effect of liquid between electrodes on the ionizing of gases. However, similar inactivation
levels and cell components damage were recorded. During direct treatment, undetectable ROS, mostly very short lived and transient species, might react immediately with cell components and be transformed. It appears cells were damaged by the relatively long lived species associated with indirect treatment, such as higher ozone levels.

After plasma discharging, the ozone concentrations in the gas phase were determined to be independent of the type of bacteria, while intracellular ROS levels were strongly correlated with both process parameter and target bacteria characteristic. This could contribute to the different reaction and diffusion patterns of ROS to the cells. Based on the absorbance results at 260 and 280 nm in Figure 6.1, ACP generated ROS could react with the cell wall rather than entering the cell in E. coli samples, whilst the ROS were accumulating inside the S. aureus cells.

E. coli samples showed a reduction of fluorescence signal of DNA correlating with treatment time in Figure 6.4. This trend elucidated that DNA damage has a plasma dose dependent pattern. There was only a trace of fluorescence signal from S. aureus samples post treatment, indicating greater DNA damage than with E. coli. It has been reported that plasma induced oxidative stress damage in S. aureus is due to intracellular oxidative reactions (Zhang, Liang, et al., 2013).

Overall, treatment time and post-treatment storage time had strong effects on inactivation efficacy against E. coli and S. aureus in this study, with a lower impact observed for mode of plasma exposure. The amount of reactive species generated, including ozone, has been shown to be correlated with inactivation efficacy (Ali et al., 2014; Arjunan et al., 2012; Brun et al., 2012; Ishaq et al., 2014; Joshi et al., 2011). Among the reactive species generated during ACP treatment, ROS contributed as major antimicrobial factors. Their concentrations were governed by plasma dose and
applied gas compositions (Han et al., 2014). The generation of ozone as an indicator of ROS showed a time-dependent pattern, while intracellular ROS had a similar trend. During penetration, ROS could react with the lipid content in the cell membrane and cause certain damage. Compared with Gram positive bacteria, the membrane of Gram negative bacteria was more vulnerable. Visible damage as a result of plasma exposure was previously observed for *E. coli* (Dobrynin et al., 2009).

A much higher intracellular ROS density detected in *S. aureus* showed the probable penetration of reactive species within the cell. At the same time, higher concentrations of reactive species overall could lead to more intracellular damage to cell components such as DNA, which was clearly noted in this study. Since the total amount of ROS generated using any system or process setting is around the same level and is independent of the target bacteria characteristics, it is apparent that less cell envelope damage may be combined with more intracellular damage.

In this study, the ACP inactivation efficacy of *E. coli* and *S. aureus* bacteria was correlated with process and system parameters (i.e. treatment time or post-treatment storage time). These determined the amount and reaction time of reactive species, which were the essential factors of antimicrobial reactions. Two different possible mechanisms of inactivation were observed in the selected Gram negative and Gram positive bacteria. Reactive species were either reacting with cell envelope or damaging intracellular components. *E. coli* was inactivated by cell envelope damage induced leakage, while *S. aureus* was mainly eliminated by intracellular damage. Additionally, the different cell damage mechanisms might due to different type of reactive species with regard to the mode of exposure. These findings are critical for the successful development of plasma applications where the system and process parameters can be nuanced in relation to the target risk characteristics presented.
Future studies focusing on cell regulatory responses by utilising knock-out strains would provide further insights on bacterial protection or enhanced sensitivity towards ACP.
Chapter 7. *E. coli* Regulatory Response Under Atmospheric Cold Plasma Stress

Five knock out mutants of oxidative stress related genes in *E. coli*, as listed in Table 2.2 and 2.3, were compared with the parent strain with respect to their inactivation patterns, ROS density and membrane integrity. Key plasma process parameters of treatment time and post-treatment storage time as well as mode of exposure were employed as variables.

Five genes associated with stress regulation and responses were studied in this section. RpoS is a well-known global regulator for stationary phase and general stress (Matsuoka and Shimizu, 2011). Two major transcriptional regulators, SoxRS and OxyR, have been found to control bacterial genetic responses to oxidative stress. SoxR can be induced by both ROS and RNS (Ding and Demple, 2000; Greenberg et al., 1990), and they trigger its regulation through *soxS* (Pomposiello et al., 2001). OxyR is induced by hydrogen peroxide and controls the expression of a group of enzymes for scavenging ROS species and the regulatory gene *oxyS* for further response (Christman et al., 1989; Mukhopadhyay and Schellhorn, 1997). DnaK is one of the most abundant constitutively expressed and stress inducible chaperones involved in cell repair and it is the central hub in *E. coli* chaperone network interfacing upstream and downstream chaperonin (Calloni et al., 2012).

By comparing the response of mutants under ACP exposure and key processing parameters, the mechanism of microbial inactivation was elucidated with respect to cellular regulation and repair.
### 7.1 Reactive species generation

ROS levels were detected using fluorescence probe DCFH-DA and are represented in Figure 7.1. The ROS signal generally decreased with post-treatment storage time, due to their oxidative reaction with cell components.

**Figure 7.1** *E. coli* mutants and parent strain ROS density assay by DCFH

Experimental conditions: 1, 3, 5 min treatment at 70 kV\textsubscript{RMS}, following 0, 1, 24 h post-treatment storage. Untreated controls followed the same preparation procedure at all
post-treatment storage times. Higher fluorescence signal detected in AFU indicates higher intracellular ROS concentration measured by DCFH DA. All experiments were carried out in triplicate and replicated at least twice.

(a) 0 h storage with direct exposure; (b) 0 h storage with indirect exposure;
(c) 1 h storage with direct exposure; (d) 1 h storage with indirect exposure;
(e) 24 h storage with direct exposure; (f) 24 h storage with indirect exposure.

Figure 7.1 a and b shows an increase of ROS levels in tandem with increasing treatment times in samples with no post-treatment storage, while in-package ozone concentrations showed the same trend in Table 7.2.

ΔdnaK with both direct and indirect exposure had much higher signal than all other mutant strains (p<0.05). However, when samples were stored for 1 h post treatment, ΔsoxS with indirect exposure had the highest ROS signal followed by ΔoxyR and ΔdnaK (Figure 7.1 c and d, p<0.05). After 24 h post-treatment storage, higher remaining ROS levels were observed from ΔsoxS than other mutants (Figure 7.1 e and f, p<0.05).

Table 7.1 In-package ozone concentration after ACP treatment at 70 kV_{RMS} in response to process parameters

<table>
<thead>
<tr>
<th>Post-treatment storage time</th>
<th>Mode of exposure</th>
<th>1min</th>
<th>3min</th>
<th>5min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ozone (ppm)</td>
<td>SD*</td>
<td>Ozone (ppm)</td>
<td>SD*</td>
</tr>
<tr>
<td>0 h Direct</td>
<td>1500</td>
<td>200</td>
<td>2600</td>
<td>231</td>
</tr>
<tr>
<td>0 h Indirect</td>
<td>1950</td>
<td>191</td>
<td>2750</td>
<td>412</td>
</tr>
<tr>
<td>1 h Direct</td>
<td>100</td>
<td>8</td>
<td>180</td>
<td>28</td>
</tr>
<tr>
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<td>130</td>
<td>26</td>
<td>258</td>
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</tr>
<tr>
<td>24 h Direct</td>
<td>ND*</td>
<td>-</td>
<td>ND*</td>
<td>-</td>
</tr>
<tr>
<td>24 h Indirect</td>
<td>ND*</td>
<td>-</td>
<td>ND*</td>
<td>-</td>
</tr>
</tbody>
</table>

ND*: Under detection limit (<1 ppm)
*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

Table 7.2 lists in-package ozone levels detected with regards to plasma treatment time and post-treatment storage time. Ozone levels strongly increased with extended treatment time (p<0.05), where 5 min (both direct and indirect) exposure to ACP generated around 4000 ppm ozone. Similar to ROS results obtained from DCFH DA assay, the detected ozone levels dropped significantly in 1 h (p<0.05), while 24 h post-treatment storage decreased the concentrations under the detection limit (<1 ppm).

**7.2 Membrane integrity**

The membrane integrity was tested using PI after 24 h post-treatment storage only (Figure 7.2). PI staining indicates the cell membrane integrity by binding with double strand of DNA, where the breakage of ds DNA in severely damaged cells will decrease the fluorescence signal. \(ΔrpoS\) mutant strain samples had a signal strength twice that of the control, while the signal of \(ΔoxyR\) mutants was 1.5 fold higher than the control (p<0.05). However, as ACP treatment causes both cell envelope damage and intracellular DNA damage in bacteria (Han et al., 2014), the detected levels of PI staining may be a combination of both effects.
Figure 7.2 E. coli mutants and parent strain cell integrity assay by PI

Experimental conditions: 1, 3, 5 min treatment at 70 kV<sub>RMS</sub>, following 24 h post-treatment storage. Untreated controls were provided following same preparation procedure with 24 h post-treatment storage time. Higher fluorescence signal detected in AFU indicates higher permeability of cell membrane. All experiments were carried out in triplicate and replicated at least twice.

Therefore, Figure 7.3 represents the membrane integrity by an alternative measurement, absorbance at 260 and 280 nm based on the release of intracellular DNA and protein by damaged cells (Virto et al., 2005).
Figure 7.3 E. coli mutants and parent strain cell integrity assay

Experimental conditions:  1, 3, 5 min treatment at 70 kV\textsubscript{RMS}, following 24 h post-treatment storage. Untreated controls were provided following same preparation procedure with 24 h post-treatment storage time and are shown as 0 min treated samples. Higher absorbance at 260 and 280 nm indicates higher leakage of DNA and greater damage of cell membrane. All experiments were carried out in triplicate and replicated at least twice.

(a) Absorbance at 260 nm with direct exposure; (b) Absorbance at 260 nm with indirect exposure; (c) Absorbance at 280 nm with direct exposure; (d) Absorbance at 280 nm with indirect exposure.
Similar trends were observed with this assay. $\Delta rpoS$ samples had significantly higher absorbance than other mutant strains ($p<0.05$), while $\Delta rpoS$ strains exposed to indirect treatment showed greater leakage than those exposed to direct treatment. However, $\DeltaoxyR$ had an absorbance equivalent to the other strains ($p>0.05$). This suggests that membrane integrity had been compromised to allow PI to enter but cell damage was not severe enough to result in DNA leakage from the cell.

7.3 Inactivation efficacy associated process and system parameters

Figure 7.4 shows inactivation patterns for $E. coli$ mutants over different ACP treatment times and post-treatment storage times.
Figure 7.4 Survival number in suspensions of *E. coli* mutants and parent strain with regards to ACP treatment time and post-treatment storage time.
Experimental conditions: 1, 3, 5 min treatment at 70 kV\textsubscript{RMS}, following 0, 1, 24 h post-treatment storage. Untreated controls were provided following same preparation procedure at all post-treatment storage times and are shown as 0 min treated samples.

(a) 0 h storage with direct exposure; (b) 0 h storage with indirect exposure;
(c) 1 h storage with direct exposure; (d) 1 h storage with indirect exposure;
(e) 24 h storage with direct exposure; (f) 24 h storage with indirect exposure;

All experiments were carried out in duplicate and replicated at least twice.

Dotted line: detection limit (1.0 Log\textsubscript{10} CFU ml\textsuperscript{-1})

When no post-treatment storage time was employed, the immediate effect of the reactive species generated was assessed. All mutant strains were similarly affected by direct and indirect exposure to plasma for 1 min (p<0.05), with some difference in response noted after 3 min exposure. However, obvious separation of effect corresponding to the mutant strain characteristics was noted after 5 min (p>0.05). \(\Delta\text{rpoS}\) was reduced below the detection limit (<1.0 Log\textsubscript{10} CFU ml\textsuperscript{-1}) after 5 min treatment with indirect exposure, while direct exposure reduced cell concentration to 1.38±0.25 Log\textsubscript{10} CFU ml\textsuperscript{-1}. Both wild type and \(\Delta\text{oxyR}\) were reduced below the detection limit (<1.0 Log\textsubscript{10} CFU ml\textsuperscript{-1}) after 5 min treatment with both modes of exposure. Similar trends of 4 Log\textsubscript{10} CFU ml\textsuperscript{-1} surviving populations were observed from other mutants, \(\Delta\text{soxR}, \Delta\text{soxS}\) and \(\Delta\text{dnaK}\) (Figure 7.4 a and b, p>0.05).

After 1 h post-treatment storage, 1 min ACP treated \(\Delta\text{soxS}\) with direct exposure had a slightly lower survival level than all other strains (p<0.05), but with 3 min treatment, both \(\Delta\text{soxR}\) and \(\Delta\text{oxyR}\) mutants exhibited the greatest resistance to plasma effects (p≤, while wild type with indirect exposure and \(\Delta\text{soxS}\) samples exposed to either mode of exposure were reduced below the detection limit (<1.0 Log\textsubscript{10} CFU ml\textsuperscript{-1}). Further
extending the treatment time to 5 min, $\Delta$soxR levels were maintained at 3.22±0.20 and 4.38±0.10 Log$_{10}$ CFU ml$^{-1}$ with direct and indirect exposure respectively, whilst the $\Delta$dnaK mutant was still detectable at 1.98±0.21 Log$_{10}$ CFU ml$^{-1}$ following direct exposure only. However, all other mutant strains were reduced below the detection limit (<1.0 Log$_{10}$ CFU ml$^{-1}$) regardless of mode of exposure (Figure 7.4 c and d). By extending post treatment storage time to 24 h, only $\Delta$soxR samples had populations surviving above the detection limit (>1.0 Log$_{10}$ CFU ml$^{-1}$) with either 3 or 5 min ACP treatment duration. With the short treatment time of 1 min, minimal further mutant strain differentiation was apparent, where ArpoS had lowest survival level of 4.88±0.10 Log$_{10}$ CFU ml$^{-1}$ with direct exposure only (p<0.05).

7.4 Discussion

The inactivation of mutant and wild type strains showed different results with regards to reduction levels, ROS densities and cell integrity. The cellular response of all strains to ACP exposure stress are discussed below with respect to process and system parameters.

Effect of treatment time

Treatment time, which dominates the exposure doses, had a positive effect on inactivation efficacy. The same trends were observed for all strains, while similar results have been widely reported by many researchers (Ghomi et al., 2009; Han et al., 2014; Kvam et al., 2012; Liu et al., 2013). The in-package ROS generation was increased with respect to extension of ACP treatment time, as represented by ozone level and ROS in Table 7.2 and Figure 7.1. Increasing RNS levels with longer treatment time was reported by Cheng et al. (2014). The overall greater amount of
reactive species led to the increasing of cell membrane permeability in Figure 7.2 and 7.3, and resulted in lower survival level (Figure 7.4).

**Effect of post-treatment storage time**

Introducing a post-treatment storage time exposed a clearer differentiation of inactivation patterns across the range of mutant strains. A post-treatment storage time facilitated prolonged and contained exposure to a range of longer lived reactive species (Figure 7.4 e and f) and had a positive effect on inactivation efficacy, by providing extended reaction time for cell lethal species.

With all post treatment storage times, the absence of RpoS increased the sensitivity of cells to ACP treatment. Comparing with wild type, a slightly lower capability for ROS clearance of *ArpoS* was only observed following 1 h post-treatment storage (Figure 7.1, p<0.05), while its cell membrane permeability was significantly increased (Figure 7.2, p<0.05). *RpoS* is known as encoding a crucial regulator and 11% of the genes it regulates are correlated to stress management (Weber et al., 2005). *katE* is regulated by *rpoS* and participates in the antioxidant defence mechanism (Jung and Kim, 2003).

With the increase of post-treatment storage time, *AsoxR* and *AsoxS* had significantly different performance. *AsoxR* mutant was the most resistant strain with 1 and 24 h post-treatment storage, while *AsoxS* showed increased sensitivity. Initially, similar ROS levels were detected, but much higher levels of ROS remained in *AsoxS* over prolonged storage after extended treatment times, which indicated the importance of SoxS for ROS scavenging. *SoxRS* has been reported as an important regulon of *E. coli* under superoxide and nitric oxide stress (Greenberg et al., 1990; Perni et al., 2007). The SoxS stimulates genes for resistance to oxidative stress and antibiotics, while SoxR removes intracellular nitric oxide by its [2Fe-2S] centres (Demple, 1996; Ding and Demple, 2000). The transcriptional initiation of *soxS* is known to be induced by
SoxR, whereas it is also regulated proteolytically by the Lon protease (Blanchard et al., 2007). The difference of ROS levels observed from this study implied that soxS is the crucial subunit in the regulon under plasma stress rather than soxR. The alternative mechanism of activating soxS without soxR is still unclear where Gaudu et al. (1997) discussed the hypothesis but it has not been verified.

The importance of dnaK gene increased gradually with extended post-treatment storage time, as observed in the increasing sensitivity of ΔdnaK samples to ACP. It indicated that the demand of the DNA repairing system occurred with extended post-treatment storage time rather than immediately after treatment. Therefore the damage of DNA could be a long-term effect of ACP treatment rather than an immediate reaction. Additionally, the highest initial ROS densities observed with the ΔdnaK mutant could be attributed to deficiencies in correlated cell components synthesis without DnaK protein, which is the central hub in the chaperone network and plays an important role in the formation of filamentous phenotype (Calloni et al., 2012; Rockabrand et al., 1995). It also interacts with membrane proteins and assists protein folding (Calloni et al., 2012). The DnaK protein also plays an important role in DNA repair by participating in the initiation of DNA replication, and is regulated by htpR (rpoH) gene (Skowyra et al., 1990). DnaK is also the major bacterial Hsp70 (heat shock protein), where the absence of dnaK has been reported to increase the cell sensitivity under both thermal and oxidative stress (Bukau and Walker, 1989; Calloni et al., 2012; Delaney, 1990; Itikawa and Ryu, 1979; Rockabrand et al., 1995). However, it has been found to be down regulated with several other chaperones after H$_2$O$_2$ treatment (Chang et al., 2002).

*Effect of mode of exposure*
As another important system parameter, indirect mode of exposure has been studied with distanced treatment or additional mesh (Dobrynin et al., 2009; Fridman et al., 2007; Okubo et al., 2004). Long-lived and recombined reactive radicals (such as peroxynitrites) led to microbiocidal effects with indirect exposure, instead of short-lived species and free radicals in discharging area with direct exposure (Dobrynin et al., 2009; Han et al., 2014). Obvious differences of ROS generation were observed from ROS density results, where indirect exposure led to much higher ROS densities than direct exposure. In-package ozone measurements after treatment had the same trend. During direct exposure treatment, discharging occurred in both gas and liquid phase, where ozone formation rates are quenched by OH• radical generation and other higher dissociation energy is required (Fridman, 2008a; Moiseev et al., 2014). Whereas, indirect exposure offered discharging in air, where higher ozone concentrations were generated. Hence, higher ROS concentration could be generated inside the solution of indirectly exposed samples by following secondary reactions.

Among the products of secondary reactions, peroxynitrites have been shown to have strong antimicrobial activities (Lukes et al., 2014; Machala et al., 2013). SurA is another gene regulated by rpoS and involved in the folding of membrane proteins (Lazar and Kolter, 1996). Thus, the absence of rpoS gene could have resulted in the incorrect folding of some membrane proteins, which further affected the cell permeability. Whilst increased membrane permeability was observed from ΔrpoS with both exposure modes compared with wild type and other mutants, an even higher leakage level was observed from ΔrpoS samples using indirect exposure. Higher ozone concentration with indirect exposure (Table 7.1) was the possible reason for this, because of its strong effect on the bacterial cell wall (Yusupov et al., 2012). However,
the effect of knocking out \textit{rpoS} gene at low cell inactivation level was not noticed in our study or Perni et al. (2007).

\textbf{Survival of \textit{ΔoxyR} mutant}

In \textit{ΔoxyR} mutant results, populations were undetectable after a relatively long treatment time (5 min) or post-treatment storage time (24 h), but otherwise this mutant survived in high numbers, 6.63 to 7.70 Log$_{10}$ CFU ml$^{-1}$. \textit{ΔoxyR} mutant also showed high readings of ROS (similar to \textit{ΔsoxS} and \textit{ΔdnaK}) after 1 h post-treatment storage with 5 min indirect treatment, while its initial ROS was detected as the average level. However, a higher cell permeability of \textit{ΔoxyR} samples was observed after 24 h post-treatment storage, which could be a result of the prolonged ROS damaging effect on the cell membrane. \textit{ΔoxyR} was found to be resistant with high surviving populations after 1 and 3 min treatment followed by 0 and 1 h post-treatment storage or 24 h storage following 1 min treatment. But, extended treatment time could dramatically reduce it under the detection limit (<1.0 Log$_{10}$ CFU ml$^{-1}$). This indicated that \textit{oxyR} gene could have crucial effects on ROS scavenging under high oxidative stress, which is shown by its high ROS signal after 1 h post-treatment storage in Figure 7.1 d. Moreover, long lived species are generated during extended storage time, such as H$_2$O$_2$, O$_3$ and HNO$_x$ (Moiseev et al., 2014), where H$_2$O$_2$ dominates the activation of \textit{ΔoxyR}. OxyR is activated by the generation of disulfide bonds resulting from oxidative species such as hydrogen peroxide, and further acts as a transcription activator and induces a series of antioxidative enzymes (Cabisco et al., 2000; Christman et al., 1989; Mukhopadhyay and Schellhorn, 1997). Without the presence of \textit{oxyR, hemH} might be an alternative mode of response under oxidative stress by encoding ferrochelatase (Zheng et al., 2001), which could explain the survival of \textit{ΔoxyR} mutants.

\textit{Overview}
Stress response system studies in *E. coli* have been extensively reported but information on the cellular response to cold plasma is still limited. The knock-out mutants used here have also been investigated against ozone treatment, where different sensitivities were observed (Patil et al., 2011). In our study, the mutant ΔsoxR has been found to be more sensitive than ΔsoxS (p<0.05) after exposure to ozone, which might due to the complex reactive species actions during plasma treatment. Sharma et al. (2009) studied the cold plasma response of *E. coli* on transcription level using micro-array. Some genes related to SOS response, oxidative repair, regulation were up-regulated significantly, which contributed to the repair of DNA damage and redox of reactive species. Furthermore, there are studies of stress response against ACP treatment on yeast and cancer cells, which have more comprehensive regulatory systems (Feng et al., 2010; Keidar et al., 2011), where a similar inactivation mechanism was observed. These mechanisms involving oxidative stress response were further elucidated in our study based on the different reactions to ACP of *E. coli* mutants deficient in key stress responsive genes.

Overall, among the investigated process and system parameters, distinctive responses were observed with respect to increasing the post-treatment storage time, while treatment time and mode of exposure showed similar effects on all strains. A proposed regulatory model is therefore presented in Figure 7.5 based on our observations.
The absence of \( rpoS \) and \( oxyR \) genes led to reduced cell envelope recovery and ROS scavenging effects, regardless of post-treatment storage time, while some genes varied in impact during the storage period, suggesting their different roles in the stress response. \( SoxS \) gene became important for cell survival by clearance of reactive species with both 1 and 24 h storage, which was not observed from \( AsoxR \), implying their different characteristics as subunits. \( DnaK \) showed its repairing function correlated to cell survival with 24 h storage. Besides general regulatory and resistance functions, the cell response against plasma generated oxidative stress could be divided into short term and long term, dominated by oxidative stress response genes and damaged repair genes, respectively. The uncovered emergency response relating to reactive species clearance and further mechanisms of cell repair provides an explanation of cell survival under plasma stress and offers promising insights for optimising ACP applications.
Chapter 8. INVESTIGATION OF ATMOSPHERIC COLD PLASMA APPLICATION ON MEAT MODEL

As a promising non-thermal industrial food decontamination technology, the ACP applications on food products have been widely studied by researchers (Deng et al., 2007; Surowsky et al., 2014; Ziuzina et al., 2014). However, the research on meat products are limited. Therefore, this chapter provides a detailed investigation of the bactericidal effect of atmospheric cold plasma on three key meat-contaminating pathogens using a complex meat model media and elucidates the role of reactive oxygen species in disinfection by ACP. Industry-relevant processing parameters that could interact with ACP efficacy and mode of action were evaluated by using MAP gases of meat products as plasma working gases as well as storage temperature. Therefore these interactive effects were assessed with regard to ACP efficacy in a meat model to provide insight for the application of ACP for meat decontamination. DCFH DA was also used in this section for ROS action mechanism studies in nutritive treatment media. However, BE has much higher background fluorescence signal than from PBS. All fluorescence data in this section were therefore calculated by subtracting the background fluorescence of respective untreated control cells.

8.1 Effect of applied voltage on ACP inactivation efficacy

Due to their heterogeneous nature and complex structure, biofilms and attached cells are characterized by an enhanced resistance when compared to their planktonic counterparts for most environmental stresses encountered in food production plants (Bridier et al., 2014; Giaouris et al., 2014). Therefore, the effect of a range of high voltage level (60, 70 and 80 kV_{RMS}) ACP treatments against key meat pathogens grown as biofilms in a meat model medium was investigated (Figures 8.1).
Figure 8.1 Biofilm inactivation efficacy with respect to applied voltage

Experimental conditions: 60, 120, 300 s treatment at 60, 70, 80 kV_{RMS}, following


24 h storage at room temperature. All experiments were carried out in duplicate and replicated at least twice.

Treatment at 60 or 70 kV_{RMS} had similar inactivation effects for *E. coli* with approximately 2 \log_{10} CFU ml^{-1} remaining after 60 s of plasma exposure (p>0.05). However, inactivation under detection limit (<1.0 \log_{10} CFU ml^{-1}) was obtained after 60 s of treatment at 80 kV_{RMS} (Figure 8.1 a). *L. monocytogenes* biofilm showed greater susceptibility, where similar inactivation patterns were found for 60, 70 and 80 kV_{RMS} treatment, with non-detectable cell concentrations after 60 s (Figure 8.1 b).

Extending treatment time from 120 s to 300 s at 60 kV_{RMS} reduced *S. aureus* biofilms significantly (p<0.05). After 60 s treatment at 80 kV_{RMS}, bacterial concentrations were reduced to 2.96±0.14 \log_{10} CFU ml^{-1}. However, extending the exposure time at 70 and 80 kV_{RMS} did not lead to further significant reductions in population density (Figure 8.1 c, p>0.05).

The fluorescent probe used for measuring ROS is more accurate when using planktonic cells in PBS and thus the positive effect of voltage on the ROS densities in liquid samples is shown in Figure 8.2. Fluorescence levels gradually increased with applied voltages of 60, 70 and 80 kV_{RMS} (p<0.05).
Figure 8.2 Intracellular ROS density assay by DCFH DA

Experimental conditions: 60 s treatment at 60, 70, 80 kV\text{RMS} in PBS and analysed without post-treatment storage. All experiments were carried out in triplicate and replicated at least twice.

Increasing voltage levels generally resulted in a higher inactivation of biofilms, reaching non-detectable levels in some cases. The applied power determines the input energy of discharge, which leads to different amounts of reactive species generated and inactivation levels (Pankaj et al., 2013; Tang et al., 2011). The detected ROS levels increased along with the applied voltages, indicating the reason for faster inactivation at higher voltages. Treatment time is another parameter governing input energy of ACP and influences microbial inactivation by generating time-dependent amounts of reactive species. Similar trends were observed in previous studies on the inactivation efficacy correlated to voltage and treatment time (Fernández et al., 2013; Han et al., 2014; Niemira et al., 2014). These results confirmed that high voltage ACP significantly reduced key meat pathogens in a nutrient rich environment and in an attached state, and that when the highest voltage level was applied, there was no further
advantage incurred with extending duration of treatment beyond 60 s, which is of relevance when retention of other fresh quality characteristics is required.

### 8.2 Effect of media and post-treatment storage temperature on plasma inactivation efficacy

Tables 8.1-8.3 show the inactivation efficacy of ACP treatment at 80 kV\textsubscript{RMS} against planktonic cells of \textit{E. coli}, \textit{L. monocytogenes} and \textit{S. aureus}, respectively, as a function of treatment time, media composition and storage temperature conditions.

**Table 8.1 Surviving cell numbers of planktonic \textit{E. coli} NCTC 12900 with respect to treatment time, media and storage condition at 80 kV\textsubscript{RMS}**

<table>
<thead>
<tr>
<th>Media</th>
<th>Plasma treatment time (s)</th>
<th>4 °C storage</th>
<th>RT storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cell density (Log\textsubscript{10} CFU ml\textsuperscript{-1})</td>
<td>SD*</td>
</tr>
<tr>
<td>3% BE</td>
<td>C\textsuperscript{1}*</td>
<td>7.11\textsuperscript{ab}</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>C\textsuperscript{2}*</td>
<td>6.95\textsuperscript{bc}</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.35\textsuperscript{b}</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>6.73\textsuperscript{c}</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>6.06\textsuperscript{d}</td>
<td>0.15</td>
</tr>
<tr>
<td>12% BE</td>
<td>C\textsuperscript{1}*</td>
<td>7.13\textsuperscript{ab}</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>C\textsuperscript{2}*</td>
<td>7.18\textsuperscript{ab}</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.32\textsuperscript{b}</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>7.12\textsuperscript{c}</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>5.79\textsuperscript{d}</td>
<td>0.92</td>
</tr>
<tr>
<td>PBS</td>
<td>C\textsuperscript{1}*</td>
<td>7.18\textsuperscript{ab}</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>C\textsuperscript{2}*</td>
<td>6.94\textsuperscript{bc}</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>ND\textsuperscript{e}</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>ND\textsuperscript{e}</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>ND\textsuperscript{e}</td>
<td>-</td>
</tr>
</tbody>
</table>

Different letters indicate a significant difference at the 0.05 level between media and treatment times.
*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log_{10} CFU ml^{-1})

C^{1*}: Control without storage; C^{2*}: Control with storage.

Table 8.2 Surviving cell numbers of planktonic *L. monocytogenes* NCTC 11994 with respect to treatment time, media and storage condition at 80 kV_{RMS}

<table>
<thead>
<tr>
<th>Media</th>
<th>Plasma treatment time (s)</th>
<th>Storage condition</th>
<th>Cell density (Log_{10} CFU ml^{-1})</th>
<th>SD*</th>
<th>Cell density (Log_{10} CFU ml^{-1})</th>
<th>SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 ºC storage</td>
<td></td>
<td></td>
<td>RT storage</td>
<td></td>
</tr>
<tr>
<td>3% BE</td>
<td>C^{1*}</td>
<td>7.23^a</td>
<td>0.06</td>
<td></td>
<td>7.23^a</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>C^{2*}</td>
<td>7.35^a</td>
<td>0.01</td>
<td></td>
<td>9.10^b</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.23^a</td>
<td>0.14</td>
<td></td>
<td>8.90^b</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>6.70^b</td>
<td>0.23</td>
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<td>8.60^b</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>5.07^c</td>
<td>0.35</td>
<td></td>
<td>7.20^a</td>
<td>0.84</td>
</tr>
<tr>
<td>12% BE</td>
<td>C^{1*}</td>
<td>7.26^a</td>
<td>0.10</td>
<td></td>
<td>7.26^a</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>C^{2*}</td>
<td>7.43^d</td>
<td>0.02</td>
<td></td>
<td>9.63^c</td>
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<td>9.00^d</td>
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</tr>
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<td>7.54^d</td>
<td>0.19</td>
<td></td>
<td>9.16^cd</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>6.18^e</td>
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<td>7.93^e</td>
<td>0.49</td>
</tr>
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<td></td>
<td>7.29^a</td>
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<tr>
<td></td>
<td>C^{2*}</td>
<td>7.08^g</td>
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<td></td>
<td>6.84^f</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>ND^h</td>
<td>-</td>
<td></td>
<td>ND^g</td>
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<td></td>
<td>ND^g</td>
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<td>ND^h</td>
<td>-</td>
<td></td>
<td>ND^g</td>
<td>-</td>
</tr>
</tbody>
</table>

Different letters indicate a significant difference at the 0.05 level between media and treatment times.

*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log_{10} CFU ml^{-1})

C^{1*}: Control without storage; C^{2*}: Control with storage.
Table 8.3 Surviving numbers of planktonic *S. aureus* NCTC 1803 with respect to treatment time, media and storage condition at 80 kV<sub>RMS</sub>.

<table>
<thead>
<tr>
<th>Media</th>
<th>Plasma treatment time (s)</th>
<th>Storage condition</th>
<th>4 °C storage</th>
<th>RT storage</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Cell density (Log&lt;sub&gt;10&lt;/sub&gt; CFU ml&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>SD&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Cell density (Log&lt;sub&gt;10&lt;/sub&gt; CFU ml&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3% BE</td>
<td>C&lt;sup&gt;1*&lt;/sup&gt;</td>
<td>7.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04</td>
<td>6.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>C&lt;sup&gt;2*&lt;/sup&gt;</td>
<td>6.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.26</td>
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<td>0.33</td>
<td>7.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>12% BE</td>
<td>C&lt;sup&gt;1*&lt;/sup&gt;</td>
<td>7.17&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.06</td>
<td>6.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>C&lt;sup&gt;2*&lt;/sup&gt;</td>
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<td>9.01&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>0.33</td>
<td>8.83&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
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<td>120</td>
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<td>8.76&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>0.05</td>
<td>8.43&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBS</td>
<td>C&lt;sup&gt;1*&lt;/sup&gt;</td>
<td>7.40&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
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<td>6.37&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>6.52&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>ND&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-</td>
<td>ND&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different letters indicate a significant difference at the 0.05 level between media and treatment times.

*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log<sub>10</sub> CFU ml<sup>-1</sup>)

C<sup>1*</sup>: Control without storage; C<sup>2*</sup>: Control with storage.

In PBS where there was little or no media based interference with the reactive species generated, all populations were reduced to non-detectable levels by 60 s treatments at both storage temperatures. Inactivation in both 3% and 12% BE was strongly reduced compared to treatment in PBS and required extended treatment times of 120 and 300 s to achieve any significant effects, which did not exceed 2 Log<sub>10</sub> CFU ml<sup>-1</sup> total.
reduction (Tables 8.1-8.3). There were minimal effects of BE percentage in terms of protection and recovery of the cells, and the presence of even 3% BE indicated that a longer treatment time at the higher voltage level would be required for the product study to overcome the strong challenge to inactivation efficacy. In Figure 8.3, the detected ROS levels from BE at either concentration were much lower than those from PBS (p<0.05) and actually decreased with treatment time (p≤0.05). Again, *S. aureus* suspensions in PBS had the highest ROS densities among all three strains for all treatment times, and *L. monocytogenes* had slightly higher levels than *E. coli* (p<0.05).

![Figure 8.3 ROS density assay by DCFH DA with respect to treatment media](image)

**Figure 8.3** ROS density assay by DCFH DA with respect to treatment media

Experimental conditions: 60, 120, 300 s treatment at 80 kV$_{\text{RMS}}$ in PBS, 3% BE and 12% BE and analysed without post-treatment storage. All experiments were carried out in triplicate and replicated at least twice.

The comparison of ACP inactivation effects on different bacteria has been previously reported (Ermolaeva et al., 2011; Klämpfl et al., 2012; Liang et al., 2012). In the
current study, the three bacteria displayed distinct differences in the detected ROS concentrations, although similar inactivation levels were achieved with the same treatment parameters. Moreover, different inactivation efficacies were achieved using PBS and BE, where strong protective effects were observed with the BE media (Figure 8.4).

![Diagram](image)

Figure 8.4 Proposed mechanism of action of ACP generated ROS in bacteria suspensions of PBS and BE

Because complete inactivation was not achieved, the high nutrient content in the BE enabled regrowth after treatment, particularly under temperature abuse conditions. Assessment of ROS showed that very low concentrations of ROS were available for contact with cells treated in the BE media (Figure 8.3) as components of the meat medium such as proteins are likely to scavenge many of the plasma generated reactive species and thus pose a protective effect against the antimicrobial action of the reactive species. At room temperature, control groups of all bacteria in BE increased by 1.87 to 2.84 Log \(_{10}\) CFU ml\(^{-1}\) during 24 h storage and populations in treated samples
exceeded the starting cell concentrations after storage (Table 8.1-8.3). However, at 4 °C storage, no significant growth of bacteria occurred during the storage period and the inactivation achieved with ACP treatment was maintained.

8.3 Effect of different media and gas composition on plasma inactivation efficacy

Modified atmosphere packaging (MAP) is commonly used in the packaging of meat products to extend product quality and shelf-life. The efficacy of ACP treatment for inactivation of meat contaminants was therefore investigated not only in air but also in two modified atmospheres commonly used for packed meat products.

Table 8.4 Surviving numbers of planktonic cells in PBS with 24 h storage at 4 °C with respect to treatment time and applied gas mixtures

<table>
<thead>
<tr>
<th>Gases</th>
<th>Plasma treatment time (s)</th>
<th>E. coli NCTC12900</th>
<th>L. monocytogenes NCTC11994</th>
<th>S. aureus NCTC1803</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell density (Log_{10} CFU ml^{-1})</td>
<td>SD*</td>
<td>Cell density (Log_{10} CFU ml^{-1})</td>
<td>SD*</td>
</tr>
<tr>
<td>MP1: 70% N₂+ 30% CO₂</td>
<td>C¹a 7.16 a 0.07</td>
<td>7.51 a 0.11</td>
<td>6.88 a 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C²b 6.57 b 0.24</td>
<td>6.93 b 0.40</td>
<td>6.52 b 0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 6.87 c 0.09</td>
<td>6.48 c 0.09</td>
<td>6.21 c 0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 5.87 d 0.03</td>
<td>5.83 d 0.06</td>
<td>5.74 d 0.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 5.74 d 0.05</td>
<td>5.21 e 0.10</td>
<td>5.39 e 0.10</td>
<td></td>
</tr>
<tr>
<td>Air</td>
<td>C¹a 7.16 a 0.07</td>
<td>7.51 a 0.11</td>
<td>6.88 a 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C²b 6.99 ab 0.07</td>
<td>6.93 b 0.40</td>
<td>6.78 a 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 6.50 b 0.23</td>
<td>6.55 bc 0.13</td>
<td>5.47 c 0.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 2.98 c 0.48</td>
<td>0.92 f 0.09</td>
<td>2.51 f 0.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 0.00 f 0.00</td>
<td>0.00 g 0.00</td>
<td>0.00 g 0.00</td>
<td></td>
</tr>
<tr>
<td>MP2: 70% O₂+ 30% CO₂</td>
<td>C¹a 7.16 a 0.07</td>
<td>7.51 a 0.11</td>
<td>6.88 a 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C²b 6.81 ab 0.10</td>
<td>6.05 h 0.17</td>
<td>6.30 a 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 6.57 b 0.08</td>
<td>5.79 d 0.02</td>
<td>5.11 c 1.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 ND* f -</td>
<td>ND* g -</td>
<td>1.71 h 0.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 ND* f -</td>
<td>ND* g -</td>
<td>ND* g -</td>
<td></td>
</tr>
</tbody>
</table>
Different letters indicate a significant difference at the 0.05 level between media and treatment times.

*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log_{10} CFU ml^{-1})

C1*: Control without storage; C2*: Control with storage.

As before, the inactivation efficacy increased with treatment time in PBS (Table 8.4), where all strains were undetectable after 60 s treatment in air. *E. coli* and *L. monocytogenes* were reduced below the detection limit (<1.0 Log_{10} CFU ml^{-1}) after 30 s treatment in high oxygen gases, which was achieved with 60 s treatment in air. *S. aureus* had the highest resistance to ACP and was reduced from 6.88±0.04 Log_{10} CFU ml^{-1} to 5.74±0.19, 2.51±0.45 and 1.71±0.36 Log_{10} CFU ml^{-1} in MP1, air and MP2 respectively after 30 s treatment (Table 8.4, p<0.05).

The beef extract maintained a strong protective effect against ACP efficacy in the modified atmospheres. One minute of plasma treatment resulted in up to 7 Log_{10} CFU ml^{-1} reduction in PBS samples, while a maximum 2.2 Log_{10} CFU ml^{-1} reduction was obtained with BE samples and extended treatment times of 300 s (Table 8.4 and 8.5). The highest inactivation levels were achieved with high oxygen content gases. The influence of gas composition on ACP treatment has been reported, where oxygen percentage played the most important role (Han et al., 2014; Laroussi and Leipold, 2004). At the same time, the generation of reactive nitrogen species has also been observed in air plasma (Jayasena et al., 2015; Moiseev et al., 2014; Price et al., 2013; Ziuzina et al., 2013), where joint effects of RNS with ROS lead to higher inactivation.
than either group alone (Boxhammer et al., 2012), therefore the use of a MAP gas combination that mitigates the production of either ROS or RNS may not yield the best antimicrobial efficacy.

Table 8.5 Surviving numbers of planktonic cells in 3% BE with 24 h storage at 4 °C with respect to treatment time and applied gas mixtures

<table>
<thead>
<tr>
<th>Gases</th>
<th>Plasma treatment time (s)</th>
<th>E. coli NCTC12900</th>
<th>Microorganisms</th>
<th>L. monocytogenes NCTC11994</th>
<th>S. aureus NCTC1803</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cell density (Log_{10} CFU ml^{-1})</td>
<td>SD*</td>
<td>Cell density (Log_{10} CFU ml^{-1})</td>
<td>SD*</td>
</tr>
<tr>
<td>MP1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70% N_{2}+</td>
<td>C_{1}^{*}</td>
<td>6.78^{a}</td>
<td>0.03</td>
<td>7.17^{a}</td>
<td>0.09</td>
</tr>
<tr>
<td>30% CO_{2}</td>
<td>C_{2}^{*}</td>
<td>7.18^{ce}</td>
<td>0.08</td>
<td>7.56^{c}</td>
<td>0.08</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>7.64^{b}</td>
<td>0.13</td>
<td>7.28^{ab}</td>
<td>0.12</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>7.49^{bc}</td>
<td>0.24</td>
<td>7.46^{bc}</td>
<td>0.06</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>7.01^{ae}</td>
<td>0.25</td>
<td>7.34^{ac}</td>
<td>0.20</td>
</tr>
<tr>
<td>Air</td>
<td>C_{1}^{*}</td>
<td>6.98^{de}</td>
<td>0.05</td>
<td>7.02^{ef}</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>C_{2}^{*}</td>
<td>7.15^{ce}</td>
<td>0.06</td>
<td>7.56^{c}</td>
<td>0.08</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>6.87^{df}</td>
<td>0.06</td>
<td>6.98^{e}</td>
<td>0.04</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>6.74^{f}</td>
<td>0.10</td>
<td>7.18^{f}</td>
<td>0.05</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>6.55^{g}</td>
<td>0.12</td>
<td>6.78^{d}</td>
<td>0.14</td>
</tr>
<tr>
<td>MP2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70% O_{2}+</td>
<td>C_{1}^{*}</td>
<td>6.98^{de}</td>
<td>0.01</td>
<td>6.99^{e}</td>
<td>0.03</td>
</tr>
<tr>
<td>30% CO_{2}</td>
<td>C_{2}^{*}</td>
<td>7.23^{ce}</td>
<td>0.05</td>
<td>7.56^{c}</td>
<td>0.08</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>6.47^{h}</td>
<td>0.14</td>
<td>6.55^{h}</td>
<td>0.22</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>5.93^{i}</td>
<td>0.11</td>
<td>5.97^{g}</td>
<td>0.03</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>5.49^{j}</td>
<td>0.17</td>
<td>4.77^{i}</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Different letters indicate a significant difference at the 0.05 level between media and treatment times.

*SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.

*ND: under detection limit (<1.0 Log_{10} CFU ml^{-1})

C_{1}^{*}: Control without storage; C_{2}^{*}: Control with storage.
Overall, little difference among the strain responses was observed, and the parameters of treatment time and gas composition dominated the increase in inactivation levels.

In our study, in-package ozone levels were measured as an indicator of the overall ROS concentration in gas phase.

![Figure 8.5](image.png)

**Figure 8.5** Applied gases associated intracellular ROS density assay by DCFH DA

Experimental conditions: 60 s treatment at 80 kV\textsubscript{RMS} in PBS with different applied gas mixtures and analysed without post-treatment storage. All experiments were carried out in triplicate and replicated at least twice.

**Table 8.6** In-package ozone concentration after ACP treatment with respect to applied gas mixtures and treatment time at 80 kV\textsubscript{RMS}

<table>
<thead>
<tr>
<th>Plasma treatment time (s)</th>
<th>MP1</th>
<th>SD\textsuperscript{*}</th>
<th>Air</th>
<th>SD\textsuperscript{*}</th>
<th>MP2</th>
<th>SD\textsuperscript{*}</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>ND\textsuperscript{*}</td>
<td>ND\textsuperscript{*}</td>
<td>533</td>
<td>231</td>
<td>1280</td>
<td>179</td>
</tr>
<tr>
<td>30</td>
<td>ND\textsuperscript{*}</td>
<td>ND\textsuperscript{*}</td>
<td>1067</td>
<td>493</td>
<td>5400</td>
<td>849</td>
</tr>
<tr>
<td>60</td>
<td>ND\textsuperscript{*}</td>
<td>ND\textsuperscript{*}</td>
<td>2450</td>
<td>496</td>
<td>12050</td>
<td>2375</td>
</tr>
<tr>
<td>120</td>
<td>ND\textsuperscript{*}</td>
<td>ND\textsuperscript{*}</td>
<td>2814</td>
<td>1619</td>
<td>12240</td>
<td>1951</td>
</tr>
<tr>
<td>300</td>
<td>ND\textsuperscript{*}</td>
<td>ND\textsuperscript{*}</td>
<td>2950</td>
<td>191</td>
<td>16480</td>
<td>2305</td>
</tr>
</tbody>
</table>

\textsuperscript{*}SD: standard deviation. All experiments were carried out in duplicate and replicated at least twice.
Both ozone levels and detected ROS concentrations were dependent on the gas composition, where ROS were barely detected in MP1 samples and no ozone was measured inside these packages (Figure 8.5, Table 8.6). A complete analysis of ROS and RNS generation and kinetics with this DBD system is detailed in Moiseev et al. (2014). A higher content of oxygen in MAP gas in this study led to a higher level of ROS generated, which improved the inactivation effect.

*S. aureus* is well-known as a multi-resistant bacteria, however, its strong resistance was only observed in biofilm results here, with similar reductions to *L. monocytogenes* and *E. coli* in liquid. *L. monocytogenes* showed higher sensitivity than *E. coli* and *S. aureus* in PBS but equivalent survival in BE media, which indicates the more critical role of the nutrient environment and availability on protection or recovery. Among the parameters studied, the target properties, including planktonic or biofilm and nutritive composition in media, were crucial for ACP inactivation. While a strong interactive effect of treatment time and gas composition was observed from PBS planktonic models, the influence of these parameters were minimized by the protection of surface properties. Hence a cell recovery modelling study was performed to further inform the product challenge study.

### 8.4 Modelling microbial recovery

The effect of environmental factors on the lag phase and growth rate of foodborne pathogens has been widely reported, including environmental pH, osmolality, temperature and sterilization treatment (Mackey and Derrick, 1982; Mellefont et al.,
2003; Robinson et al., 1998). \( N_{turb} \) of three strains were determined and tabulated in Table 8.7 before ACP treatment.

### Table 8.7 \( N_{turb} \) from the calibration curves

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>( N_{turb} ) from calibration curve</th>
<th>( N_{turb} ) experimental data</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E. ) coli NCTC 12900</td>
<td>7.89</td>
<td>7.05</td>
</tr>
<tr>
<td>( L. ) monocytogenes NCTC 11994</td>
<td>8.39</td>
<td>8.62</td>
</tr>
<tr>
<td>( S. ) aureus ATCC 1803</td>
<td>8.13</td>
<td>7.04</td>
</tr>
</tbody>
</table>

Table 8.8 represents the results of \( \mu_{\text{max}} \) with different ACP treatment times for the three bacteria. An overall decrease of the growth rate was observed for extended treatment times.

### Table 8.8 Kinetic parameters of the bacteria after different ACP treatment times at 80 kV\(_{\text{RMS}}\) and 24 h storage at 4 \(^\circ\)C

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Treatment time (s)</th>
<th>( \mu_{\text{max}} ) (h(^{-1}))</th>
<th>SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E. ) coli NCTC 12900</td>
<td>C</td>
<td>1.26</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.09</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.59</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.21</td>
<td>0.04</td>
</tr>
<tr>
<td>( L. ) monocytogenes NCTC 11994</td>
<td>C</td>
<td>0.35</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.17</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.28</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>( S. ) aureus NCTC 1803</td>
<td>C</td>
<td>0.86</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.46</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.46</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.31</td>
<td>0.34</td>
</tr>
</tbody>
</table>

SD*: standard deviation. All experiments were carried out in duplicate and replicated at least twice.
A similar trend after DBD-ACP treatment is reported in Millan Sango et al. (2015) for 
*E. coli*. This tendency was also observed in our study, where the $\mu_{\text{max}}$ was found to be 
ACP treatment time dependent with *E. coli* showing more significant changes of $\mu_{\text{max}}$ 
than the Gram positive bacteria. The cell damage caused by ACP treatment resulted in 
a longer recovery time during refrigerated storage.

**8.5 Meat product challenge**

Treatment of lamb chops inoculated with *E. coli* reduced the bacterial load by about 
0.6 $\log_{10}$ CFU g$^{-1}$ with little growth observed in either the control or plasma treated 
sample in the first 24 h post treatment. A reduction of up to 0.5 $\log_{10}$ CFU g$^{-1}$ for 
treated lamb compared to controls was retained over the course of the study (Figure 
8.6). Albeit small, this difference was significant for most days of testing and suggests 
that the initial bacterial reduction is maintained and the remaining bacteria grew at a 
rate comparable to the control (p<0.05 on days 0, 1, 4 and 11).

![Figure 8.6 Reduction of *E. coli* surface inoculated on lamb chop](image)

Experimental conditions: 60 s treatment at 80 kV$_{\text{RMS}}$ with post-treatment storage at 4 
°C. All experiments were carried out in duplicate and replicated at least twice.
*E. coli* levels on pork loin showed a significant reduction of culturable cells by almost 2 \( \log_{10} \) CFU g\(^{-1}\) immediately after plasma treatment (Figure 8.7). *E. coli* concentrations increased in both samples over the next 24 h but lower levels were maintained in treated samples and this trend continued for the remainder of the 14 days of storage (p<0.05 on days 0, 1 and 14).

![Figure 8.7 Reduction of *E. coli* surface inoculated on pork loin](image)

Experimental conditions: 60 s treatment at 80 kV\(\text{RMS}\) with post-treatment storage at 4 °C. All experiments were carried out in duplicate and replicated at least twice.

Surface inoculation of turkey slices with *S. aureus* gave bacterial concentrations above 7 \( \log_{10} \) CFU g\(^{-1}\). Treatment for 60 or 300 s reduced bacterial growth in the first 24 h post-treatment to the same extent and an average 1 \( \log_{10} \) CFU g\(^{-1}\) difference was maintained over 35 days of storage (Figure 8.8). Importantly, no significant differences were observed between the effects of 60 and 300 s treatment.
Experimental conditions: 60 s treatment at 80 kV\textsubscript{RMS} with post-treatment storage at 4 °C. All experiments were carried out in duplicate and replicated at least twice.

Compared to previously obtained inactivation efficiencies for the treatment of microorganisms in simple buffer solutions, inactivation on meat surfaces showed limited efficacy. The influence of the complex matrix consisting of a range of proteins and lipids needs to be taken into account in addition to effects of the sample geometry on ACP mode of action. Thick meat slices, uneven surfaces and multiple layers will provide more opportunity for bacteria to adhere, internalize and escape direct plasma exposure while also providing a diffusion barrier to reactive species. Although plasma treatment did show a reduction of meat product challenge microorganisms, the efficacy was insufficient to reduce challenges to acceptable levels (>2 Log\textsubscript{10} CFU g\textsuperscript{-1} deemed unacceptable for \textit{E. coli} and >4 Log\textsubscript{10} CFU g\textsuperscript{-1} or \textit{S. aureus}). Moreover, the safety limits of industrial processors can be lower (1 Log\textsubscript{10} CFU g\textsuperscript{-1} for \textit{E. coli} and 2 Log\textsubscript{10} CFU g\textsuperscript{-1} for \textit{S. aureus}). Whilst high inoculation levels were used in these studies, the inactivation efficacy demonstrated on meat products here would require further
hurdles to ensure product safety. However, high voltage ACP treatment may inactivate low levels of pathogen contamination on complex and nutritious surfaces such as meat, and as such represents an additional processing measure with the advantage of in package treatment mitigating against recontamination events.

Overall, the process and system parameters of ACP treatments were found to govern the inactivation efficacy by generating different amounts of reactive species. Gram positive and negative bacteria showed significant differences in the quantities of reactive oxygen species generated which could imply different damaging patterns, although ACP had similar antimicrobial efficacy against both. However, the nutrient content of food products could provide protective effects against ACP treatment with reduced inactivation levels due to the decreased ROS levels, while food surface properties may also affect the bactericidal action. Maintaining refrigerated storage temperature will maintain the microbial reductions achieved ACP treatment. These results indicate both the potential and the limitations of the use of ACP for bacterial inactivation on meat products and can help to develop and optimize treatment strategies in the future.
Chapter 9. OVERALL DISCUSSION AND CONCLUSIONS

ACP provides challenging effects against a wide range of microbes, mainly by the generation of cell-lethal reactive species (Basaran et al., 2008; Kayes et al., 2007; Klämpfl et al., 2012). By discharging in air, groups of reactive species are generated, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), ultraviolet (UV) radiation, energetic ions and charged particles (Cullen and Milosavljević, 2015; Moiseev et al., 2014).

Therefore, DBD ACP was investigated as a microbicidal process governed by processing and system parameters. The microbial responses were studied with regard to the mechanism of inactivation. The technology was further assessed for application to a food model and products, where promising results were obtained.

The effects of process parameters such as voltage levels, treatment time, mode of exposure and post-treatment storage time were initially investigated and optimized for a high-voltage di-electric barrier system.

The generation of reactive species could be enhanced by increasing input voltage or extending treatment time, which is observed from the ozone measurements and ROS assays in this study. The mode of exposure is a novel parameter due to our experimental design. The reported indirect/remote treatment in other previous studies referred to no direct contact with electron beam but reactive species had interaction with samples during exposure. However, the ACP equipment used in this design provided a possibility of post-treatment reaction with reactive species inside package.

Without the liquid sample in the discharging area, greater amounts of reactive species were detected with indirectly exposed samples. Direct exposed samples contacted with relatively short-live species, electrons, charged particles and UV radiation besides ROS/RNS. With indirect exposed packages, samples reacted mainly with recombined
and long-live reactive species. Although the detected ROS levels were higher from indirect packages, the effect of short-lived species and charged particles were apparent in conjunction with other parameters, such as low voltage or short treatment time. The possible reason might be that these species are the primary product of discharging. Their effects can be observed at those circumstances, where long-lived species have not dominated the inactivation mechanism. Similarly, relatively long-life species also played an important role during post-treatment storage procedure. In-package species further reacted with bacterial components and enhanced inactivation efficacy.

Applied gas mixtures were also evaluated as a relevant industrial processing factor, which strongly interacted with the generation of reactive species. Oxygen percentage in applied gases has been found as the crucial factor of inactivation due to the generation of ROS. Interestingly, the presence of oxygen can significantly enhance the dissociation of N₂ molecules, where CO₂ had much lower effect on this. Hence, the synergy of gas compositions could have better inactivation efficacy than pure gases. Microbial inactivation by non-thermal plasma is not only determined by process parameters but was also dependant on system and product characteristics. The nutritive contents of food product, such as proteins, lipid, antioxidants, have a scavenging effect on ROS, while food surface geometry might reduce the exposure extent of food pathogens to ACP. Furthermore, nutrient-rich substrates could enable cell recovery as discussed in Chapter 8, especially abused temperature. All of these factors might limit the applicability of ACP for food preservation.

In food industry application, the gas composition of MAP can be adjusted according to required microbicidal effect of ROS/RNS. Traditionally, MAP was used for maintaining colour and moisture and avoiding further contamination of food products. After in-package ACP treatment, a post-treatment procedure can be easily achieved
during delivery and general storage. Although this could improve the decontamination efficacy, no extra cost or treatment was required comparing with traditional method. Refrigerated storage conditions might be employed when applying ACP treatment for nutritive food products, especially.

Moreover, biofilms commonly exist on contaminated food surfaces, which have stronger resistance than planktonic cells. The formation of exopolymers, quorum sensing and other molecular features increases the resistance of biofilm to microbicidal processes. ACP treatment has been proven to be effective for inactivating biofilms, although lower efficacy was obtained than for planktonic cells.

Different bacterial formats were used in this study, including biofilm, planktonic cells (in PBS or nutritive media) or surface inoculated food products. PBS cell suspension is the most important as a model for plasma-liquid system study. Sample volumes of 100 µl, 3 ml and 10 ml were used in different experimental designs. Oehmigen et al. (2011) reported the decreasing efficacy of plasma treatment along with increased volume, which is mainly due to the reactive species effect. From the previous results, it is reasonable to believe that similar amount of reactive species could be generated under same process and system parameters. Increasing of solution volume would increase the diffusion time for reactive species reaching bacterial surfaces, where short-lived species can be depleted or converted to more stable species (as per reactions described in Chapter 1). Moreover, reactive species in liquid phase might be diluted with the increasing of solution volume and microbial cell numbers, which is the main reason the reduced inactivation number.

As suggested from the microbial inactivation results, the decontamination efficacy could be enhanced by operating process and system parameters. Extension of treatment time and post-treatment storage time in combination with storage condition and
applied MAP gas mixtures could lead to useful efficacy for the food industry. In our study of meat products, adding plasma treatment to the existing preservation technologies of Refrigeration and MAP did have a positive significant effect on microbial control, which was evident over the shelf-life period of the study. This effect was not significantly related to the plasma treatment time (extending treatment time of sliced turkey from 1 min to 5 min in Chapter 8). Process parameters could be further optimised by comprehensive consideration with regards to industrial processing requirements. However, the amount of available nutritive components and surface geometry can influence the treatment outcome, while food quality after treatment should also be considered. Thus, in considering the development of ACP technology for the food industry and selection of products where the technology can be most gainfully employed, close attention should be paid to the food surface characteristics, moisture content and geometry and the nature of the microbial contamination in order to ensure useful efficacy at an appropriate energy and cost demand.

The microbial inactivation mechanisms of ACP treatment were also well studied for both Gram negative and positive bacteria. The results revealed there are different patterns of damage on Gram negative and positive bacteria due to their structural difference. Both cell leakage assay and SEM images showed cell envelope disruption of two types of \textit{E. coli} cells, standard strain and resistant strain with high virulence. Two types of Gram positive bacteria were observed to have severe DNA damage compared to \textit{E. coli}. As cellular response to this damage, correlated genes were upregulated under ACP stress. The up-regulation of genes involved in oxidative stress response and induction of SOS response genes were observed by Sharma et al. (2009) after plasma exposure, while genes responsible for housekeeping functions, ion transport, and metabolism were down-regulated. Five knock-out mutants were chosen
in this study to investigate those modified genes effects during post-treatment storage procedure after ACP treatment. The surviving mechanism of bacteria under plasma stress was partly revealed with regards to post-treatment storage time. Five mutants showed different responses as general response, short-term and long-term responses. Regulator factor rpoS was determined to have a general effect during the 24 h post-treatment storage time. DNA repairing gene dnaK only showed a long-term effect, while oxidative response genes were found to be important immediately after treatment.

Overall a number of conclusions regarding the use of DBD ACP as a microbicidal process and its application for food products can be made from this study:

- DBD-ACP system has been proved effective for microbial inactivation against both Gram negative and positive bacteria. It is promising for developing an economical and effective plasma treatment for industrial application. Careful selection of product type is likely to have a strong impact on success.

- Growth media composition may affect microbial inactivation efficacy due to cross protection of acid stress response induced by metabolic products.

- Process and system parameters, including voltage level, treatment time, applied gas mixtures (especially oxygen content) and mode of exposure, have been proven to govern the inactivation efficacy by generating various amount of reactive species. Moreover, post-treatment storage procedure of in-package system provided reaction time for long-lived species, which enhanced the microbicidal effect.

- Different inactivation mechanisms were observed from Gram negative and positive bacteria samples, regardless of their resistance and virulence. Gram
negative bacteria were inactivated due to cell leakage induced by cell envelope damage, while Gram positive bacteria had mainly intracellular damage. Interaction mechanisms of ROS with cell components were considered as the main reason of different damaging patterns.

- Cell responses were observed as general regulatory, short term and long term stress responses, dominated by general regulators, oxidative stress response genes and damage repair genes, respectively. Cellular surviving mechanisms include intracellular ROS scavenging, maintaining of cell integrity and DNA repairing.

- Inactivation efficacy is influenced by characteristics of samples. While the complex structure of biofilms might increase the resistance against plasma treatment, nutritive composition in liquid can also provide a protective effect for microorganisms. Moreover, the influence of the complex product matrix consisting of e.g. a range of proteins and lipids in meat products needs to be taken into account in addition to effects of the sample geometry on ACP mode of action.

- ACP treatment showed a promising effect on food products, where microbial recovery was also reduced following ACP treatment. However, the investigation of storage temperature indicated the necessity of refrigerated condition of food product storage in conjunction with ACP treatment to prevent regrowth.

Following DBD-ACP investigation applied in this study, some suggestions for future studies include:
Because of limitations of the package, only direct exposure was studied with respect to individual reactive species. The liquid diagnostic with both modes of exposure should be accomplished.

To represent no storage, short and long storage time, post-treatment storage procedures of 0, 1 and 24 h were applied throughout the whole study. However, the reaction mechanisms and conversion between short-lived and long-lived species were not known. It would be worthwhile to assess the mechanism during storage at intervals over the 24 h with individual reactive species assays.

Cellular response mechanism study can be further studied on transcription and expression level, with RT PCR and protein analysis.

Intracellular damage was represented by DNA damage in this study. Further research may involve other intracellular components to indicate intracellular changes, such as LDH, Alkaline Phosphatase, GAPDH activities or RNase, thiol groups, NADPH concentrations.

Microbial safety of ACP treated food product was investigated in this study. However, the associated quality, health risks and flavour need to be evaluated in an appropriate food model.

As a result of cell damage, microbial invasion factors such as lipid A or protein A could be degraded. Subsequent invasion level to mammalian cells might be investigated.
Reference


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Konesky, G., 2009. Dwell time considerations for large area Cold Plasma decontamination, Proc. SPIE 7304, Chemical, Biological, Radiological, Nuclear, and Explosives (CBRNE) Sensing X. International Society for Optics and Photonics, pp. 73040N-73040N.


APPENDIX

Peer reviewed publications


International journal of food microbiology, 210, 53-61. DOI: 10.1016/j.ijfoodmicro.2015.05.019.


**Book Chapter**


**Under Review**

Congress and Conferences


Han, L., Heslin, C., Boehm, D., Cullen, P. J. & Bourke, P. (2014) Atmospheric cold plasma inactivation of *Escherichia coli*: Role of ROS in different media and gases. Safefood Knowledge Networks Conference, Dublin

Han, L., Ziuzina, D., Cullen, P. J. & Bourke, P. (2014) Atmospheric cold plasma inactivation of *Listeria monocytogenes*: Role of ROS in different media. Safefood Knowledge Networks Conference, Dublin

Han, L., Patil, S., Cullen, P. J. & Bourke, P. (2014) Inactivation mechanism of atmospheric cold plasma against *Escherichia coli* and *Staphylococcus aureus* in liquid. 5th International Conference on Plasma Medicine, ICPM5 [Nara (Japan), 18-23 May, 2014]
