COLD ATMOSPHERIC PLASMA: STUDIES ON INACTIVATION MECHANISMS IN FOOD-BORNE PATHOGENS AND LABORATORY STRAINS

By

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Originality certificate

This is to certify that I am responsible for the work submitted in this thesis and the original work is my own and the work of others whenever used are fully acknowledged as specified in the references. This thesis including the original work contained has not been submitted to any other institutions.

Omar Elhenshir

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ABBREVIATIONS

% vol/vol	percent volume per volume
% wt/vol	percent weight pr volume
°C	degree centigrade
Amp	Ampicillin
BHI	Brain Heart Infusion
bp	base pair
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
F.W	Formula weight
g	gram
h	hour
Km	Kanamycin
Km^R	Kanamycin resistant
kb	kilobases
kDa	kiloDalton
kV	kiloVolt
L	Litre
LB	Luria Bertani
M	Molar
mA	miliampere
mg	miligram
MHz	Mega hertz
min	minutes
ml	mililiters
mm	milimeters
mM	milimolar
n	number of replicates
N	Normality
Ø	Diameter

OD_{600}	optical density at a wavelength of 600 nanometers
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RO	reverse osmosis
rpm	rounds per minute
s	seconds
SD	standard deviation
Str	Streptomycin
Str^R	Streptomycin resistant
Tet	tetracycline
TSB	tryptic soy broth
UV	ultraviolet
V	Volt
µg	microgram
µl	microliter
µm	micrometer

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ABSTRACT

Food-borne disease remains accountable for high levels of morbidity and mortality in the world. The World Health Organization (WHO) estimated that food-borne and water-borne diarrhoeal diseases kill about 2.2 million people annually (SGM, 2013).

In this thesis, studies on microbial inactivation were carried out using Cold Atmospheric plasma (CAP) generated by a radiofrequency (RF) source at RF = 4.6 MHz. The operating gas used was a mixture of helium and oxygen

The study has investigated the effect of CAP on different strains of food-borne bacteria. These included strains/ mutants of *S.* Typhimurium, *L. monocytogenes* and *E. coli*. The result has shown that CAP has the capability to inactivate a wide range of food-borne bacteria. However, *S.* Typhimurium LT2, *E. coli* O157: H7 and *E. coli* H10407 showed more tolerance to CAP treatment than *L. monocytogenes* strains and *E. coli* K-12 MG1655. This result led to further investigations into the role of lipopolysaccharide (LPS) structure in the tolerance to CAP treatment. To investigate the role of LPS structure, derivatives of *E. coli* K-12 which lack the O-antigen and isogenic mutants of them (core polysaccharide defective) were used. In addition to these strains and mutants, partially and fully restored LPS derivatives of *E. coli* K-12 MG1655 and *S.* Typhimurium TA98 which is a mutant of *S.*Typhimurium LT2 were also used. No significant differences in the sensitivity to CAP treatments between these mutant and their parental strains were found except between *S.* Typhimurium TA98 and its parental

strain. S. Typhimurium TA98 was missing many other genes, therefore it was not comparable to the parental strain. The results suggest no role for the LPS structure regions in the resistance to CAP treatment.

The study has also investigated the role of oxidative stress in bacterial inactivation. Mutants of *E. coli* K-12 BW25113 were a single gene of the OxyR regulated genes has been deleted, were used. Three mutants were found significantly ($P < 0.05$) more sensitive to CAP treatments than other strains. These mutants were (Δ*dps*), (Δ*grxA*) and (Δ*sufC*). The gene deleted from each mutant is hydrogen peroxide inducible and OxyR regulated (Zheng *et al.*, 2001a). Since each of these genes has an important role in the resistance to oxidative stress, the result suggests the involvement of oxidative stress in inactivation by CAP treatments.

To confirm these results and to find out whether the CAP treatment caused DNA damage or mutagenic effects, the Ames strains were used. Exposure to CAP treatment for 5 and 10 seconds caused a significant ($P < 0.05$) increase in the number of revertant colonies of *S.* Typhimurium TA100 and *S.* Typhimurium TA102. The results suggest that CAP caused base-pair substitution in the DNA of *S.* Typhimurium TA100 (Barnes *et al.*, 1982) and transition/transversion mutations in the DNA of *S.* Typhimurium TA102 (Levin *et al.*, 1982a) and potentially in the DNA of other bacteria. It can be concluded that CAP inactivates bacteria due to oxidative stress damage and induces mutation in the bacterial DNA. Modification of the Ames strain test was successfully validated. This finding highlights some concerns about application of CAP in dental and wound treatments.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. MICROBIOLOGICAL FOOD SAFETY

Food safety is an essential public health concern. Despite intensive efforts to ensure the safety and quality of food, by international organizations and governmental and non governmental authorities worldwide, World Health Organization (WHO) statistics indicate that food-borne diseases account for high morbidity and mortality levels in the world. Food-borne diseases are caused by the consumption of food or drink that contains agents which cause diseases. Bacteria, viruses and parasites are the most common agents involved in food-borne diseases. Toxic chemicals also can be one of these agents. Food-borne and water-borne diarrhoeal diseases kill about 2.2 million people each year as estimated by the World Health Organization (SGM, 2013). According to Center for Deaease Control and prevention (CDC), about 17% (48 million) of the population of the United States of America become ill each year with food-borne diseases resulting in about 128,000 cases of people being hospitalized and about 3,000 deaths annually. The top five pathogens causing food-borne disease which led to hospitalization in the United States are: nontyphoidal *Salmonella* (35%), Norovirus (26%), *Campylobacter* spp. (15%), *Toxoplasma gondii* (15%) and *E. coli* (STEC) O157 (8%). However, the top five pathogens causing food-borne diseases resulting in death are: nontyphoidal *Salmonella* (28%), *Toxoplasma gondii* (24%), *Listeria monocytogenes* (19%), Norovirus (11%) and *Campylobacter* spp. (6%) (CDC, 2011). Similar statistics for food-borne diseases in the United Kingdom were also reported by the Food Standards Agency (FSA).

The FSA estimates that one million people in the U.K. suffer from food-borne diseases each year, resulting in 20,000 being hospitalized and 500 deaths (FSA, 2012). This figure is estimated to cost the UK economy about £2 billion annually (FSA, 2012). Among the five main pathogens which were monitored (*Campylobacter*, *L. monocytogenes*, Norovirus, *E. coli* O157 and *Salmonella*), *Campylobacter* was found to account for the highest number of food-borne disease cases (52%) and highest hospitalization rate (90%). However, *Listeria monocytogenes* was found to account for the highest rate (32%) of death (FSA, 2012). Moreover, it was estimated that about 33% of food produced globally for human consumption is lost or wasted. Spoilage is one of the causes of these losses (Gustavsson *et al.*, 2011).

Therefore, most of the food preservation methods are designed to inactivate, reduce, or inhibit, microbial growth in/on food. However, obviously, from the statistics presented above, preservation or control methods available to the food industry have some limitations. The limitations of these methods are most likely consequences of the many challenges that food manufacturers and distributors face with respect to the assurance of food safety.

Global changes in the environment, changes in lifestyles and new scientific findings occasionally deliver new challenges to the food industry, as well as to other sectors in society. The current challenges to the food industry can be related to the following causes 1) Emergence of strains of pathogens that are resistant to conventional methods of food preservation and/or carry drug resistance, 2) Cross contamination from surfaces and equipment, 3) Capability of some microorganisms to form biofilms, 4) Increasing consumer demand for ready-to-eat

fresh produce, and globalization of the food industry and 5) Limitations of conventional sterilization methods in treating certain foods.

1.1.1. Emergence of resistant strains of pathogens

Emergence of strains of pathogens that are resistant to one or more of the available inactivation methods, disinfectants and antimicrobials has been observed in the last few years. Resistance of some microoganisms to high hydrostatic pressure processing has been documented. Smelt (1998) reported that spores of *B. megaterium* have shown resistance to high hydrostatic pressure processing. Similarly a mutant of *L. monocytogenes* Scott A, AK01, was isolated and characterized as piezotolerant (Karatzas and Bennik, 2002). In the same way mutants of *Escherichia coli* MG 1655 were identified as resistant to inactivation by high hydrostatic pressure (Hauben *et al.*, 1997). Furthermore, resistance to antibiotics and detergents such as Quaternary Ammonium compounds (QACs) is one of the major challenges to both public health and the food industries. Multidrug resistance in the clinically important Enterobacteriaceae has emerged, and the choice of antibiotics with potential activity against these strains has become very limited (Paterson 2006). Therefore, action to prevent and reduce the spread of multidrug-resistant Enterobacteriaceae strains throughout the world must be taken. Similarly antimicrobial resistance in nonfermenting Gram negative bacteria which are also clinically important has become a serious threat (McGowan 2006).

1.1.2. Cross contamination from surfaces and equipment

Surfaces, that are usually used in the food manufacturing or preparation areas, can be contaminated by pathogenic or spoilage bacteria. Many of these bacteria can attach to these surfaces and become less sensitive to sanitizers (Frank and Koffi, 1990). Investigation of the *Listeria monocytogenes* contamination at a poultry processing plant revealed that carcasses acquire *Listeria* mainly from contaminated surfaces and equipment. This investigation has also found that although Listerias were not detected on chicken skin from the neck (obtained from freshly slaughtered birds), or from caecal samples, 50% of oven-ready chicken carcasses were found contaminated with *L. monocytogenes* prior to packaging (Hudson and Mead, 1989). Similarly it was found that *Salmonella* Enteritidis, *Staphylococcus aureus* and *Campylobacter jejuni* were easily transmitted from wet kitchen sponges to stainless steel surfaces and from these surfaces to cucumber and chicken fillet slices. The transfer rates varied from 20% to 100% and these bacteria can survive on these surfaces for substantial periods of time (Kusumaningrum *et al.*, 2003). In another investigation, *L. monocytogenes* was found to be more transferable to food from stainless steel surfaces than from polyethylene surfaces. Regardless of the surface, biofilms have a tendency to transfer more *L. monocytogenes* to foods than do attached cells. Among foods, transferability of *L. monocytogenes* to bologna was easier than to cheese (Rodríguez and McLandsborough, 2007). Recently the potential of transferring *E. coli* O157:H7 from stainless steel surfaces to food products was shown by Silagyi *et al.* (2009). Their findings indicate that *E. coli* O157:H7 strongly attaches to a

variety of food products which include raw products (beef, pork, chicken and turkey), fresh produce (cantaloupe, lettuce, alfalfa sprouts, carrots, spinach and green beans) and ready-to-eat products (salami, ham and turkey ham) even after rinsing the food samples with water.

1.1.3. Ability of some microorganisms to form biofilms

The ability of some microorganisms to attach to surfaces of materials that are widely used in the food processing environment is well documented. It has been found that *L. monocytogenes* attaches easily to surfaces of stainless steel and buna-n (nitrile) rubber materials. *Listeria* also has the ability to survive for long periods on these materials and even multiplies on stainless steel under favourable conditions (Wong, 1998). Furthermore, it has been reported that *Enterobacter sakazakii* has shown the ability to grow in infant formula milk at refrigerator temperature and can form biofilms on surfaces such as latex, silicone and, to a lesser extent, stainless steel (Iversen *et al.*, 2004). These materials are normally used in making infant-feeding utensils and in preparation areas, as well as in infant formula production facilities (Iversen *et al.*, 2004). Recently, it has been reported that *E. coli* O157:H7 has shown the ability to grow well in a variety of broths including meat broths, poultry broths, and on fresh produce broths (cantaloupe, lettuce, carrot, and spinach). Moreover, it has shown a better capability for forming biofilms in meat and poultry broths than in produce broths and significantly higher growth in pork broth than in other broths. *E. coli* O157:H7 can also form biofilms in alfalfa sprout broth compared to other produce broths, even though the strain did

not grow well in the alfalfa broth, which means the biofilm formation is not directly related to the levels of growth (Silagyi *et al.*, 2009).

1.1.4. Increasing consumer demand for ready-to-eat fresh produce

It has been documented that consumer demand for natural, fresh, and minimally processed foods is increasing, whereas modern food businesses are under pressure to produce products with an increased shelf life. For instance, the production and delivery of high quality and safe muscle foods (meat and poultry) will remain the critical issue for U.S. agriculture (Mahapatra *et al.*, 2005). Bhagwat (2006) has reported that sales of fresh-cut-produce increased by two figures in the past few years and is expected to continue growing over the next decade. In addition, packaged salads have been identified as the second- fastest-selling item in U.S. grocery, trailing only bottled water.

1.1.5. Technical limitations of conventional physical sterilization methods

Although some conventional methods for sterilization are well established and effective in inactivating a wide range of microorganisms, sometimes they are inadequate due to technical issues. Of these methods, heat treatments which are very effective in destroying a wide range of microorganisms can only be applied to materials that do not get damaged by heat or moisture and heat (if moist heat is used) (Laroussi, 1996). Similarly, sterilization by gases is an efficient method of microbial inactivation. Ozone (O_3) is one of the gases used to decontaminate water

and to prevent food spoilage, but it has some drawbacks as it needs to be generated on site, and is toxic to humans even at low concentrations (Laroussi, 1996 and Mahapatra *et al.*, 2005). Sterilization methods by electromagnetic radiation, which include microwaves, ultraviolet radiation, gamma-rays, and X-rays, are another choice for inactivation of microorganisms (Laroussi, 1996). Nevertheless, irradiation treatments can cause softening and discolouration of some fruits, an unpleasant taste in milk, and can cause the development of off-flavours in certain protein-rich foods. Moreover, the major factor that limits their application is low consumer acceptability, due *inter alia* to a association with radioactivity (Mahapatra *et al.*, 2005). Additionally, high pressure treatment is an effective treatment and has the advantages of low energy input and the stability of small molecules against ultrahigh pressure. Thus, it will have less effect on the quality of vitamin-rich foods than heat treatment. Nevertheless, the treatment could only be used as a substitute to heat pasteurization as bacterial spores need very high pressures (> 1000 MPa) to be inactivated (Smelt, 1998).

Clearly, these challenges put the search for new methods that inactivate different types of microorganism, with no effects on the quality demanded by consumers, as well as fulfilling the safety and quality requirements of regulatory authorities, as now top of food industry priorities. Researchers in some institutes have identified cold atmospheric plasma (CAP) as a potential method for food preservation. It seems CAP is a promising method for inactivation of a wide range of microorganisms. This method will be reviewed in the subsequent section of this study.

1.2. COLD ATMOSPHERIC PLASMAS

1.2.1. What is plasma?

Plasma is the fourth state of matter. It consists of particles, in permanent interactions, which include photons, electrons, positive and negative ions, atoms, free radicals and excited or non-excited molecules (Foest *et al.*, 2006; Moreau *et al.*, 2008). Electrons and photons are often considered as light species unlike the other particles, which are considered as heavy species (larger than the mass of the proton). Therefore, the term "plasma" is considered to describe a state of matter in which the heavy species are neutral or ionised particles which is a consequence of energetic transfers to the gases in plasma (Moreau *et al.*, 2008). The term "plasma" was introduced by Irving Langmuir (Tonks, 1967; Goldston and Rutherford, 1995). Plasma constitutes more than 99% of the universe (Denes and Manolache, 2004; Foest *et al.*, 2006; Tendero *et al.*, 2006). Objects such as the Sun and other stars, interstellar matter, cometary and planetary atmospheres, and the terrestrial ionosphere, are all in the plasma state. Natural plasmas on the earth include lightning and flames (Denes and Manolache, 2004; Foest *et al.*, 2006).

Providing a solid material with thermal energy, sufficient to break the crystal lattice structure, generally causes a liquid to be formed. When the liquid is given enough thermal energy which causes the atoms to vaporize off the surface faster than they re-condense, a gas is formed. When the gas is given enough thermal energy, collisions between atoms occur and this results in loss of their electrons, leading to the formation of plasma (Goldston and Rutherford, 1995). However, not all substances can endure all these conversion processes due to differences in their

specific molecular structure. Inorganic compounds usually exist in solid, liquid or gaseous phases or a mixture of these because they are thermally more stable than organic compounds which upon heating will melt and decompose, or will endure thermal decomposition without melting if they have higher molecular weights (Denes and Manolache, 2004).

1.2.2. Generation of plasmas

Laboratory plasmas can be generated by applying energy to gas. This energy could be delivered in the form of thermal energy, electric current or electromagnetic radiation (Braithwaite, 2000; Tendero *et al.*, 2006). The atmospheric plasmas described in this report are produced using electrical energy. The electric field applied to the gas mixture transmits energy to the gas electrons. Collisions between gas molecules cause transfer of electronic energy from the charged to the neutral species in the gas flow. These collisions can be divided into two types:

1- Elastic collisions: these do not affect the internal energy of the neutral species however, these collisions cause a little increase in their kinetic energy.

2- Inelastic collisions: these do modify the electronic structure of the neutral species when the electronic energy is high enough. They produce excited species or ions if they are energetic enough (Tendero *et al.*, 2006).

The lifetime of the majority of the excited species in plasma is very short, and they return to a lower, stable energy level by emitting a photon. However, ''metastable species'' of ionized gases, which are also found in plasmas, do have a longer lifetime than other excited species, since their decay, characterized by the emission

of radiation is hampered, as there are no allowed transitions departing from the particular state: decay can only occur by energy transfers through collisions with other molecules (Tendero *et al.*, 2006).

1.2.3. Types of plasma

The form of energy used to generate and the amount of energy transferred to the plasma are responsible for the temperature and the electron density of plasma. Therefore, these two parameters (temperature and electron density of plasma) are used to classify plasmas into different groups presented in Figure 1.1 (Tendero *et al.*, 2006).

The atmospheric plasma sources described in this thesis are presumed to be positioned, in Figure 1.1, near the glow discharges and the arcs. In these plasma groups, a differentiation between Local thermodynamic (or thermal) equilibrium plasmas (LTE) and Non-local thermodynamic equilibrium plasmas (non-LTE) can be made (Foest *et al.*, 2006; Tender *et al.*, 2006; Moreau *et al.*, 2008). A summary of the differences between each of these two different plasmas is given in Table 1.1.

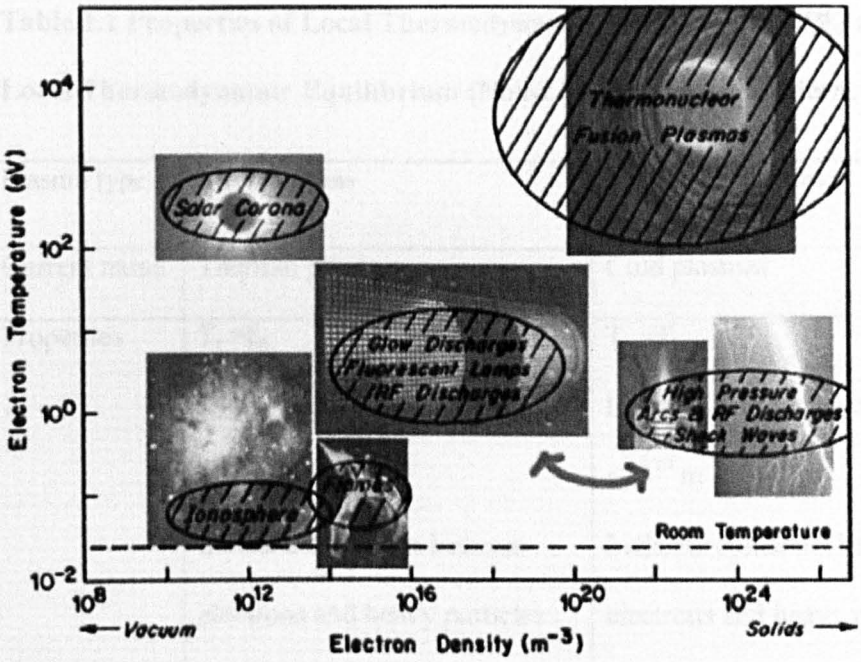

Figure 1.1: Plasma classification based on electron density and temperature.

(Taken from Tendero *et al.*, 2006).

Table 1.1 Properties of Local Thermodynamic Equilibrium (LTE) and Non Local Thermodynamic Equilibrium (Non-LTE) plasmas (Tendero, 2006).

Plasma type	LTE plasmas	Non-LTE plasmas
Current name	Thermal plasmas	Cold plasmas
Properties	$T_e=T_h$ High electron density: 10^{21}–10^{26} m^{-3} Inelastic collisions between electrons and heavy particles create the plasma reactive species whereas elastic collisions heat the heavy particles (the electron energy is thus consumed).	$T_e \gg T_h$ Lower electron density: $<10^{19}$ m^{-3} Inelastic collisions between electrons and heavy particles induce the plasma chemistry. Heavy particles are slightly heated by a few elastic collisions (that is why the electron energy remains very high).
Examples	Arc plasma (core) $T_e = T_h \sim 10{,}000$ K	Glow discharges $T_e \sim 10{,}000 - 100{,}000$ K $T_h \sim 300 - 1000$ K

Note: T_e = Electron temperature and T_h = Heavy particles temperature.

Cold plasmas have the advantage that the average electron energy is in the range of 1-5 eV (1 eV=$1.6x10^{-19}$ J or 11,600K), thus, the dissociation of molecules and excitation or ionization of atoms and molecules of the discharge gases occurs without requiring the heavy species to be as energetic. Consequently, the cost of energy used to generate the plasma is lower, and additionally glass or metal discharging vessels can be used to generate plasmas with no need for heavy cooling, which is required for thermal plasmas (Moisan *et al.*, 2001). In some kinds of pulsed discharges, the temperature of the gas can even be equal to ambient temperatures (Moisan *et al.*, 2001). Initially cold plasmas could only be produced under lower pressure conditions which needed complex and expensive vacuum equipment for initiating and sustaining the discharges. This equipment is expensive and has low efficiency (Denes and Manolache, 2004). Subsequently, equipment which has the capability to produce sustainable discharges at atmospheric pressure and low temperatures (cold atmospheric plasmas) has been developed in a number of laboratories (Denes and Manolache, 2004).

Cold atmospheric plasmas (CAP) can be generated using any of the following excitation frequencies: below 1 MHz; Radio frequency (1 MHz - 1 GHz); or Microwave (higher than 1 GHz). The excitation frequency is critical as it affects the behaviour of electrons and ions. Therefore CAP has been classified, based on the frequency used, into three types, which are:

1) Direct current and low frequency discharges.

2) Radio frequency ignited plasmas.

3) Microwave discharges.

However, researchers usually use different terms based on the configuration of the system they use. Systems which are the most widely used in microbial inactivation are:

1. Dielectric barrier Discharges (DBD) plasmas

Dielectric barrier Discharges are designed to employ a dielectric barrier in the gas gap for the discontinuation of electric currents and prevention of spark formation. Generally dielectric barrier discharges operate at frequencies between 0.05 and 500 kHz. Since they are generated at robustly non-equilibrium conditions at atmospheric pressure and reasonably high power levels and do not require sophisticated pulsed power supplies, they have a wide industrial application (Fridman *et al.*, 2005).

2. Corona discharge

A corona discharge is a weakly luminous electric discharge which normally ouccurs at atmospheric pressure near sharp points, edges, or thin wires if the electric field is sufficiently large. Corona discharges require relatively high voltages to be ignited (Fridman *et al.*, 2005).

3. Radio-frequency discharges (RF)

Radio-frequency discharges are plasmas produced by subjecting a gas to an oscillating electromagnetic field generated by an induction coil surrounding the reactor or by separate electrodes arranged on the external surface of the reactor (Moreau *et al.*, 2008).

4. Gliding arc discharges

Gliding arc discharges are plasma generated using a reactor composed of two or more curved metallic electrodes raised to a large potential difference (9 kV, 100mA in open conditions). In the gap between the electrodes a gas which is normally humid air is introduced (Moreau *et al.*, 2008).

These types of CAP have been employed to inactivate a wide range of microorganisms. The effect of CAP on food microorganisms will be reviewed in the next section of this chapter.

1.3. EFFECTS OF COLD PLASMA ON FOOD MICROORGANISMS

1.3.1. Introduction

Man-made plasmas are produced mainly for technological applications, which include high pressure and fluorescent lamps, plasma torches used for cutting metals, welding arcs, the ignition spark in an internal combustion engine, and low-pressure plasmas used in etching silicon wafers during fabrication of microelectronic devices (Foest *et al.*, 2006). These plasmas are mainly hot plasmas or low pressure plasmas and have limited application for the treatement of foods. However, advances in the production of cold plasma at atmospheric pressure have encouraged scientists to employ CAP in various fields, as this represents a novel technology. For example, in the field of material science, cold plasma has been used to modify the surface properties of some materials. Low temperature plasma

effectively modifies the adhesion properties of wool, cotton, and polyamide 6 fabrics. This modification ultimately enhances the antibacterial activity of these materials, which are widely used for wound dressing (Canal *et al.*, 2009). Similarly, cold plasma has been utilized to improve the smoothness and hydrophilicity of stainless steel surfaces. This improvement resulted in a reduction of 90% in the capability of *L. monocytogenes* to attach and form biofilms on plasma-treated stainless steel compared with untreated stainless steel, so bacterial contamination on surfaces used in food processing environments could be reduced by plasma polishing of the surface (Wang *et al.*, 2003). In the same way, Lee *et al.* (2010) have used atmospheric pressure plasma to control the hydrophobic and hydrophilic properties of the surface of poly-ether sulfone (PES) films. This led to the conclusion that atmospheric pressure plasma is a useful technology in manufacturing of flexible protein adhesive chips with uniform biomolecular adhesive properties.

The advancements in equipment that produce CAP have also motivated scientists to utilize this promising technology in the field of microbial inactivation. Therefore, in this section, the inactivation capability and the proposed modes of action of CAP will be reviewed.

1.3.2. Inactivation capability

Inactivation of microorganisms is commonly measured by D-value. This term was principally used by researchers studying heat sterilization but researchers in the

field of plasma inactivation of bacteria have also adopted this terminology (Laroussi, 2002). The D-value is the time, at a given temperature, required to reduce the number of microorganisms by 90% i.e. one logarithmic cycle (Jay, 2005; Adams and Moss, 2008). It is equal to the negative reciprocal of the slope of the inactivation curve, where the inactivation of microorganisms is a logarithmic function of time (Jay *et al.*, 2005; Adams and Moss, 2008).

Microbial inactivation using atmospheric plasmas goes back to 1968 when Menashi published a patent, which for the first time described the capability of atmospheric plasmas to sterilize surfaces (Menashi, 1968). A pulsed radiofrequency field (RF) and argon gas were used to produce plasma at atmospheric pressure in order to sterilize the inner surface of glass vials. The RF field was imposed in the bottle by inserting a wire inside the bottle and wrapping a coil around it which acted as the reference electrode to close the RF circuit (a corona-type discharge). It was found that in a fraction of a second, vials containing 10^6 spores had been sterilized (Menashi, 1968). Research in this field has increased considerably in the past few years, probably driven by the microbial challenges which were mentioned earlier in this chapter. Consequently, inactivation of microorganisms by CAP has been documented by many researchers in the last few years. Laroussi (1996) reported that CAP generated by RF effectively inactivates *Pseudomonas fluorescens* within a few minutes and is more effective than UV radiation. Montenegro *et al.* (2002) reported that a pulsed nonthermal plasma system operating at a frequency of less than 100 Hz with 4000 pulses of 9000 V peak voltage effectively reduces the count of *Escherichia coli* O157:H7 by 5 log cfu/ml within 40 seconds if the treatment time is considered as pulse number divided by pulse frequency.

Similarly, Lee *et al.* (2006) reported that CAP generated using helium/oxygen and AC power at 10 kHz and 6kV effectively inactivates *Escherichia coli, Staphylococcus aureus*, and *Saccharomyces cerevisiae* deposited on nitrocellulose filter membranes and spores of *Bacillus subtilis* deposited on polypropylene plates. The D-values were 18 sec, 19 sec, 115 sec and 14 min respectively. Scanning electron micrographs have shown substantial damage to *E. coli* cells, intermediate damage to S. *aureus* cells, peeling of *S. cerevisiae* cells and shrinkage of *B. subtilis* spores. Moreover, Morris and co-workers (Morris *et al.*, 2007) have found that cold air plasma generated by a dielectric barrier discharge using atmospheric air as the operating gas has significant capability to inactivate vegetative cells of *Geobacillus stearothermophilus* and both vegetative cells and spores of *Bacillus cereus*. Nevertheless, CAP was reported by these workers to have no significant capability to inactivate *Geobacillus stearothermophilus* spores. Recently, it has been shown that *Salmonella* Stanley and *Escherichia coli* O157: H7, inoculated on to golden delicious apples, could be significantly inactivated using CAP generated by a gliding arc using dried filtered air at different flow rates (10, 20, 30 or 40 litre/min). The higher flow rate was found to be more effective than other flow rates (Niemira and Sites, 2008). Moreover, CAP generated by parallel-plate dielectric-barrier discharge has the capability to decontaminate *E. coli* and *S. aureus*, in less than 10 and 7 seconds respectively (Ma *et al.*, 2008). CAP, generated using a gas mixture of He and O_2 at a fixed flow rate of 5 litre min^{-1} and 25 ml min^{-1} respectively and with a peak-to-peak voltage of 16 kV, has been shown to effectively reduce microbial counts, from 10^6 cfu/cm^2, of *Pantoea agglomerans* and *Gluconacetobacter liquefaciens* deposited on melon and mango skins, to less than

the detection limit (equivalent to at least 3 log cfu/cm^2) after 2.5 seconds of exposure, whilst the same treatment reduced the counts of *E. coli* to below the detection limit after only 5 seconds. *S. cerevisae* inoculated onto mango and melon showed higher resistance to CAP and reached the same level of reduction after 10 and 30 seconds of exposure respectively (Perni *et al.*, 2008a). The authors also found that increasing the voltage from 12 kV to 16 kV enhances the inactivation capability.

Furthermore, inactivation of *Escherichia coli* type 1, *Saccharomyces cerevisiae*, *Gluconobacter liquefaciens*, and *Listeria monocytogenes* Scott A deposited on 0.2μm-pore-size Whatman polycarbonate membrane filters and on cut fruit surfaces using CAP generated by an AC voltage of 8 kV at 30 kHz has been studied by Perni *et al.* (2008b). They found that CAP efficiently reduced the microbial load on the filter membranes. CAP reduced the counts of *G. liquefaciens*, *L. monocytogenes*, *E. coli* and *S. cerevisiae* to lower than the detection limit (1×10^3 cfu/cm^2) in 1.5, 1.5, 5, 30 seconds respectively. On the other hand, the capability of CAP to inactivate microorganisms was noticeably reduced for microorganisms on the cut fruit surfaces due to the migration of microorganisms from the outer surface of the fruit tissue to its interior. Recently, Song *et al.* (2009) evaluated the efficiency of CAP, generated using He gas at 10 litre/min and a power range between 75 and 150W, in inactivation of mixed cultures of *Listeria monocytogenes* strains (ATCC 19114, 19115, and 19111, LMC) inoculated onto sliced cheese and ham. They found that CAP has the capability to inactivate mixed cultures of *L. monocytogenes*, however the inactivation capability was food type and power dependant and D-values obtained ranged from, 17.27 to 476.19 seconds. Similarly,

Yun *et al.* (2010) found that atmospheric pressure plasma effectively inactivates *L. monocytogenes* attached to different types of material that are used for disposable food containers. However, achieving satisfactory inactivation levels for each material needs appropriate treatment conditions which should be taken into consideration. More recently, Wang *et al.* (2012) have reported that cold plasma generated using a direct-current, atmospheric-pressure air cold plasma microjet, effectively inactivates *Salmonella* cells which were directly deposited on fresh fruit and vegetable slices in a very short time (one second).

Besides the efficient inactivation of vegetative cells and spores, there are some positive indications in the literature that CAP can remove biofilms. Biofilms are bacterial populations fixed in a matrix which mainly consists of polysaccharides with some proteins. Bacteria in biofilms have resistance mechanisms different from those of bacteria that are planktonic (Stewart and Costerton, 2001). Nonetheless, atmospheric pressure glow discharges (APGD) have shown the capability to effectively inactivate *Pantoea agglomerans*, embedded in biofilms. CAP inactivation was more effective than UV irradiation. Moreover, the treatment caused no significant discolouration of bell pepper samples on which biofilms were formed (Vleugels *et al.*, 2005). Comparable results have been published recently on the effect of CAP, generated using a He and N_2 at flow rates of 20.4 L/min and 0.305 L/min respectively, on *Chromobacterium violaceum* bacterial biofilms by Vandervoort *et al.* (2008). They employed Atomic force microscope (AFM) to visualize the damage caused by CAP to the biofilm. Their findings demonstrate that

CAP effectively removed 90% of culturable cells of *Chromobacterium violaceum* from bacterial biofilms.

In addition to all these wide ranging antimicrobial capabilities, CAP have also shown promising results in the inactivation of proteins. Deng *et al.* (2007) employed a laser induced fluorescence technique to investigate effects of CAP, generated by an atmospheric pressure glow discharge (APGD) jet struck inside a He-O_2 channel and ejected into the ambient air, on proteins deposited on stainless-steel surfaces, using bovine serum albumin (BSA) as a model protein. They found that CAP is capable of reducing surface protein by 4.5 logs (molecules/mm^2) through protein destruction and degradation.

The mechanisms by which CAP inactivates this wide range of microoganisms are still under investigation. Some researchers have reported on some proposed mechanisms. These mechanisms will be reviewed in the next section.

1.3.3. Proposed inactivation mechanisms

Researchers studying the microbial inactivation mechanism of CAP have taken two approaches. The first addresses the mechanism through identification of plasma constituents that have the most important role in the inactivation process. The second approach addresses the inactivation mechanism through detection of the occurrence of defects and damage to specific sites or molecules in the microorganisms.

1.3.3.1. Plasma constituents

Laroussi (2005) indicated that CAP constituents (inactivation agents) which might have a contribution in the CAP inactivation mechanism are principally the reactive species, ultraviolet radiation (UV), and charged particles (electrons and ions).

1.3.3.1.1 The role of reactive species

Of the reactive species, oxygen species have been predicted to be the main agents to be involved in the microbial inactivation. Nagatsu *et al.* (2005) studied the effect of low pressure oxygen plasma on *Bacillus stearothermophilus* spores. They used a perforated stainless steel plate to prevent the passage/transfer of electrons and ions, allowing only the neutral species and any UV photons to pass the filter. Their conclusion was that the CAP inactivation mechanism might be attributed to a chemical etching reaction on the spores by reactive oxygen species. Deng *et al.* (2006) have also found, using scanning electron microscopy and fluorescence microscopy, that CAP caused the complete rupture of the membrane of *Bacillus subtilis* spores. Meanwhile they employed optical emission spectroscopy with inactivation kinetic studies to identify the inactivation agents. This enabled them to demonstrate that spore destruction was most likely caused because of reactive oxygen species combined with a small contribution from heat, UV photons, electric field and charged particles. Similarly, Ross *et al.* (2006) have studied the effect of low pressure plasma inactivation using different ratios of O_2/N_2 in the gas mixture on *Geobacillus stearothermophilus* spores. They found that the best inactivation

results were obtained when the ratio of atomic oxygen to nitrogen is 95% O_2: 5% N_2. They also found that the gas mixture, which produced the highest yield of UV photons, demonstrated a low sterilization capability. Therefore, they believed the inactivation mechanism was through an etching mechanism by reactive particles and mainly oxygen atoms. More substantiation of the role of oxygen species in microbial inactivation has been documented by Perni *et al.* (2007) in their study on the effect of CAP generated using either He gas alone or a He/O_2 mixture. They employed optical emission spectroscopy and *E. coli* K-12 MG 1655 and its Δ*rec*A, Δ*rpo*S and Δ*sox*S mutants to identify the constituent which has the most important role in microbial inactivation. Their findings indicated that inactivation is mainly due to the effect of oxygen atoms accompanied by lesser effects due to OH radicals, UV photons, singlet oxygen metastables, and nitric oxide. In addition to this work, Lim *et al.* (2007) have concluded that atomic oxygen radicals have a significant function in microbial inactivation of *Bacillus atrophaeous* spores using CAP generated using Ar/O_2. Moreover, Deng *et al.* (2007) employed optical emission spectroscopy and protein inactivation kinetics to identify the main plasma constituent which causes protein destruction and degradation. They have found that the major plasma constituents (which cause protein damage) are excited atomic oxygen and excited nitrogen oxides. They also demonstrated that a synergistic effect between these two constituents is possibly the main cause of protein destruction. More indication of the involvement of oxygen species in the microbial decontamination by CAP were found by Xin *et al.* (2008). They have investigated the effect of different gases (oxygen, nitrogen and air) on the inactivation efficiency of non thermal plasma against bacteria (which commonly causes serious pipe

corrosion in oil field injection water system). Oxygen has been identified as the most efficient CAP gas followed by air and nitrogen (Xin *et al.*, 2008). Moreover, enhancement in CAP capability to inactivate a pathogenic bacterium (*Escherichia coli*) and a range of food spoilage microorganisms (*Saccharomyces cerevisae, Pantoea agglomerans*, and *Gluconacetobacter liquefaciens*) has been observed, when levels of oxygen atoms are increased (Perni *et al.*, 2008a). Recently, Ma *et al.* (2008) have studied the effect of CAP generated by parallel-plate dielectric-barrier discharge on two different species of bacteria (*E. coli* and *S. aureus*). They observed cellular damage using transmission electron microscopy and have also measured the concentrations of K^+, protein, and nucleic acid leakage from the cells. They found that reactive oxygen species (ROS) has the major effect on cellular inactivation. They hypothesized that ROS cause oxidization of the cell membrane followed by damage to the protein and nucleic acid inside the cells.

1.3.3.1.2 The role of UV photons

The effects of UV radiation on microorganisms are well documented. The work done by Norman in 1954 (see Laroussi and Leipold, 2004), indicates that UV radiation causes dimerization of thymine bases in DNA strands of microbial cells. Consequently, the bacteria lose their ability to replicate properly. The optimum effect of UV on bacteria is known to occur at the wavelength range of 220 - 280 nm and doses of several mWs/cm^2. Laroussi and Leipold (2004) evaluated the role of UV radiation in inactivation of microorganisms by CAP generated by Dielectric Barrier Discharges (DBD) in air. They conducted spectroscopic and absolute power

measurements to quantify the effect of UV in the process of inactivation. Their results indicate that no significant UV emission occurred below 285 nm when the power density was less than 50 $\mu W/cm^2$ and essentially is not affected by flow rate of the air therefore, their expectation was UV has no significant role in the inactivation process by CAP in air. Moreover, Ross *et al.* (2006) and Deng *et al.* (2008) have reported that UV photons emitted from CAP have little effect on microbial inactivation. Similar results were also obtained by Deng *et al.* (2006). They employed a UV-transparent glass as a barrier for other plasma constituents in order to investigate the effect of UV radiation emitted from CAP on *Bacillus subtilis* spores. They found a minor reduction of 0.2 log cfu/cm^2 after 10 minutes exposure to the atmospheric-helium plasma, in contrast to a reduction of 4 log cfu/cm^2 obtained by the same treatment in the absence of UV-transparent glass.

1.3.3.1.3 The role of charged particles

According to Laroussi (2002) the average energy of electrons in cold plasmas is around one electron volt (~ 1.6×10^{-19} Joule) and the temperature of ions is near ambient temperature. In such conditions of high pressure and low temperature, it is expected that bombardment of bacterial cells by charged particles is unlikely to cause any significant effects. As a consequence most researchers have neglected to consider in their studies the effects of charged particles in cold plasmas on microbial inactivation.

1.3.3.2 Plasma targets

Little work has been done to identify sites in microorganisms that are targeted by CAP inactivation agents. However, there are a few published papers indicating that particular sites in microoganisms are targets of CAP constituents. It has been suggested that membrane lipids are probably the most susceptible molecules to CAP (Montie *et al.*, 2000). This susceptibility may be due to the membrane lipid location, which is near the cell surface, as membrane lipids are sensitive to reactive oxygen species. Gram negative bacteria are likely to be more susceptible to CAP, since they have a unique outer membrane. Macromolecular leakage was observed from the Gram negative bacterium, *E. coli*, after 10 seconds of exposure to CAP and the cells were rapidly and severely fragmented. This damage was most likely due to destruction of its outer membrane. By contrast, no leakage was observed from *S. aureus* (which is a Gram positive bacterium) during the same time of treatment. Montie *et al.* (2000) have also suggested that the thick polysaccharide layer of the *S. aureus* cell wall is not susceptible to chemical change; however it permits diffusion of ROS to the cytoplasmic membrane which is susceptible to ROS. Alteration of cytoplasmic membrane lipids in both types of bacteria leads to the release of macromolecules and ultimately death of the cells. Moreover, Laroussi *et al.* (2002) employed the Biolog (Hyward, CA) GN2^{TM} 96 well microtitre plate growth system to investigate the effect of sublethal doses of CAP, generated by the resistive-barrier discharge (RBD), on the heterotrophic pathways of *Escherichia coli*. They found that CAP induced significant changes in the utilization of some substrates (L-fucose, D-sorbitol, and D-galacturonic acid) and a decrease in

utilization of methyl pyruvate, dextrin, and D-lactic acid. These changes were attributed to possible alteration in enzyme activity or alteration of the permeability of the membrane. They also observed morphological changes in Gram positive and Gram negative bacteria (*E. coli* and *B. subtilis*) using scanning electron microscopy. Their results indicate that CAP treatment caused severe damage to *E. coli* cells. However, the morphology of *B. subtilis* cells had not been changed. Furthermore, Yasuda *et al.* (2008) studied the effect of CAP on *Escherichia coli* and bacteriophage-λ. They monitored changes in the biological components during the course of inactivation. They observed minor and slow degradation in membranes, proteins, and DNA during the course of inactivation and extraordinary degradation was observed when complete sterilization was achieved. They also found, from the analysis of Green Fluorescent Protein (GFP) coding plasmid pGLO (5.4 kb in size) transfected into *E. coli*, that CAP has the ability to denaturate proteins without altering the peptide bonds. The permanent denaturation of proteins was presumed to have a significant function in the microbial inactivation process.

Cold atmospheric pressure plasma (CAP) has a very good capability to inactivate a wide range of microorganisms. It is a very promising technology which could be employed by the food industry to ensure production of safe and high quality foods; however the microbial inactivation mechanisms of CAP are still not completely understood and there are some concerns about the safety of this technology. Therefore, the aims of this study were:

1) Establishing standard inactivation conditions for food-borne pathogens.

2) Understanding the mechanism by which CAP inactivates microorganisms.

3) Identification of sites and molecules in bacteria that are targets for the CAP species.

4) Assessment of the safety of this technology.

The hypotheses to be tested in this study are:

1) CAP has direct and indirect effects on cells of bacteria.
2) The indirect effects are mainly related to oxidative stresses due to reactive oxygen species in the plasma.
3) Gram positive bacteria may have higher resistance to CAP treatments than Gram negative bacteria as they have differences in external cell wall structure.
4) The death of bacterial cells is most likely the result of permanent damage in certain important sites in the DNA of these bacteria.
5) CAP treatments could cause gene alteration which might lead to mutations.

CHAPTER 2
MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals and media

All chemicals and media used in this study are shown in Table 2.1.

Table 2.1. Chemicals and media used in this study.

Chemical	Supplier
Agar Bacteriological (Agar No. 1) LP0011	Oxoid, Ltd UK
Ampicillin	Fisher Scientific, UK Ltd
Brain Heart Infusion broth (CM1135)	Oxoid, Ltd UK
Citric acid anhydrous ($C_6H_8O_7$)	Sigma-Aldrich
Crystal violet	Fisher Scientific, UK Ltd
D-Biotin (F.W.244.3)	Sigma-Aldrich
D-Glucose, anhydrous	Fisher Scientific, UK Ltd
Ethidium Bromide	Fisher Scientific, UK Ltd
Hydrochloric acid	Fisher Scientific, UK Ltd
Kanamycin	Melford Laboratories Ltd.
L-Histidine. HCL. H_2O (F.W.209.63)	Sigma-Aldrich
Magnesium sulphate ($MgSO_4.7H_2O$)	Fisher Scientific, UK Ltd
Maximum Recovery Diluent (MRD) CM0733	Oxoid, Ltd UK
Nutrient Broth No.2 (CM0067)	Oxoid, Ltd UK
Potassium phosphate, dibasic, anhydrous (K_2HPO_4)	Fisher Scientific, UK Ltd

Table 2.1. continued

Sodium ammonium phosphate ($NaHNH_4PO_4.4H_2O$)	Acros Organic
Sodium chloride (NaCl)	Fisher Scientific, UK Ltd
Streptomycin	Fisher Scientific, UK Ltd
Tetracycline	Sigma-Aldrich
Tryptone	Fisher Scientific, UK Ltd
Yeast extract	Oxoid, Ltd UK
1kb DNA ladder	Promega, Co. USA
ReddyMix™ PCR Master Mix	Thermo Scientific (ABgene)
Bacto Agar	Difco
Oligonucleotides	Eurofins MWG Operon.
Agarose	Sigma-Aldrich
Tris base	Sigma-Aldrich
Glacial acetic acid	Fisher Scientific, UK Ltd
EDTA	Fisher Scientific, UK Ltd
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich

2.1.2. Bacterial strains/mutants

The strains/mutants of bacteria used in this study and their suppliers are listed in Table 2.2.

Table 2.2. Strains/mutants of bacteria used in the study.

No	Strain	Antibiotic required	References	Supplier /obtained from
1	*E. coli* K-12 MG 1655 (CGSC 7740) F-, λ^-, *rph-1*	non	Blattner *et al.* (1997), (CGSC, 2013)	Food microbiology Group, UoN obtained from Yale Stock Centre
2	*E. coli* HS (commensal)	non	Rasko *et al.* (2008)	Food microbiology Group, UoN
3	*E. coli* O157: H7 Sakai (Δ*stx1*, Δ*stx2*)	non	Hayashi *et al.* (2001), Pacheco-Gomez *et al.* (2012)	Food microbiology Group, UoN Gift from Dr Martin Goldberg University of Birmingham
4	*E. coli* H10407 (ETEC)	non	Crossman *et al.* (2010)	Food microbiology Group, UoN
5	*E. coli* K-12 BW25113 (CGSC 7636) F-, Δ*(araD-araB)567*, Δ*lacZ4787*(::rrnB-3), λ^-, *rph-1*, Δ*(rhaD-rhaB)568*, *hsdR514*	non	Baba *et al.*(2006), (CGSC, 2013)	Gift from Dr. Douglas F. Browning, UoB

Table 2.2. continued

6	*E. coli* K-12 BW25113 (Δ *ahpC*) as BW25113, but: *ahpC*::Km	Km (50 μg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
7	*E. coli* K-12 BW25113 (Δ *katG*) as BW25113, but: *katG*::Km	Km (50 μg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
8	*E. coli* K-12 BW25113 (Δ *grxA*) as BW25113, but: *grxA*::Km	Km (50 μg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
9	*E. coli* K-12 BW25113 (Δ *flu*) as BW25113, but: *flu*::Km	Km (50 μg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
10	*E. coli* K-12 BW25113 (Δ *ahpF*) as BW25113, but: *ahpF*::Km	Km (50 μg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
11	*E. coli* K-12 BW25113 (Δ *dps*) as BW25113, but: *dps*::Km	Km (50 μg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
12	*E. coli* K-12 BW25113 (Δ *fur*) as BW25113, but: *fur*::Km	Km (50 μg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
13	*E. coli* K-12 BW25113 (Δ *sufC*) as BW25113, but: *sufC*::Km	Km (50 μg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB

Table 2.2. continued

14	*E. coli* K-12 BW25113 (Δ *sufB*) as BW25113, but: *sufB*::Km	Km (50 µg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
15	*E. coli* K-12 BW25113 (Δ *sufD*) as BW25113, but: *sufD*::Km	Km (50 µg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
16	*E. coli* K-12 BW25113 (Δ *yaiA*) as BW25113, but: *yaiA*::Km	Km (50 µg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
17	*E. coli* K-12 BW25113 (Δ *rfaE*) as BW25113, but: *rfaE*::Km	Km (50 µg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
18	*E. coli* K-12 BW25113 (Δ *lpcA*) as BW25113, but: *lpcA*::Km	Km (50 µg/ml)	Baba *et al.*(2006)	Gift from Dr. Douglas F. Browning, UoB
19	*E. coli* K-12 W3110 (CGSC 4474) F-, λ^-, *IN(rrnD-rrnE)1*, *rph-1*	non	Hayashi *et al.* (2006), (CGSC, 2013)	(Heurlier, K., personal communication)
20	*E. coli* K-12 W3110 (Δ *rfaC*) as W3110, but : *rfaC*::Km	Km (50 µg/ml)	(Heurlier, K., personal communication)	(Heurlier, K., personal communication)
21	*E. coli* K-12 DFB1655 L5 (MG1655 with pJP5603/*wbbL* plasmid integrated downstream of the IS5 element)	Km (50 µg/ml)	Browning *et al.* (2013)	Gift from Dr. Douglas F. Browning, UoB

Table 2.2. continued

22	*E. coli* K-12 DFB1655 L9 (MG1655 with pJP5603/*wbbL* plasmid integrated upstream of the IS*5* element)	Km (50 µg/ml)	Browning *et al.* (2013)	Gift from Dr. Douglas F. Browning, UoB
23	*E. coli* K-12 MG 1655 Str^R (Spontaneous streptomycin-resistant mutant of MG1655)	Str (100 µg/ml)	Møller *et al.* (2003)	Gift from Dr. Paul S. Cohen, Cohen, University of Rhode Island
24	*E. coli* K-12 MG 1655 MD42 Str^R Km^R (P1 mini-Tn*5* Km::*waaQ*)	Str (100 µg/ml) &Km (50 µg/ml)	Møller *et al.* (2003)	Gift from Dr. Paul S. Cohen, University of Rhode Island
25	*L. monocytogenes* ATCC 23074 (4b)	non	(Murray *et al.*) Pirie ATCC® 23074™	Food microbiology Group, UoN
26	*L. monocytogenes* WSLC 1042 (4b)	non	Loessner and Busse (1990)	Food microbiology Group, UoN
27	*L. monocytogenes* EGD-e (1/2a)	non	Murray *et al.* (1926)	Food microbiology Group, UoN
28	*L. monocytogenes* 00054-0305 (1/2b)	non	Vatanyoopaisarn (1999)	Food microbiology Group, UoN
29	*S. enterica* serovar Typhimurium LT2 (Δ*spvC*)	non	McClelland *et al.* (2001)	Food microbiology Group, UoN

Table 2.2. continued

30	*S. enterica* serovar Typhimurium TA98 *hisD3052*, (Δ *rfa*), *bio chlD uvrB gal*, pKM101	Amp (24 μg/ml)	McCann *et al.* (1975)	Moltox, Inc. (Molecular Toxicology, Inc), US
31	*S. enterica* serovar Typhimurium TA100 *hisG46* , (Δ *rfa*), *bio chlD uvrB gal*, pKM101	Amp (24 μg/ml)	McCann *et al.* (1975)	Moltox, Inc. (Molecular Toxicology, Inc), US
32	*S. enterica* serovar Typhimurium TA102 *hisG428*, (Δ *rfa*), pKM101, pAQ1	Amp (24 μg/ml) & Tet (2 μg/ml)	Levin *et al.* (1982a)	Moltox, Inc. (Molecular Toxicology, Inc), US
33	*S. enterica* serovar Typhimurium TA1535 *hisG46*, (Δ *rfa*), *bio chlD uvrB gal*	non	Ames *et al.* (1973)	Moltox, Inc. (Molecular Toxicology, Inc), US
34	*S. enterica* serovar Typhimurium TA1537 *hisC3076*, (Δ *rfa*), *bio chlD uvrB gal*	non	Ames *et al.* (1973)	Moltox, Inc. (Molecular Toxicology, Inc), US

UoN = University of Nottingham and UoB= University of Birmingham.

Table: 2.3. Oligonucleotides used in the study.

No.	Primer name	Oligonucleotide sequence (5'--------3')	Targeted Gene	Annealing temperature	Product size bp	Reference
1	For-*dps*-OE	TTA ATC TCG TTA ATT ACT GGG ACA (24)	*dps*	52.5 °C	597	This study
2	Rev-*dps*-OE	AGC CGC TTT TAT CGG GTA CT (20)				
3	For-*flu*-OE	GGA AGA CGG TGA ACA ACG AT (20)	*flu*	52.5 °C	985	This study
4	Rev-*flu*-OE	ACA GAC ATA CCG GCA ACC TC(20)				
5	For-*fur*-OE	CAT TTA GGC GTG GCA ATT CT (20)	*fur*	52.5 °C	669	This study
6	Rev-*fur*-OE	AAA TGA TCA GGC GGT GAA AG (20)				
7	For-*katG*-OE	CCA TAA CAC CAC AGC CAC TG (20)	*katG*	55 °C	1055	This study
8	Rev-*katG*-OE	TAA TTT CCG GTG CGT CTA CC (20)				
9	For-*sufB*-OE1	CAT CGT GCG GTA ATT GTG AC (20)	*sufB*	55 °C	813	This study
10	Rev-*sufB*-OE1	CGA TGT GGA TCA TCT TGG TG (20)				

Table: 2.3. continued

11 12	For-*sufC*-OE Rev-*sufC*-OE	TTA AGC CTC GAC GTT CAT CC (20) CAT CGG CGA CCA CTT TTA AT (20)	*sufC*	52.5 °C	502	This study
13 14	For-*sufD*-OE Rev-*sufD*-OE	GGA GAG ATA TCC CCA CAG CA (20) GCA ACT GTC GGC TGT TAC AA (20)	*sufD*	52.5 °C	703	This study
15 16	For-*grxA*-OE Rev-*grxA*-OE	CCT CTG CAA AGT GAG CCT TC (20) GGT TAC TAA GCG CGG TGT TC (20)	*grxA*	52.5 °C	534	This study
17 18	For_*oxyR*_OE Rev_*oxyR*_OE	GAA GCA CAG ACC CAC CAG TT (20) AGC GCT GGC AGT AAA GTG AT (20)	*oxyR*	52.5-58.5 °C	353	This study
19 20	For_*eaeA*_OE Rev_*eaeA*_OE	AGG CTT CGT CAC AGT TG (17) CCA TCG TCA CCA GAG GA (17)	*eaeA*	51-58.5 °C	570	China, *et al.* (1996); Chen and Griffiths (1998)
21 22	For-*uspA*-OE2 Rev-*uspA*-OE2	CCG ATA CGC TGC CAA TCA GT (20) ACG CAG ACC GTA GGC CAG AT (20)	*uspA*	51- 58.5 °C	884	Chen and Griffiths (1998)

Table: 2.3. continued

23 24	For-*rfaC* Rev-*rfaC*	AAG TGC GTA AAG GTG ACG (18) CGC CAC TAA CTA TCC CTA (18)	*rfaC*	65 °C	1.6	(Heurlier, K., personal communication)
25 26	For-OE-*waaQ* Rev-OE-*waaQ*	AAA ATC ATT CGG GTC GGA TT (20) GCC TTC CCA CGA CTG TGT AT (20)	*waaQ*	54.4 °C	1348	This study
27 28	For-*lpcA* Rev-*lpcA*	TAG CAC CTG CCC GTA CTT CT (20) CAT TAT TCG GCC TAC GGT TC (20)	*lpcA*	54 °C	785	This study
29 30	For-*uvrB* Rev-*uvrB*	TTG CAA TAC ACC CGT AAC GA (20) CCA GGA TGT CGA CCA CTT TT (20)	*uvrB*	56.4 °C	1258	This study
31 32	For-*bioD* Rev-*bioD*	CCG CAA CGC AGT TAG GTT AT (20) AGG GGG CTA AGG TCC AGA TA (20)	*bioD*	56.4 °C	598	This study
33 34	For-*spvC* Rev-*spvC*	ACT CCT TGC ACA ACC AAA TGC GGA (24) TGT CTC TGC ATT TCG CCA CCA TCA (24)	*spvC*	56 °C	571	Chiu and Ou (1996)

Table: 2.3. continued

35	For-*rfaE*	AAG TGG CTG GTG GAA GAA TG (20)	*rfaE*	54 °C	1526	This study
36	Rev-*rfaE*	CGA GAT CTG TGA ACC GCT TT (20)				

2.2. MICROBIOLOGICAL AND MOLECULAR METHODS

2.2.1. Preparation of bacterial cultures

2.2.1.1 Working cultures

2.2.1.1.1. Escherichia coli strains/mutants

Working cultures of all *E. coli* strains were maintained by streaking the strains on LB agar plates which then were incubated at 37 °C for 24 h. The grown cultures were stored at 4 °C for a short period of time (maximum of 2 weeks). Kanamycin, Streptomycin, or Kanamycin and Streptomycin were added to the LB medium as required for each strain.

2.2.1.1.2. Listeria monocytogenes strains

Working cultures of all *Listeria monocytogenes* strains were maintained by streaking the strains on BHI agar plates which then were incubated at 37 °C for 24 h. The grown cultures were stored at 4 °C for a short period of time (maximu 2 weeks).

2.2.1.1.3. Salmonella strains/mutants

Working cultures of all Ames strains were maintained by streaking the strains on MG agar plates, supplemented with Biotin and an excess of histidine. The inoculated plates were then incubated at 37 °C for 48 h covered by aluminium foil. The grown cultures were stored at 4 °C for a short period of time

(maximum 2 weeks). Ampicillin or Ampicillin and Tetracycline were added to the medium as required per each strain. *Salmonella enterica* serovar Typhimurium LT2 was maintained by streaking the strain on LB agar plates which then were incubated at 37 °C for 24 h. The grown culture was stored at 4 °C for a short period of time (maximum 2 weeks).

2.2.1.2. Frozen stock cultures

For long term storage of the cultures of all strains used in this study, frozen stock cultures were made, from 18-24 h old or 48 h in the case of Ames strains, cultures grown on agar plates. The frozen stocks were made by transferring a loop-full of bacteria into cryovials containing cryopreservative fluids (Microbank®, Pro-Lab Diagnostics U.K.). The inoculated vials were tightly closed and inverted 4-5 times to mix the bacteria and to let them become bound to the porous beads. Excess cryopreservative fluid was removed from vials by aspiration with a Gilson P1000 tip and then the vials were kept at -80 °C. When a fresh culture was needed, one bead was taken aseptically using a sterile tooth pick and streaked onto the appropriate agar plates or placed into broth medium.

2.2.2. Confirmation of strains' and mutants' identities

2.2.2.1. *Escherichia coli* strains/mutants

All *E. coli* strains and mutants used in this study were tested by PCR assay, as will be described hereafter in section 2.2.4, to confirm their identities using primers which have been documented to be specific for the *E .coli* universal stress protein gene (*uspA*) and can be used to differentiate the generic *E. coli*

from other Gram negative bacteria (Chen and Griffiths, 1998). Another set of primers, which have been reported by China *et al.* (1996); Chen and Griffiths (1998) to be specific for the virulence gene (*eaeA*) which encodes the outer membrane protein intimin and was found to be useful in differentiating EPEC/EHEC from other *E. coli* strains was used to confirm the identity of *E. coli* O157:H7.

The mutants were also tested by PCR assay using primers which were designed in this study specifically to confirm the mutations in the genes of interest. Table 2.3 shows the sequences of all primers, used in this study, including their annealing temperatures and the sizes of expected PCR products.

2.2.2.2. *Listeria monocytogenes* strains

All *Listeria monocytogenes* strains used in this study were obtained from the stock cultures of the Food microbiology group, Nottingham University, UK. They were recently confirmed by a colleague in the laboratory (El Emam, M. personal communication). Therefore, no further confirmation tests were carried out on them in this study.

2.2.2.2. *Salmonella* strains/mutants

All *Salmonella* strains/mutants used in this study were tested by PCR assay, as will be described hereafter in section 2.2.4, using primers which have been published by Chiu and Ou (1996) and which are specific for the virulence gene (*spvC*) which is on the *Salmonella* virulence plasmid and appropriate to be used to differentiate *Salmonella* strains from other strains of bacteria. In

addition to this more confirmatory tests were also carried out on these strains as will be described later in section 2.2.5.

2.2.3. Primer design and preparation

2.2.3.1. Primer design

All oligonucleotides used in this study (except those obtained from published papers) were designed using online available databases. The *Coli*BASE database was used to search for the sequences of the genes of interest (Chaudhuri, 2008). The gene sequences obtained by these searches were then inputted into the Primer3Plus™ software which was used to select primer pairs to detect the given template sequence (Untergasser *et al.*, 2007).

2.2.3.2 Primer preparation

Oligonucleotide stock solutions of 100 pmol/µl concentration were made by adding the required volume of sterile Milli-Q water as described in the oligonucleotide synthesis reports provided by the supplier (Eurofins MWG Operon), to each primer tube. Working solutions were made by transferring 15 µl of the stock solutions into a sterile 1.5-ml Eppendorf tube containing 100 µl of sterile Milli-Q water. All oligonucleotide solutions were stored at -20 °C.

2.2.4. Polymerase Chain Reaction (PCR) assays

Single and Multiplex PCR assays, depending on the purposes of the PCR experiments, were used in this study. The purpose of each PCR assay will be explained hereafter in the results section for each PCR assay.

2.2.4.1. PCR protocol

The PCR assays were performed by preparing a PCR mixture containing the following ingredients:

Ingredient	Volume	Final concentration
ReddyMix ™ PCR Master Mix (2X)	10 µl	1X
Forward primer (working solution)	0.45 µl	2.25% (v/v)
Reverse primer (working solution)	0.45 µl	2.25% (v/v)
Sterile Milli-Q water	To make 20 µl	-
Total volume	20 µl	-

The volumes above were used for a single PCR reaction; however, the volumes of the PCR mixtures were adjusted depending on the number of the PCR reactions in each experiment, and then aliquots of 20 µl were transferred into each PCR tube. A tiny amount of bacterial colony was transferred into each PCR tube and mixed with the buffer. This was done by touching a Gilson yellow tip attached to a pipette to a fresh colony grown on agar plates, and then transferring the culture to the PCR mixture. The PCR tubes were placed into the block in the PCR machine (C1000 ™ thermal cycler, BIO-RAD) which was programmed to operate the following PCR cycles as described by McPherson and Møller (2006).

Step1	Initial Denaturation	10 min at 94 °C	1 cycle
Step *2.*	Denaturation	1 min at 94 °C	30 cycles
	Annealing	1 min at X* °C	
	Extension	2 min at 72 °C	
Step 3.	Final extension	5 min at 72 °C	1 cycle

X* = annealing temperature of each primer as listed in Table 2.3

The final concentration of ReddyMix™ PCR Master Mix in each reaction which was (1X) contained

0.625 units	ThermoPrime Taq DNA Polymerase
75mM	Tris-HCL (pH 8.8 at 25 °C)
20mM	$(NH_4)_2SO_4$
3.5mM	$MgCl_2$
0.01% (V/V)	Tween20
0.2mM	Each of dATP, dCTP, dGTP and dTTP

Precipitants and red dye for electrophoresis

2.2.4.2. PCR product separation and visualization (Agarose gels)

The PCR products were size/charge separated using the gel electrophoresis technique as described by Tiwari *et al.* (2009). The gel was prepared at a concentration of 1% (w/v) agarose in 1X Tris-acetate buffer and contained 5 µl/100 ml of Ethidium Bromide solution (10 mg/ml). Once the gel had become solid, it was transferred into a horizontal gel electrophoresis tank which was filled with a running buffer (1X Tris-acetate EDTA). Seven µl of each PCR product was loaded into the gel wells and 7 µl of molecular markers (5 µl of 1kb DNA ladder (100 µg/ml) (Promega, Co.) and 1 µl of Blue/orange (6X)

loading dye) (Promega, Co.) were also loaded into the first or last wells of the gel. The gel was run at 80V for about 60 - 90 min (depending on the gel size). Once the DNA had been separated, the bands were visualized and photographed using the Gel Documentation System (Bio-Rad) integrated with Quantity One 1-D Analysis Software (Bio-Rad).

2.2.5. Confirmation of the Ames strains' specific characteristics

All mutants (Ames/*Salmonella* strains) used in this study were tested beside the parental strain *S.* Typhimurium LT2 for histidine dependency, biotin dependency, histidine and biotin dependency, the *rfa* marker, deletion of the *uvrB* gene, presence of plasmid pKM101 and presence of plasmid pAQ1 (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

2.2.5.1. Histidine dependency

The histidine dependency of the strains was tested by streaking a loop-full of overnight culture onto MG plates supplemented with an excess of biotin. Plates were covered with aluminium foil and incubated upside down at 37 °C for 24 h. Strains which were unable to grow and form colonies on these plates were considered to be histidine-dependant strains (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

2.2.5.2. Biotin dependency

The biotin dependency of the strains was tested by streaking a loop-full of overnight culture onto MG plates supplemented with an excess of histidine. Plates were covered with aluminium foil and incubated upside down at 37 °C for 24 h. Strains which were unable to grow and form colonies on these plates were considered to be biotin-dependant strains (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

2.2.5.3. Histidine and biotin dependency

The histidine and biotin dependency of the strains was tested by streaking a loop-full of overnight culture onto MG plates supplemented with an excess of histidine and biotin. Plates were covered with aluminium foil and incubated upside down at 37 °C for 24 h. All strains should be able to grow on and form colonies on these plates (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

2.2.5.4. Phenotypic *rfa* marker test

The *rfa* marker test was used to confirm the deletion of the *rfa* genes from the mutants (the Ames *Salmonella* strains and derivatives of *E. coli* K-12 strains) alongside their parental strains and as described by Ames *et al.* (1973); Marton and Ames (1983); Mortelmans and Zeiger (2000). The test was performed by transferring 100 µl of overnight culture to a tube containing 3 ml of molten top agar, held at 45 ± 2 °C, and mixed well, and then the contents of the tube were

poured and distributed evenly on to either MG plates supplemented with excess of histidine and biotin or on to nutrient agar plates. Once the overlay had set, a crystal violet disc prepared fresh by adding 10 µl of 0.1% crystal violet solution to a sterile filter paper disc (6mmØ) was placed in the centre of each plate aseptically. Plates were covered with aluminium foil and incubated upside down at 37 °C for 12 h. A zone of growth inhibition around the crystal violet disc was considered a positive result, indicating that the strain has been mutated in the *rfa* gene.

2.2.5.5. Deletion of the *uvrB* gene

Deletion of the *uvrB* gene in the mutants (Ames strains) was tested alongside the parental strain *S.* Typhimurium LT2 which is wild type *uvrB*$^{+}$. The deletion was confirmed by biotin-dependency because the *uvrB* mutation in these mutants stretches across the *bio-uvrB* genes therefore, they are biotin dependant and cannot grow in the abcence of biotin (Marton and Ames, 1983; Mortelmans and Zeiger, 2000). Moreover, the PCR assay, as previously described, was employed to amplify DNA fragments from the *uvrB* and *bioD* genes using the primers which were designed to target these genes (see Table 2.3). Absence of the amplicons, specific for the *uvrB* and *bioD* PCR products, on the gel images were considered positive results (the *bioD* and *uvrB* genes were deleted).

2.2.5.6. Presence of plasmid pKM101 (ampicillin resistance)

The presence of plasmid pKM101 was tested by streaking a loop-full of overnight culture of the Ames strains onto MG plates supplemented with excess of histidine, biotin and 24 μg/ml of ampicillin. Plates were covered with aluminium foil and incubated upside down at 37 °C for 24 h. Strains which were able to grow and form colonies on these plates were considered positive; the strains had maintained the plasmid pKM101 (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

2.2.5.7. Presence of plasmid pAQ1 (tetracycline resistance)

The presence of plasmid pAQ1 was tested by streaking a loop-full of overnight culture of the Ames strains onto MG plates supplemented with an excess of histidine, biotin, 24 μg/ml of ampicillin, and 2 μg/ml of tetracycline. Plates were covered by aluminium foil and incubated upside down at 37 °C for 24 h. Strains which were able to grow and form colonies on these plates were considered positive; the strains had maintained the plasmid pAQ1 (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

2.2.6. Testing the effects of cold atmospheric plasma on strains/mutants

2.2.6.1. Sensitivity test

The sensitivity of different bacterial strains/mutants of *E. coli*, *L. monocytogenes* and *S.* Typhimurium to CAP, were tested using an agar plate approach. Overnight cultures of each strain/mutant were prepared by

transferring a single colony into a 30-ml Universal tube (Sterilin, Ltd) containing 5 ml Luria Bertani (LB) for *E. coli* and *S.* Typhimurium or 5 ml Brain Heart Infusion (BHI) for *L. monocytogenes*. The inoculated tube was incubated at 37 °C with shaking at 200 rpm for 16 -18 h. The optical density of overnight culture was measured at 600 nm. An appropriate volume from the overnight broth culture was transferred into a 30-ml sterile universal tube (Sterilin, Ltd) containing 10 ml of sterile media (LB/BHI) in order to prepare a diluted overnight broth culture of final OD_{600nm} = 0.1 ± 10%). From the diluted overnight broth culture, serial dilutions were made and then from the 10^{-3} dilution, 100 µl of culture was transferred onto LB/BHI agar plates and evenly spread over the surface of the agar. Based on the results of preliminary experiments, this procedure gives average colony counts on the control plates in the range between 500 and 1000 cfu/plate. A minimum of three plates for each treatment were used. Four plates without any treatment were used for the negative control. Other plates were treated with cold atmospheric plasma for different periods of time.

All treatments with CAP were performed by placing the seeded plate with the lid removed under the plasma jet leaving a distance of 10 ± 1 mm between the end of the plasma jet and the surface of the agar, then switching the plasma on and starting counting down the time needed for each treatment. In order to see if the prevention of ionized gas diffusion from the plate into the atmosphere during treatments had an effect on microbial inactivation, a hole with a diameter of about 8 mm was made in the centre of a Petri dish lid which was then used to cover the plates while they were treated. Plasma treatments were carried out using the plasma rig at Sutton Bonington campus, University of

Nottingham (made by Plasma and Pulsed Power Group at Loughborough University). Schematic diagrams for the plasma rig and plasma pen are shown in Figure 2.1.a and 2.1.b. The plasma was generated using a mixture of He and O_2 gases at flow rates of 7 SLM (standard litres per min.) and 35 SCCM (standard cubic centimetres per min.) respectively, with a radiofrequency (RF) = 4.6 MHz, and a peak to peak voltage = 8.6 – 9.6 kV. The temperature of the plasma plume was measured before any experiments using temperature indicator strips (30 to 60 °C, reversible) (OMEGA, UK). It was always ~ 40 °C.

The treated and control plates of *E. coli* and *Salmonella* strains were incubated upside down at 30 °C for 24 h whereas the plates inculcated with *Listeria* were incubated upside down at 37 °C for 24 h. The colonies on the plates were counted using the **a**COLyte colony counter (Synbiosis, USA). The counter allows counting of any zone of any size on the plate. A template plate was divided into five different zones. The zone size needed was pre-adjusted by the software of **a**COLyte colony counter using the template plate as a guide to position the zone at fixed places required then, the temple plate was replaced by the plate to be counted (see Figure 2.2.b). The colonies growing in each zone were counted and survival curves were produced. However, unless the sizes of zones are reported in the results the counts on the whole plate were used to produce the survival curves. The percentage of survivors (survival %) was calculated using the following equation:

Survival % = $N_1 / N_0 \times 100$

Where, N_1 is the average number of colonies grown on the treated plates and N_0 is the average number of colonies grown on the control plates.

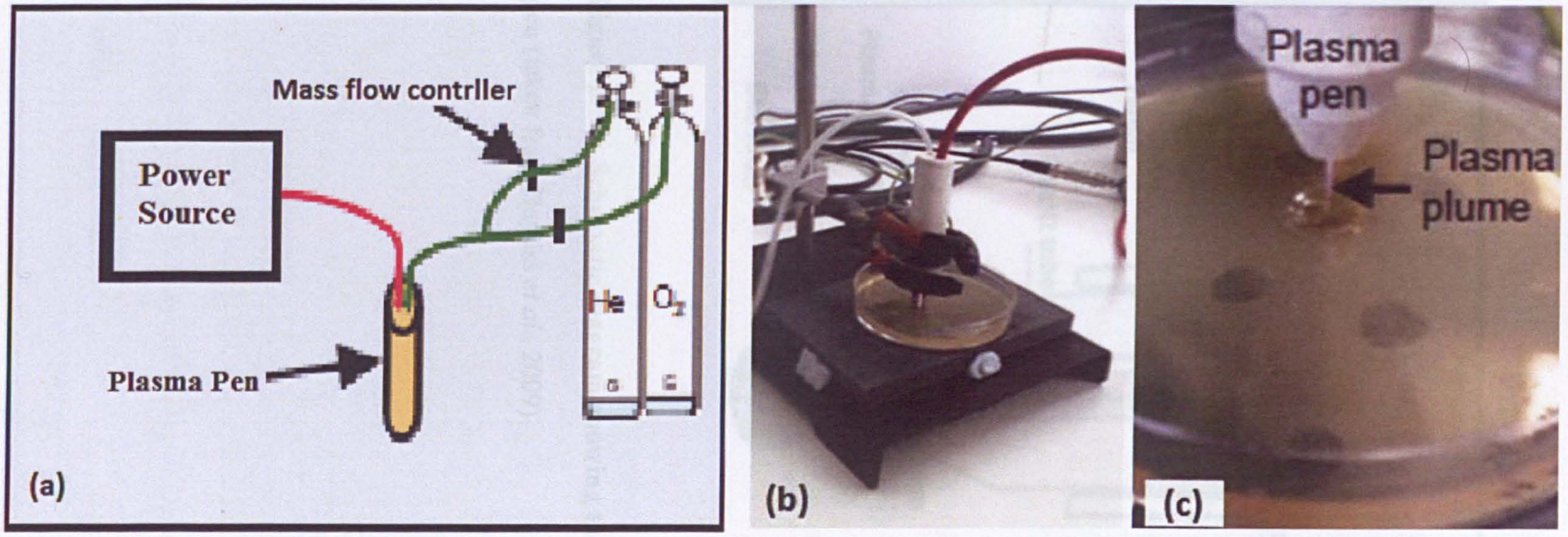

Figure 2.1.a: Outline schematic diagram for the plasma rig (a) and digital images of the plasma pen and agar plate was being treated (b) and (c).

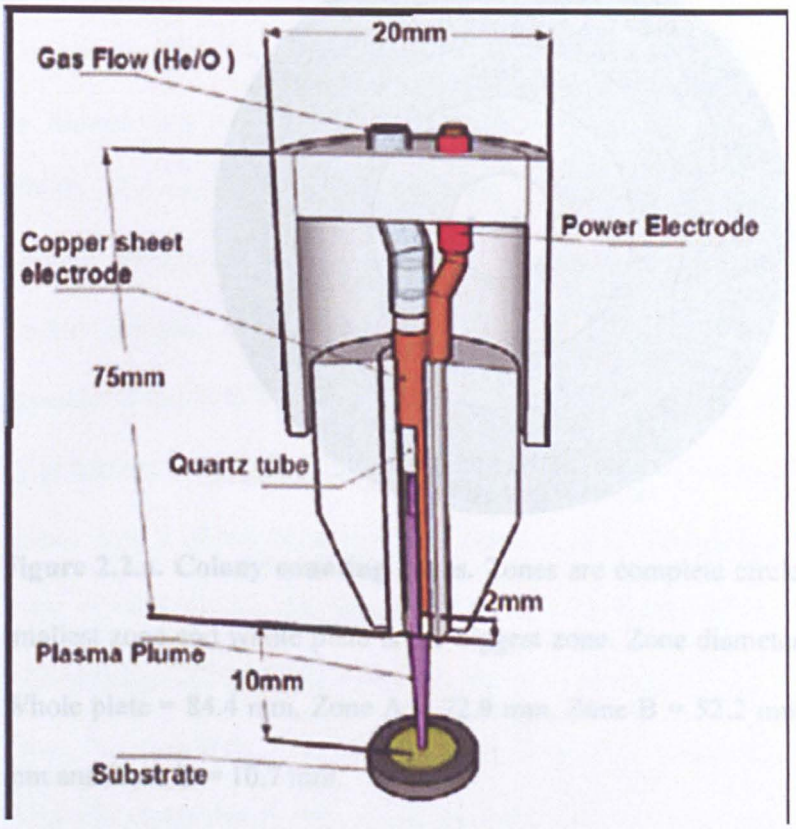

Figure 2.1.b: Schematic diagram showing the main components of the plasma pen (taken from Bayliss *et al.*, 2009)

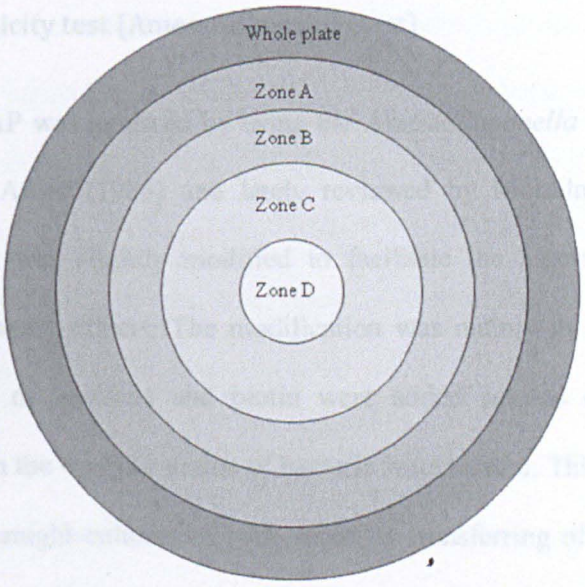

Figure 2.2.a. Colony counting zones. Zones are complete circles. Zone D is the smallest zone and whole plate is the biggest zone. Zone diameters in millimetres: Whole plate = 84.4 mm, Zone A = 72.9 mm, Zone B = 52.2 mm, Zone C = 31.4 mm and Zone D = 10.7 mm.

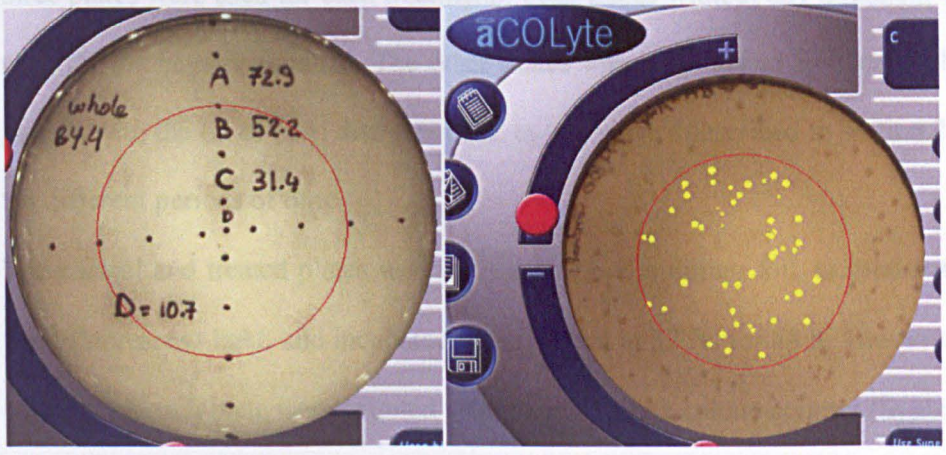

Figure 2.2.b: Image of zone B, template plate (left) and plate being counted (right).

2.2.6.2. Mutagenicity test (Ames/*Salmonella* test)

The safety of CAP was assessed by using the Ames/*Salmonella* test as described by Marton and Ames (1983) and lately reviewed by Mortelmans and Zeiger (2000). The test was slightly modified to facilitate the assessment of CAP's possible mutagenicity effects. The modification was mainly in the way that the limited amounts of histidine and biotin were added as was described in the appendix 2 and in the way the strains of bacteria were treated. This was carried out by preparing overnight cultures of each strain by transferring of a single healthy colony into a 30-ml Universal tube (Sterilin, Ltd) containing 5 ml of Nutrient Broth (Oxoid No. 2). The inoculated tubes were incubated at 37 °C for 16-18 h with shaking (200rpm) until the cells reached a density of ~1.0 to 2.0 $\times 10^8$ cfu/ml. From this overnight culture 100 µl of cells were transferred onto each plate (MGLHB agar) and evenly spread over the surface of the agar. A minimum of three plates were used for each treatment. Four plates without any treatment were used for the negative control. The other plates were exposed to cold atmospheric plasma with the lid of Petri dish removed, as was earlier described in section 2.4.1, for different periods of time.

The control and treated plates were covered with aluminium foil, to protect them from fluorescent light, and incubated upside down at 37 °C for 48 h. The revertant colonies on each plate were counted using the aCOLyte colony counter (Synbiosis, USA) and reversion curves were produced.

A CAP treatment (treatment time) that showed a high revertant rate was retested alongside positive controls (mutagenic chemicals) and negative controls (no

treatment). Sodium azide and Mitomycin C were used as positive controls in this test. Sterile filter paper disc (6 mmØ, Whatman), was placed in the centre of each plate (MGLHB agar), which was seeded with 100 µl of overnight culture as described earlier in this section, then 10 µl of the positive control (mutagenic) solution was transferred to the disc. Plates were incubated and counted as described previously in this section.

2.2.7. Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics 21 software (IBM, 2012). One way analysis of variance (ANOVA) was used to determine whether there are any significant differences between the means of treatments. To find out which treatment was significantly different from other treatments, a Tukey HSD post-hoc test was performed (IBM, 2012).

CHAPTER 3

EFFECT OF COLD ATMOSPHERIC PLASMA ON FOOD-BORNE PATHOGENIC BACTERIA

3. EFFECT OF COLD ATMOSPHERIC PLASMA ON FOOD-BORNE PATHOGENIC BACTERIA

3.1. INTRODUCTION

Food-borne bacteria are one of the major causes of food-borne diseases across the world, for example bacteria are among the top five major causes of food-borne disease in the United States and United Kingdom (CDC, 2011; FSA, 2012). Bacteria are classified into two main groups, Gram positive and Gram negative bacteria, based on their reaction with the Gram stain (Halebian *et al.*, 1981). The reaction depends on the bacterial cell wall constituents (Bartholomew and Mittwer, 1952; Murray *et al.*, 2009). Although these two bacterial groups have similar internal cellular structures they have different external structures (see Figure 3.1). Internally, the cytoplasm of Gram positive and Gram negative bacteria cells contains the chromosome which is a single, double-stranded DNA circle that is contained in a discrete area of the cell called the nucleoid. Cells also contain the mRNA, ribosomes, proteins, and metabolites. Some bacteria also contain small, often circular, extrachromosomal DNAs known as plasmids which are most commonly found in Gram negative bacteria. Many plasmids confer resistance to one or more antibiotics which make them useful markers (Murray *et al.*, 2009).

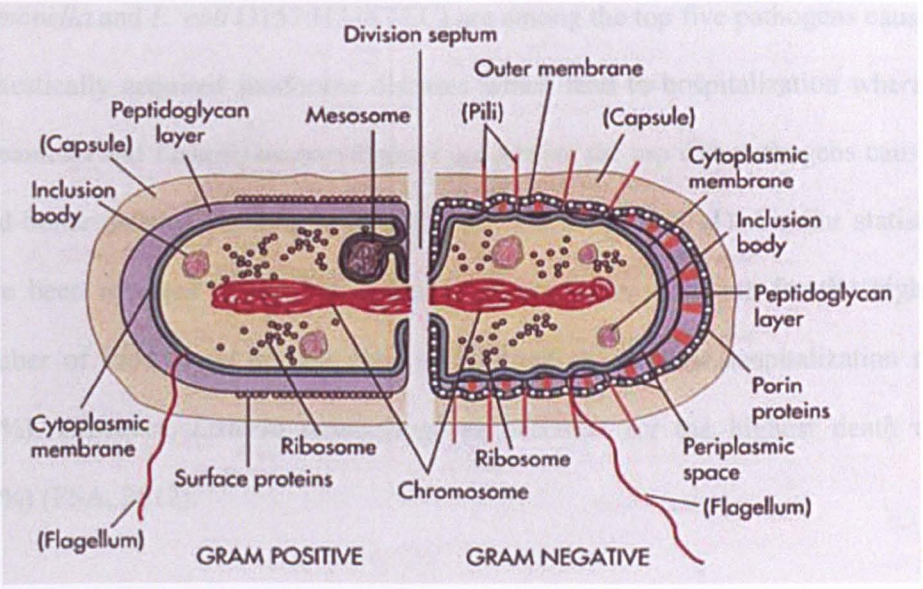

Figure 3.1: Gram positive and Gram negative bacteria. A Gram positive bacterium (left). A Gram negative bacterium (right). Structures in brackets do not exist in all bacteria (Murray *et al*., 2009).

Externally, the Gram positive bacteria have a thick cell wall composed of primarily peptidoglycan (15-50 nm) surrounding the cytoplasmic membrane. The peptidoglycan is a mesh-like exoskeleton which is permeable enough to allow diffusion of metabolites to the plasma membrane. In contrast, Gram negative bacteria have a more complicated cell wall which is composed of two layers external to the cytoplasmic membrane. The first layer is a thin layer of peptidoglycan (2-7 nm) which surrounds the cytoplasmic membrane. The second layer is the outer membrane, which is a distinctive feature of Gram negative bacteria. The space between the cytoplasmic membrane and the internal surface of the outer membrane is referred to as the periplasmic space (Murray *et al*., 2009).

Salmonella and *E. coli* O157:H7 (STEC) are among the top five pathogens causing domestically acquired foodborne diseases which lead to hospitalization whereas, *Salmonella* and *Listeria monocytogenes* are among the top five pathogens causing food-borne disease resulting in death in the US (CDC, 2011). Similar statistics have been reported in the UK, where *Campylobacter* accounts for the highest number of food-borne disease cases (52%) and the highest hospitalization rate (90%). However, *Listeria monocytogenes* accounts for the highest death rate (32%) (FSA, 2012).

Salmonella is a Gram negative, rod-shaped, motile, non-sporeforming, facultative anaerobic bacterium, which belongs to the *Enterobacteriaceae* family (Snowball, 2002; Murray *et al.*, 2009). The genus *Salmonella* is composed of two species which are *S. enterica* and *S. bongori*. Bacteria belonging to either species are pathogenic to humans. *S. enterica* represents more concern to public health and is divided in to six subspecies. One of the subspecies is *S. enterica subsp. enterica* which is composed of numerous serotypes including *S.* Enteritidis and *S.* Typhimurium. *Salmonella* can cause typhoid fever and nontyphoidal salmonellosis diseases. The first disease has a higher mortality rate than the latter (Murray *et al.*, 2009; Hammack, 2012).

Escherichia coli is a Gram negative, mostly motile, rod-shaped, non-sporeforming facultative anaerobe, which belongs to the family *Enterobacteriaceae*. *E. coli* is widespread in human colonic flora, and usually enters and colonizes the gastrointestinal tract of infants within hours of their birth. Some species are

beneficial to their hosts such as the commensal *E. coli* HS (Nataro and Kaper, 1998; Murray *et al.*, 2009). However, six *E. coli* pathotype groups are recognized as disease causing in humans: these include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohamorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). The first four groups are widely known as causative agents of food-borne or water-borne disease, especially the enterohamorrhagic *E. coli* (EHEC) group (Murray *et al.*, 2009; Feng, 2012).

Listeria monocytogenes is a Gram positive, motile (by means of flagella), rod-shaped, facultative bacterium. *L. monocytogenes* can cause listeriosis, a serious disease in humans and animals. The disease has high mortality in immunocompromised individuals, the elderly, pregnant women, unborn children, and neonates (Murray *et al.*, 2009; Yan *et al.*, 2010). *L. monocytogenes* has the ability to grow at a wide range of temperatures (1 - 45 °C), can tolerate osmotic stress, and can endure mild preservation treatments. These characteristics make this bacterium very difficult to eliminate from the food chain (Karatzas and Bennik, 2002; Chen, 2012).

The aims of this chapter were to investigate whether the difference in the external structure between the Gram positive and Gram negative bacteria had an effect on the sensitivity of the strains to cold atmospheric plasma treatment. It also aimed to find out whether the susceptibility of food-borne pathogenic bacteria to CAP treatments is similar to the non pathogenic laboratory strains. Therefore, different

strains of *E. coli* and *Salmonella* Typhimurium LT2 strains representing Gram negative bacteria, and different strains of *Listeria monocytogens* representing Gram positive bacteria (see Table 2.2) were used.

3.2. RESULTS

3.2.1. Confirmation of the *E. coli* strains/mutants' identities

Multiplex PCR assays were performed as described earlier in section 2.2.4 in order to confirm the identities of all *E. coli* strains used in this study. Two sets of primers (see Table 2.3) which were specifically designed to target the *usp*A gene of the *E. coli* strains (Chen and Griffiths, 1998) and the *eae*A gene of *E. coli* O157: H7 (China *et al.*, 1996) were used. The agarose gels showing the PCR products amplified from the *uspA* multiplex PCR assay is presented in Fig. 3.2.a and 3.2.b. The agarose gels show that PCR products of 884 bp were amplified from the *uspA* gene of all *E. coli* strains/mutants used in this study and a PCR product of 570 bp was amplified only from the *eaeA* gene of the *E. coli* O157: H7 strain. In contrast, no PCR products were obtained from *S.* Typhimurium LT2 and the negative control (PCR mixture with no added template DNA).

These results confirm that all *E. coli* strains/mutants used in this study belonged to the species *Escherichia coli* (Chen and Griffiths, 1998). It also confirms that the *E. coli* O157: H7 strain used in this study possessed the characteristic *eaeA* gene of EPEC/EHEC strains (China *et al.*, 1996).

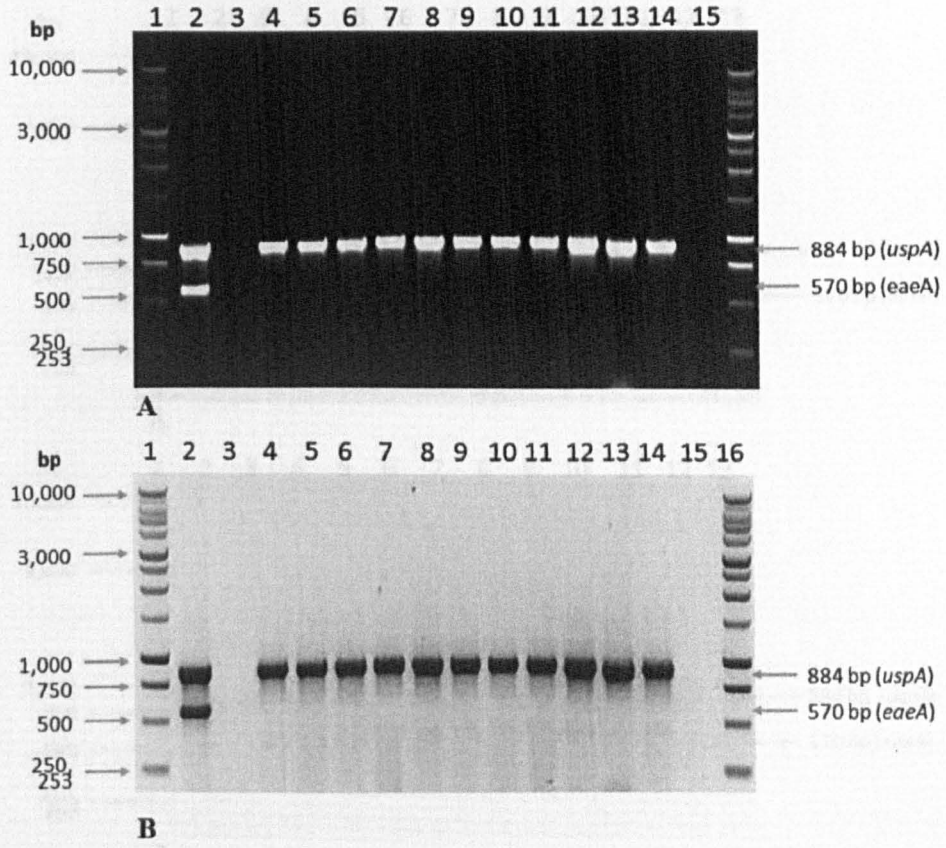

Figure 3.2.a: Agarose gel showing PCR products amplified from *E. coli* strains using primers specific for the *usp*A genes. (B = inverted image of A). Lane1, 1kb DNA ladder; lane 2, *E. coli* O157:H7; lane 3, *S.* Typhimurium LT2; lane 4, *E. coli* BW25113 (Δ*dps*); lane5, *E. coli* BW25113 (Δ*flu*); lane 6, *E. coli* BW25113 (Δ*fur*); lane7, *E. coli* BW25113 (Δ*grx*A); lane 8, *E. coli* BW25113 (Δ*kat*G); lane 9, *E. coli* BW25113 (Δ*suf*B); lane 10, *E. coli* BW25113 (Δ*sufC*); lane 11, *E. coli* BW25113 (Δ*sufD*); lane 12, *E. coli* W3110 (Δ*rfa*C); lane 13, *E. coli* BW25113 (Δ*rfa*E); lane 14, *E. coli* BW25113 (Δ*lpc*A); lane 15, negative control (PCR mixture with no added template DNA); lane 16, 1kb DNA ladder. The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 90 minutes.

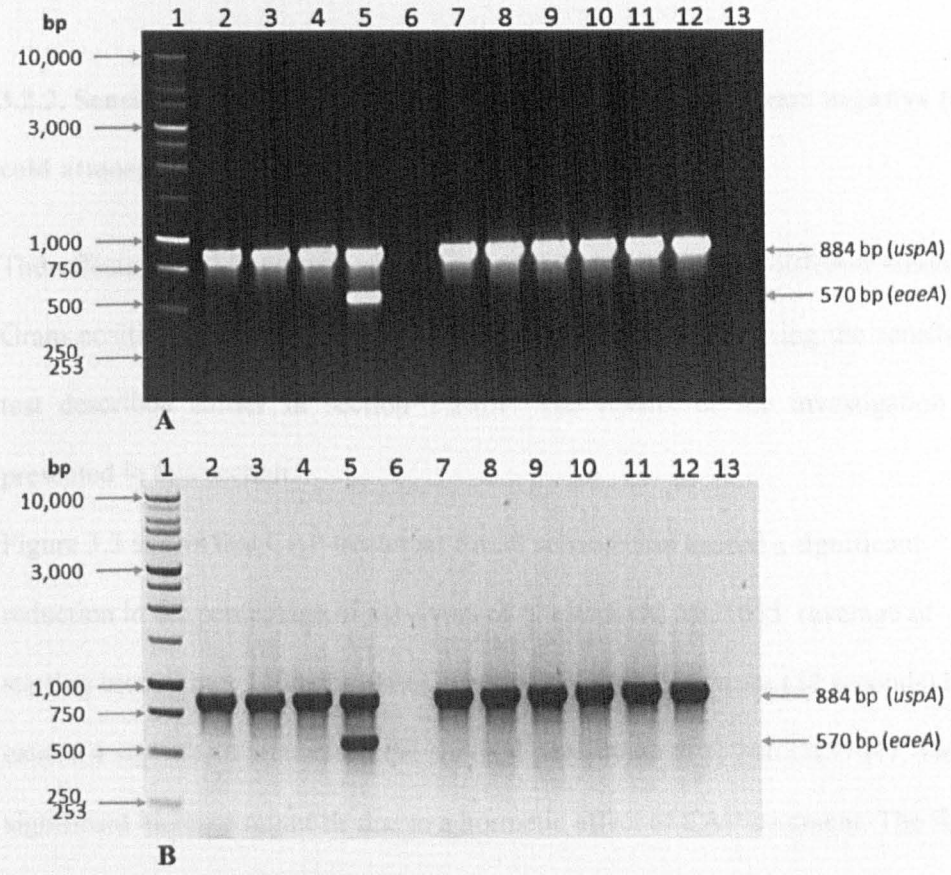

Figure 3.2.b: Agarose gel showing PCR products amplified from *E. coli* strains using primers specific for the *usp*A and *eae*A genes. PCR roduct (B = inverted image of A). Lane 1, 1kb DNA ladder; lane 2, *E. coli* BW25113; lane 3, *E. coli* MG1655; lane 4, *E. coli* CFTO73 (UPEC); lane 5, *E. coli* O157: H7(ETEC); lane 6, *S.* Typhimurium LT2; lane 7, *E. coli* W3110; lane 8, *E. coli* H10407 (ETEC) ; lane 9, *E. coli* HS (Commensal); lane 10, *E. coli* MG1655 (Δ*oxy*R); lane 11, *E. coli* DFB1655 L5; lane 12, *E. coli* DFB1655 L9; lane 13, negative control (PCR mixture with no added template DNA. The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 90 minutes.

3.2.2. Sensitivity of different strains of Gram positive and Gram negative to cold atmospheric plasma

The effects of cold atmospheric plasma (CAP) treatments on different strains of Gram positive and Gram negative bacteria were investigated using the sensitivity test described earlier in section 2.2.6.1. The results of the investigation are presented in this section.

Figure 3.3 shows that CAP treatment for 30 seconds has caused a significant reduction in the percentage of survivors of *E. coli* K-12 MG1655 (average of starting inoculum = 1300 cfu/plate), whereas the same treatment (30 seconds) has caused a significant increase in the survival percentage of *E. coli* O157:H7. This significant increase might be due to a hormetic effect of CAP treatment. The figure also shows that CAP treatment up to 1 min has not significantly affected the percentage of survivors of *E. coli* O157:H7 and the reduction were only significant when 2 minutes of exposure to CAP treatment were applied. This indicates that *E. coli* K-12 MG1655 is more susceptible to CAP treatment than *E. coli* O157: H7.

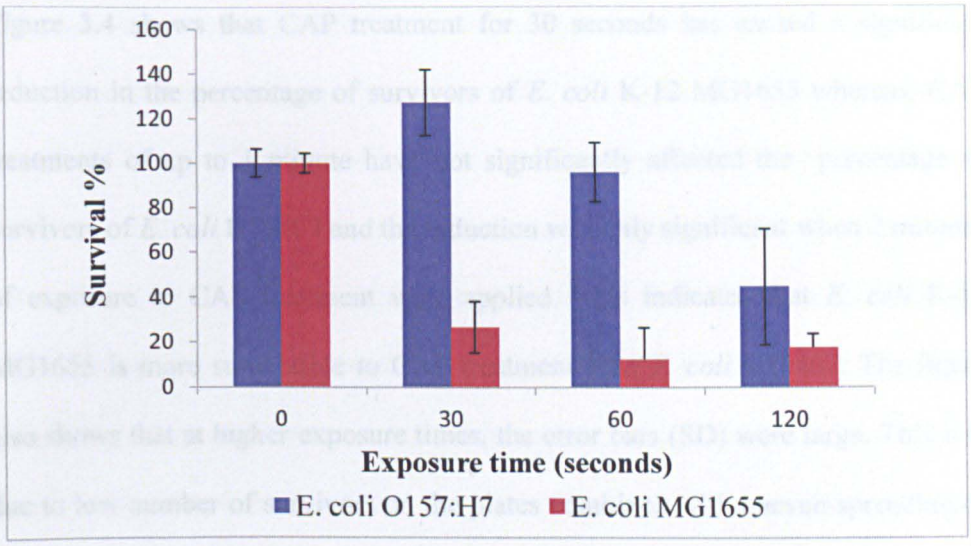

Figure 3.3: Effect of different exposure time to CAP treatment on survival of *E. coli* strains (*E. coli* O157:H7 and *E. coli* MG1655). (Mean survival % ± 1 SD, n = 3 replicates).

Figure 3.4 shows that CAP treatment for 30 seconds has caused a significant reduction in the percentage of survivors of *E. coli* K-12 MG1655 whereas, CAP treatments of up to 1 minute have not significantly affected the percentage of survivors of *E. coli* H10407 and the reduction was only significant when 2 minutes of exposure to CAP treatment were applied. This indicates that *E. coli* K-12 MG1655 is more susceptible to CAP treatment than *E. coli* H10407. The figure also shows that at higher exposure times, the error bars (SD) were large. This was due to low number of survivors on the plates combined with uneven spreading of colonies on the plates.

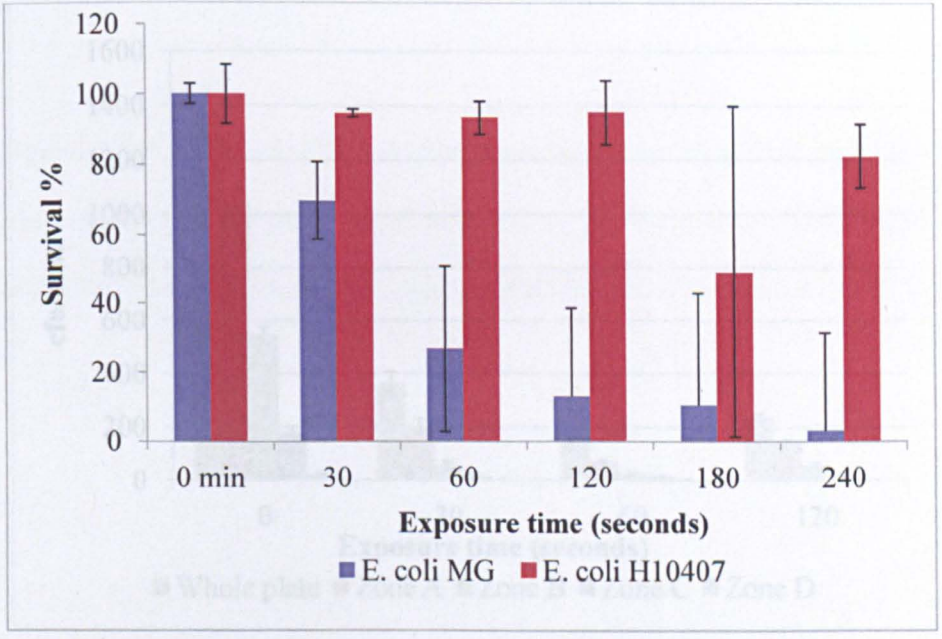

Figure 3.4: Effect of different exposure time to CAP treatment on survival of *E. coli* strains (*E. coli* H10407 and *E. coli* MG1655). (Mean survival % ± 1 SD, n = 3 replicates).

Although it was clearly observed in preliminary experiments that the zone of bacterial inactivation extends beyond the point of contact with the plasma plume, the rate of inactivation did not increase exponentially with time. The hypothesis tested was that this effect was seen because the diffusion of plasma species was not sufficient to cover the surface area of the whole plate. Therefore, colonies in different concentric zones surrounding the point of the plasma plume contact on the plate were counted as was described in section 2.2.6.1. Figures 3.5.a and 3.5.b show that the rate of inactivation of *E. coli* K-12 MG1655 has a similar trend when the colony counts of different zones were used.

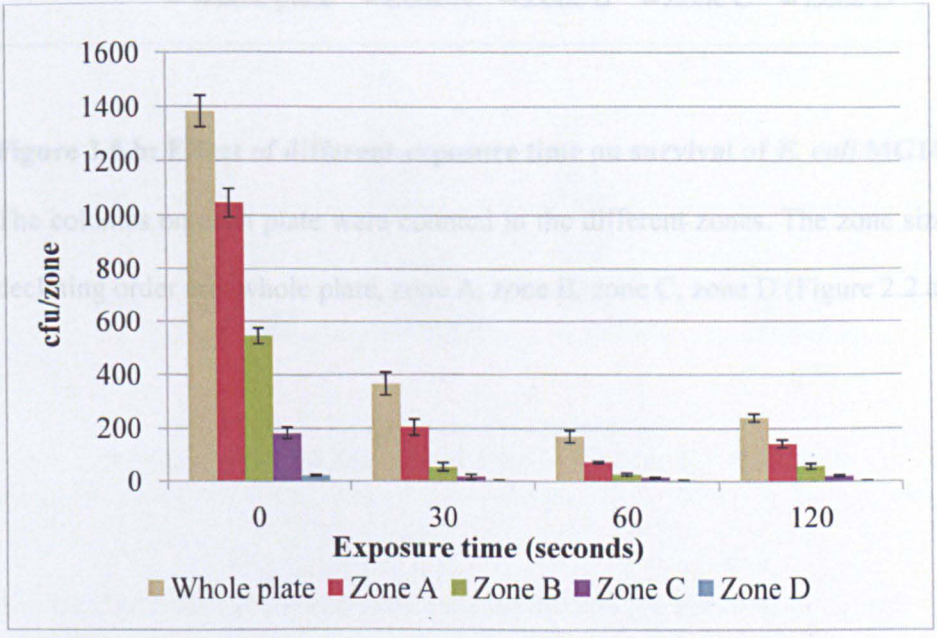

Figure 3.5.a: Effect of different exposure times on survival of *E. coli* K-12 MG1655. (Mean survival % ± 1 SD, n = 3 replicates). The colonies on each plate were counted in different zones. The zone sizes in a declining order are: whole plate, zone A, zone B, zone C, zone D (Figure 2.2.a).

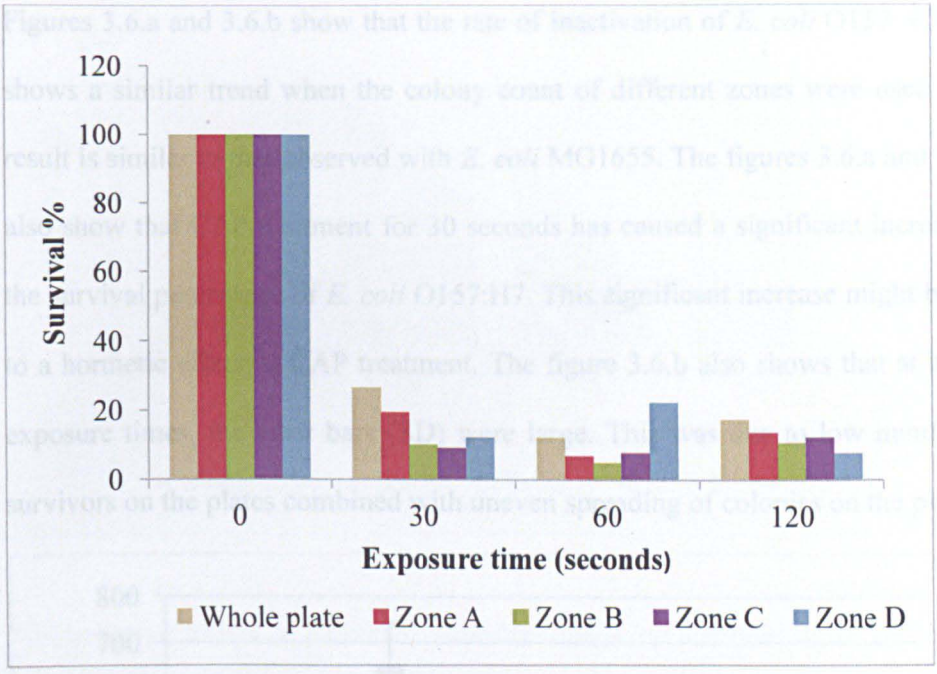

Figure 3.5.b: Effect of different exposure time on survival of *E. coli* MG1655).

The colonies on each plate were counted in the different zones. The zone sizes in declining order are: whole plate, zone A, zone B, zone C, zone D (Figure 2.2.a).

Figures 3.6.a and 3.6.b show that the rate of inactivation of *E. coli* O157: H7 also shows a similar trend when the colony count of different zones were used. This result is similar to that observed with *E. coli* MG1655. The figures 3.6.a and 3.6.b. also show that CAP treatment for 30 seconds has caused a significant increase in the survival percentage of *E. coli* O157:H7. This significant increase might be due to a hormetic effect of CAP treatment. The figure 3.6.b also shows that at higher exposure times, the error bars (SD) were large. This was due to low number of survivors on the plates combined with uneven spreading of colonies on the plates.

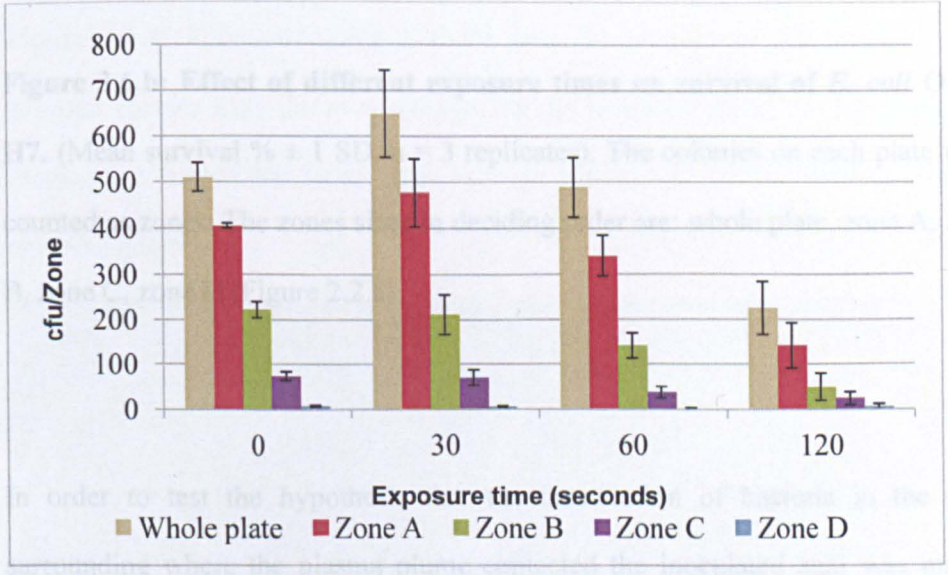

Figure 3.6.a: Effect of different exposure times on survival of *E. coli* O157: H7. (Mean survival % ± 1 SD, n = 3 replicates). The colonies on each plate were counted as zones. The zones sizes in deciding order are: whole plate, zone A, zone B, zone C, zone D (Figure 2.2.a).

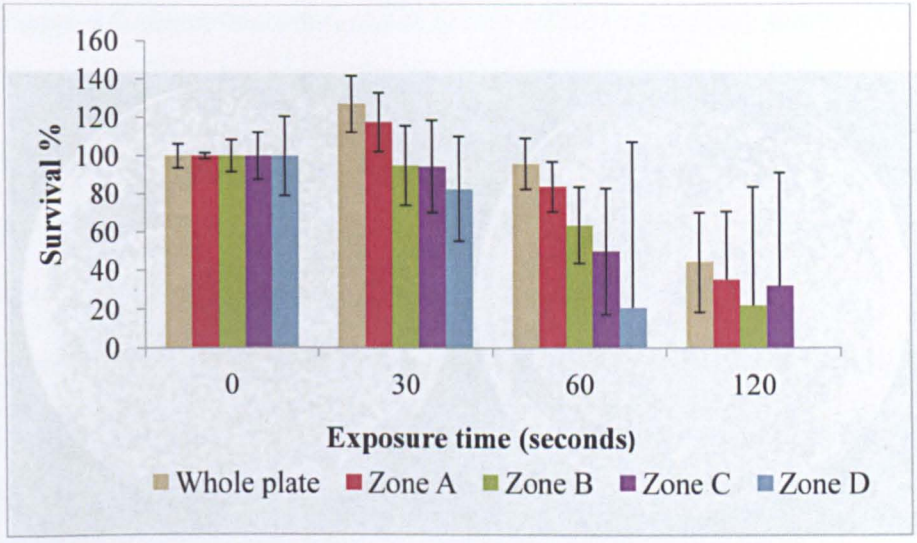

Figure 3.6.b: Effect of different exposure times on survival of *E. coli* O157: H7. (Mean survival % ± 1 SD, n = 3 replicates). The colonies on each plate were counted as zones. The zones sizes in deciding order are: whole plate, zone A, zone B, zone C, zone D (Figure 2.2.a).

In order to test the hypothesis that the inactivation of bacteria in the zone surrounding where the plasma plume contacted the inoculated agar was mainly due to gaseous plasma species, a hole was made in the Petri dish lid as described earlier in 2.2.6.1. The plasma plume was directed to pass through the hole. Figure 3.7.a shows the inactivation zones of *E.coli* K-12 MG1655 exposed to CAP for only 30 seconds and Figure 3.7.b show the inactivation zones of *E. coli* O157: H7 exposed to CAP for 2 minutes. The zone size on plates which was treated through the hole in the lid was larger than that on plates treated with lid removed. This suggests that ionized gas has an important role in the inactivation mechanism.

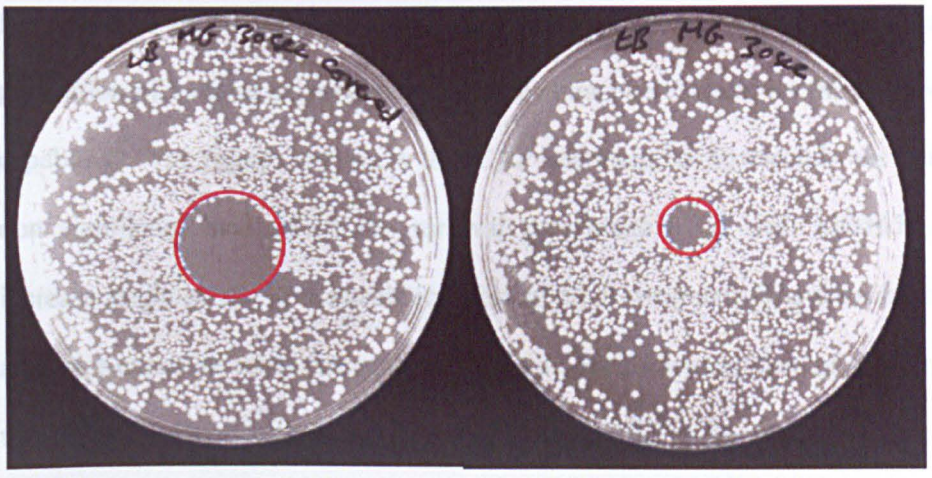

Figure 3.7.a: Effect of using a Petri dish lid to prevent diffusion of CAP gaseous species into the atmosphere, on the inactivation of *E. coli* MG1655. Plates were treated for 30 seconds, through the hole in the lid (left) or with lid removed (right).

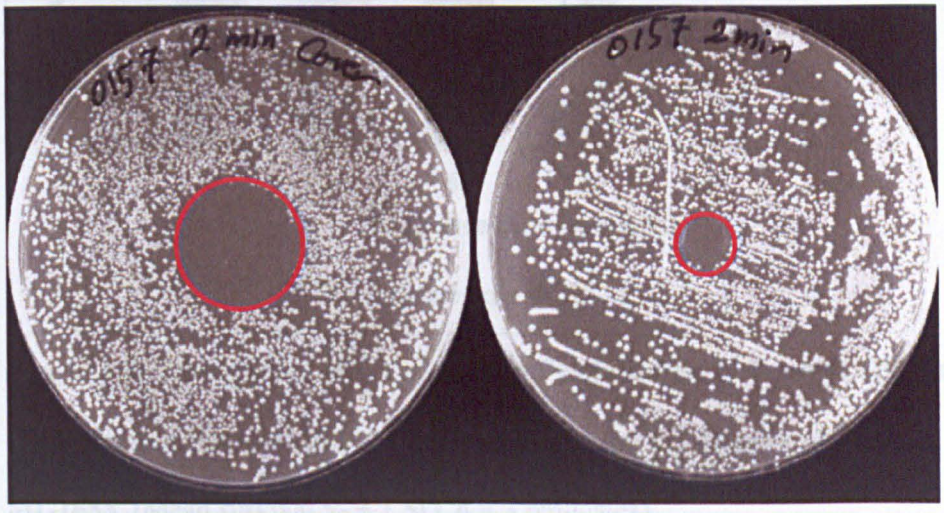

Figure 3.7.b: Effect of using a Petri dish lid to prevent diffusion of CAP gaseous species into the atmosphere, on the inactivation of *E. coli* o157: H7. Plates were treated for 2 minutes, through the hole in the lid (left) or with lid removed (right).

Figure 3.8 shows that treatment of *E. coli* MG1655 with CAP through the hole in the Petri dish lid enhanced the inactivation rate slightly. This also indicates that the prevention of ionized gas diffusing into the atmosphere improves the inactivation capability. . The figures also show that CAP treatment for 30 seconds has caused a non significant increase in the survival percentage of *E. coli* MG1655. This increase might be due to a hormetic effect of CAP treatment. The error bars (SD) at higher exposure times were large. This was due to low number of survivors on the plates combined with uneven spreading of colonies on the plates.

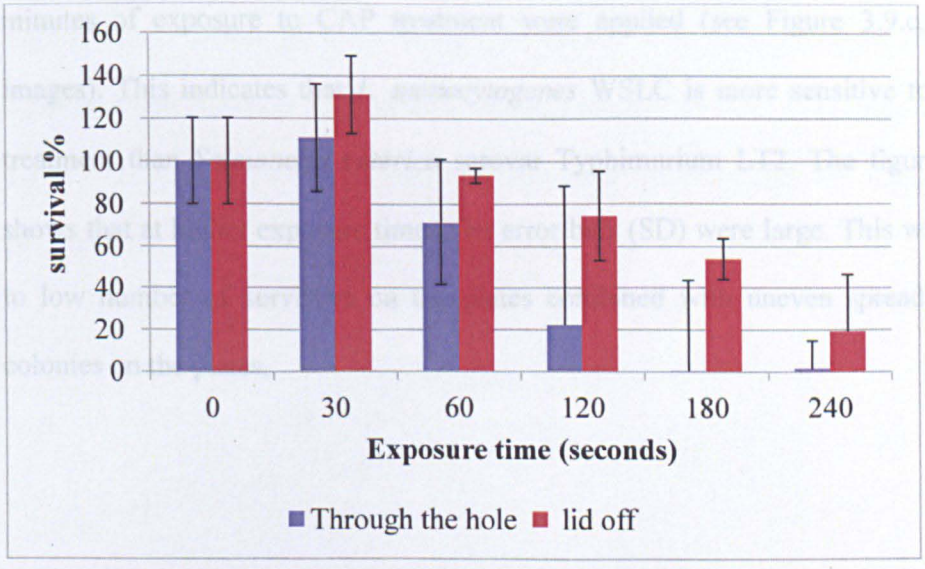

Figure 3.8: Time course effect of using a Petri dish lid to prevent diffusion of CAP gaseous species into the atmosphere, on the inactivation of *E. coli* MG1655. (Mean survival % ± 1 SD, n = 3 replicates).

To investigate whether the difference in cell wall composition between Gram positive and Gram negative bacteria has an effect on the susceptibility of the bacteria to inactivation by CAP, the sensitivity of different strains of Gram positive and Gram negative bacteria to CAP treatment was tested as described in section 2.2.6.1. Figure 3.9.a shows that CAP treatment for 30 seconds has caused a significant reduction in the percentage of survivors of *L. monocytogenes* WSLC 1042 (4b) (see Figure 3.9.b, plate images) whereas CAP treatments up to 1 minute did not cause a significant reduction in the percentage of survivors of *Salmonella enterica* serovar Typhimurium LT2. The reduction was significant only when 2 minutes of exposure to CAP treatment were applied (see Figure 3.9.c, plate images). This indicates that *L. monocytogenes* WSLC is more sensitive to CAP treatment than *Salmonella enterica* serovar Typhimurium LT2. The figure also shows that at higher exposure times, the error bars (SD) were large. This was due to low number of survivors on the plates combined with uneven spreading of colonies on the plates.

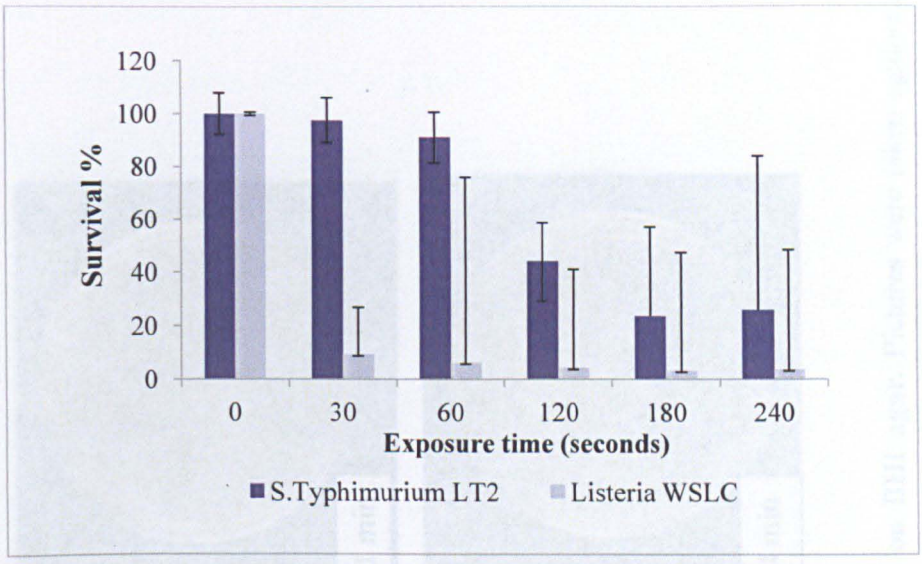

Figure 3.9.a: Effect of CAP treatment on survival of *Salmonella enterica* serovar Typhimurium LT2 and *L. monocytogenes* WSLC 1042 (4b). (Mean survival % ± 1 SD, n = 3 replicates). Only positive error bars for *Listeria* are presented here.

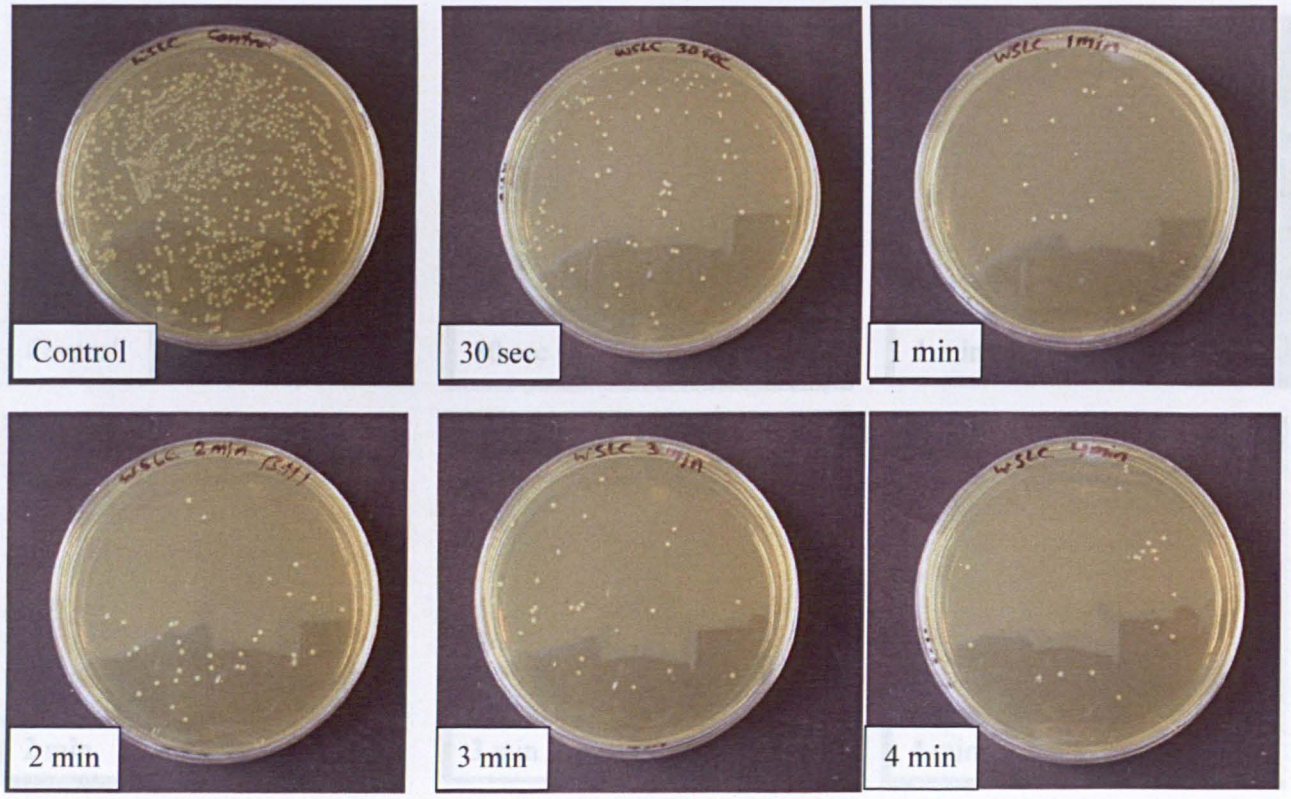

Figure 3.9.b: Effect of CAP treatments on *L. monocytogenes* WSLC 1042 (4b) grown on BHI agar. Pictures were taken against a black background. (The dark colour which appears on the images of the plates is a camera reflection).

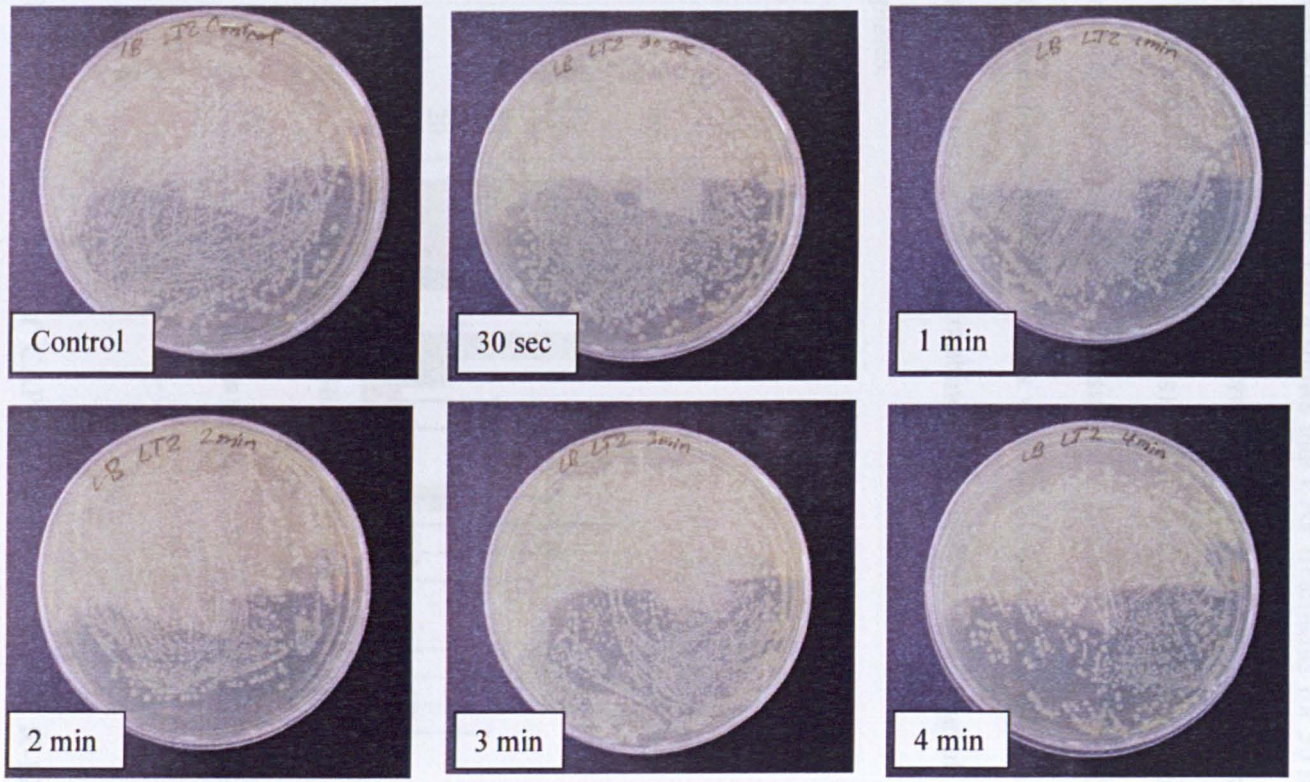

Figure 3.9.c: Effect of CAP treatments on *Salmonella enterica* serovar Typhimurium LT2 grown on LB agar. Pictures were taken against a black background. (The dark colour which appears on the images of the plates is camera reflection).

The effects of CAP treatments on different strains of *L. monocytogenes* (*L. monocytogenes* ATCC 23074 (4b), *L. monocytogenes* EGD-e (1/2a) and *L. monocytogenes* 00054-0305 (1/2b)) and *E. coli* K-12 MG1655 were investigated. Figure 3.10.a shows that CAP treatments for 30 seconds caused a significant reduction in the percentage of survivors of all strains (see Figure 10.b and 10.c, plate images). This indicates that sensitivity of all *L. monocytogenes* strains used is similar to that of *E. coli* MG1655. The error bars (SD) at higher exposure times were large. This was due to a low number of survivors on the plates combined with uneven spreading of colonies on the plates.

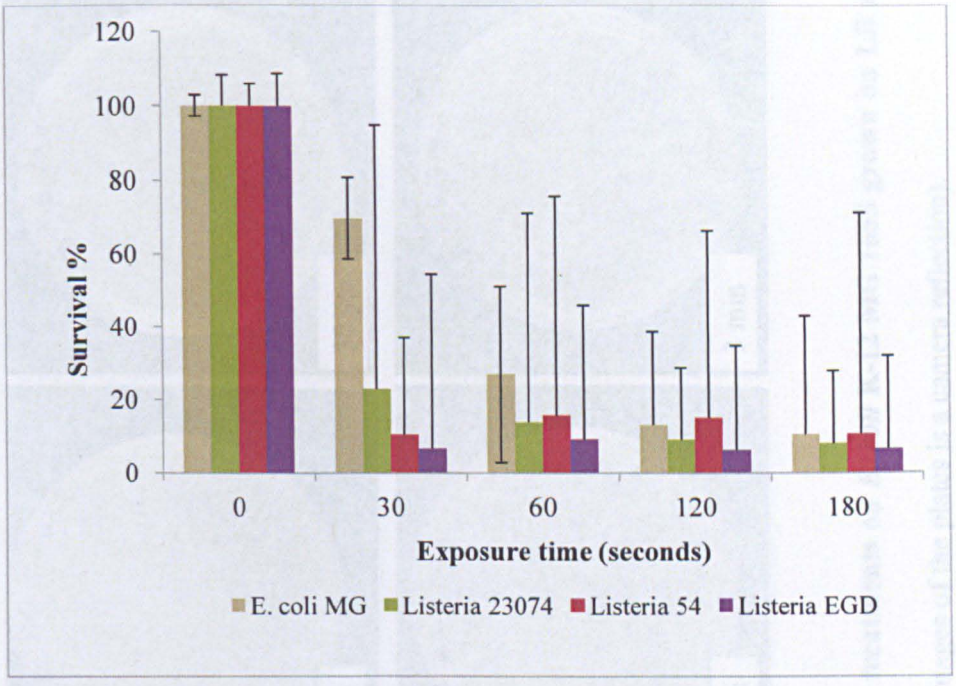

Figure 3.10.a: Effect of CAP treatment on *E. coli* K-12 MG1655 and different strains of *Listeria monocytogenes* (*L. monocytogenes* ATCC 23074 (4b), *L. monocytogenes* EGD-e (1/2a) and *L. monocytogenes* 00054-0305 (1/2b)). (Mean survival % ± 1 SD, n = 3 replicates).

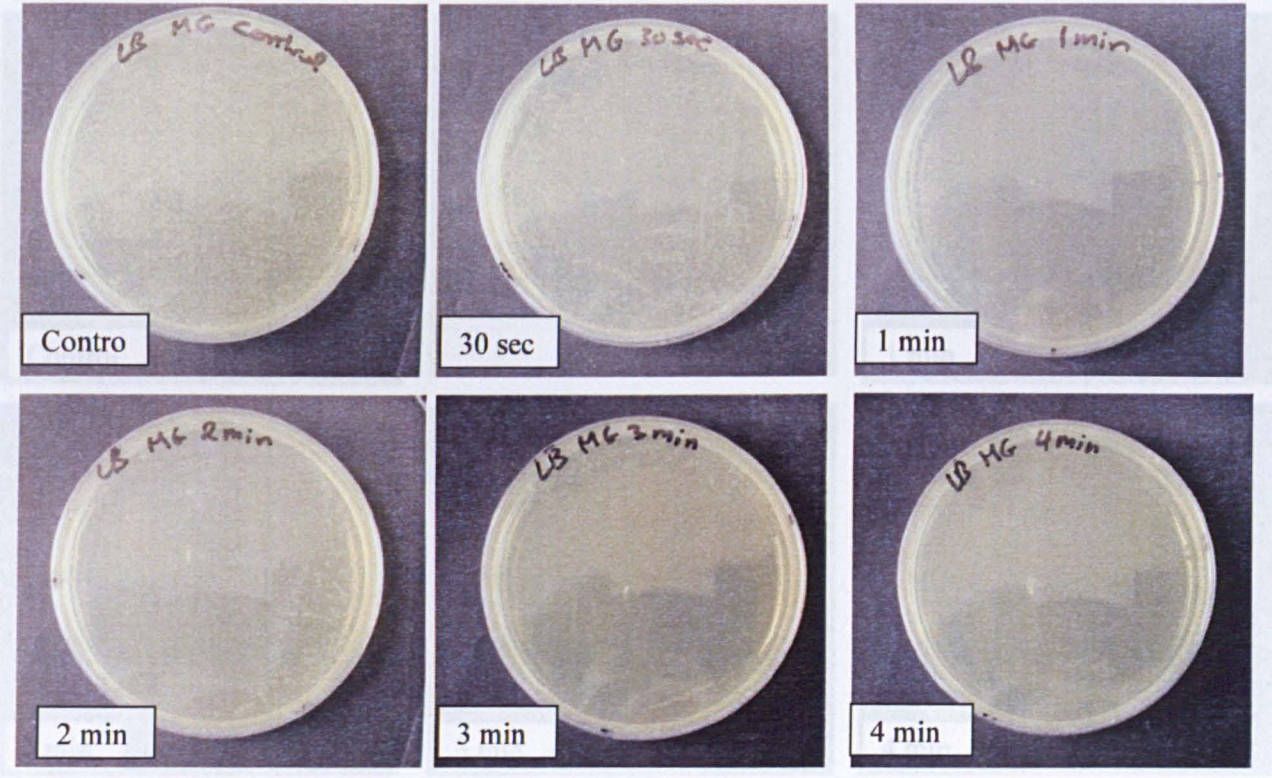

Figure 3.10.b: Effect of CAP treatments on *E. coli* K-12 MG 1655 grown on LB agar. Pictures were taken against black background. (The dark colour which appears on images of the plates is a camera reflection).

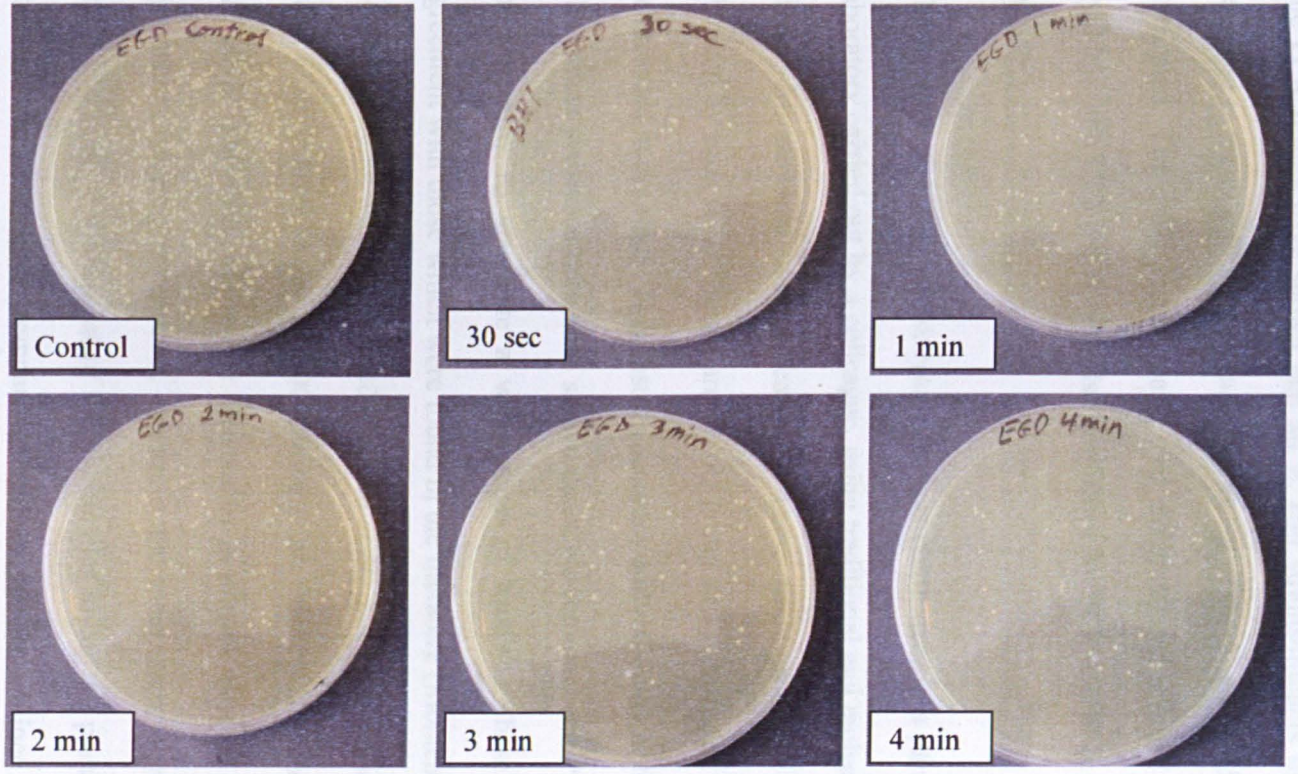

Figure 3.10.c: Effect of CAP treatments on *L. monocytogenes* EGD-e grown on BHI agar. Pictures were taken against black background. (The dark colour which appears on images of plates is a camera reflection).

3.3. DISCUSSION

The PCR results have shown that all *E. coli* strains/mutants used in this study belonged to the *E. coli* species and the identity of the *E. coli* O157: H7 strain was confirmed. The PCR results have also differentiated *E. coli* strains from *S.* Typhimurium LT2 which was also confirmed by PCR assays in chapter 6 of this thesis.

The identity of *Listeria monocytogenes* was confirmed, in a parallel study in the laboratory carried out by a colleague, using traditional and molecular methods (El Emam, M. personal communication). These findings ensure that the results obtained by each strain/mutant are for the group and species that were intended to be included in the study. This ensures accuracy and reliability of the results.

The results presented here have shown that Cold Atmospheric Plasma (CAP) has the capability to inactivate a wide range of bacteria. These findings are in agreement with those which were found in the literature (Montenegro *et al.*, 2002; Lee *et al.*, 2006; Morris *et al.*, 2007; Perni *et al.*, 2007; Niemira and Sites, 2008; Perni *et al.*, 2008b; Ma *et al.*, 2008; Song *et al.*, 2009; Yun *et al.*, 2010; Wang *et al.*; 2012). However, due to the differences in plasma systems and in treatment approaches (directly on to fruit, vegetable and meat slices or membrane filters, static exposure or movement of the membranes under the plume) used in these studies, it was difficult to compare between the results obtained in this study and other studies in terms of inactivation rates. The results obtained in this study with *E.* coli K-12 MG1655 when compared with that obtained with *E. coli* O157: H7

and *E. coli* H10407 indicate that *E. coli* K-12 was found to be more susceptible to CAP treatments. This susceptibility was expected to be as a result of differences in lipopolysaccharide (LPS) structure between *E. coli* K-12 MG1655 and *E. coli* O157: H7 and *E. coli* H10407 as it is well known that *E. coli* K-12 MG1655 strain lacks the O-antigen, the outer part of its LPS structure (Klena, *et al.*, 1992; Liu and Reeves, 1994; Hobman *et al.*, 2007; Browning *et al.*, 2013). Once the results obtained with *S.* Typhimurium LT2 was compared with that of *L. monocytogenes* WSLC 1042, the latter was found to be more sensitive to CAP treatments. Since *L. monocytogenes* WSLC 1042 is Gram positive and *S.* Typhimurium LT2 is Gram negative (Murray *et al.*, 2009), the lower susceptibility of *S.* Typhimurium may be related to the effect of the LPS structure, as *L. monocytogenes* WSLC 1042 has no LPS structure which is a unique characteristic of Gram negative bacteria (Caroff and Karibian, 2003; Murray *et al.*, 2009) and was found to be very important in making the cell wall of this group impermeable to antibiotics such as novobiocin, spiramycin, and actinomycin D (Tamaki *et al.*, 1971) and to hydrophobic dyes, detergents, fatty acids, phenols, and polycyclic hydrocarbons (Raetz and Whitfield, 2002). The LPS and enterobacterial common antigen (ECA) in STEC O157:H7 and *Salmonella* were also found to be essential to the resistance of these strains to acetic acid and other short chain fatty acids (Barua *et al.*, 2002). This finding and that obtained when *E. coli* K-12 MG1655 was compared with *E. coli* O157: H7 and *E. coli* H10407 (ETEC) led to further investigation into the role of LPS in the resistance to CAP treatments. Therefore, the sensitivity of *E. coli* K-12 MG1655 which is Gram negative and lacking the O-antigen structure was compared with the sensitivity of different Gram positive strains of *L.*

monocytogenes (*L. monocytogenes* ATCC 23074 (4b), *L. monocytogenes* EGD-e (1/2a) and *L. monocytogenes* 00054-0305 (1/2b)). The results obtained did not show any significant differences between the sensitivity of the groups. In view of the results obtained using *E. coli* strains (with and without complete O-antigens) and using *S.* Typhimurium LT2 and *L. monocytogenes* WSLC 1042, this finding suggests that the O-antigen region of LPS structure may play an important role in the resistance of Gram negative bacteria that have complete O-antigen to CAP while the outer core and inner core regions of LPS may have no role. To test these hypotheses further experiments were suggested in order to investigate the role of LPS structure in bacterial resistance to CAP treatments. These experiments will be described in the next chapter, the role of lipopolysaccharides in bacterial resistance to cold atmospheric plasma.

In most of the studies on bacterial inactivation by CAP, the bacterial cells were treated either by placing them on polycarbonate membrane filters or on slices of fruits, vegetable or meat, with the samples in a static or moving state such as in those carried out by Perni *et al.*, 2007; Perni *et al.*, 2008b; Ma *et al.*, 2008; Song *et al.*, 2009; Yun *et al.*, 2010; Wang *et al.*; 2012. This approach has the advantages of high starting cell counts, high inactivation rates and fewer variations between the replicates. However, it does not allow differentiation between the direct and indirect effects of CAP therefore, in this study the treatment of bacteria with CAP were carried out using an agar plate approach in which bacteria were plated onto agar plates then the plates were placed in a static position under the plasma pen. The plume was pointed towards the middle of plate and single treatment for each

exposure time was given, i.e no treatment was divided in to two or more exposure times. This approach was chosen to investigate the peripheral effect of CAP treatments. Inactivation zones much larger than the zones that corresponded with the point of contact with the plume were observed at the beginning of this study. This observation was attributed to the diffusion of plasma species. To confirm this observation, the experiment in which bacteriae cells were treated with CAP through the hole in a Petri dish was conducted. The result of this experiment showed that the inactivation zones on plates treated using this approach were as much as twice as big as those on plates treated with the lid being removed. Excluding the effect of temperature, as the plume temperature was always ~ 40 ^{o}C, and UV effects as it was found has no effect under similar conditions (Laroussi and Leipold, 2004; Deng *et al.*, 2006; Ross *et al.*, 2006; Perni *et al.*, 2007), then the result indicates that the inactivation of bacteria outside the direct contact area of the plume was mainly due to reactive species diffusion over the agar surfaces. The reactive species might have inactivated the cells through oxidative stress. To further explore the role of oxidative stress in the inactivation of bacteria, the sensitivity of *E. coli* strains with deletions in one of the genes involved in oxidative stress resistance, to CAP treatment were tested as will be described in chapter 5 of this thesis. In addition to that the Ames *Salmonella* test which can detect agents that cause DNA alteration specifically through oxidative stress will be addressed in chapter 6.

The agar plate approach used in this study has the disadvantage that rate of inactivation did not increase exponentially with time. The hypothesis which was

tested is whether this effect was seen because of the diffusion of plasma species which was not sufficient to cover the surface area of the whole plate. For that reason, colonies in different concentric zones surrounding the point of plasma plume contact on the plate were counted. The results showed that the inactivation rates of *E. coli* K-12 MG1655 and *E. coli* O157: H7 have a similar trend when the colony counts from different zones were compared. Therefore, in the next chapters, the colony counts on the whole plate were used for three reasons which are:

1) They have a better capability to test the sensitivity of strains, as the more sensitive cells will be inactivated more easily even when it is far from plume.

2) There are fewer variations between replicates than other zones.

3) They can be applied to test the effects of CAP on Ames strains.

CHAPTER 4

THE ROLE OF LIPOPOLYSACCHARIDES IN BACTERIAL RESISTANCE TO COLD ATMOSPHERIC PLASMA

4. THE ROLE OF LIPOPOLYSACCHARIDES IN BACTERIAL RESISTANCE TO COLD ATMOSPHERIC PLASMA

4.1. INTRODUCTION

The outer membrane (OM) of Gram negative bacteria is composed of two kinds of lipids, phospholipids and lipopolysaccharide (LPS) (Nikaido, 1996). LPS consists of three regions: 1) the interior region, composed of hydrophobic lipid A, which is a polar lipid and contains six or seven saturated fatty acids; 2) the middle region, composed of core polysaccharides, which links lipid A with the O-antigen and 3) the exterior region, the O antigen, which is a polysaccharide structure made of repeating units of oligosaccharide usually 3-6 sugars (O units; usually repeated 10-30-fold) as shown in Figure 4.1 (Reeves, *et al.*, 1996; Nikaido, 1996; Raetz and Whitfield, 2002; Caroff and Karibian, 2003). The inner part of the core polysaccharides and the backbone of lipid A enclose a large number of charged groups which are mainly anionic groups (Nikaido, 1996).

The LPS of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium have been the most thoroughly studied of the Gram negative LPS (Caroff and Karibian, 2003). Mutations in LPS structure affect the appearance of colonies of LPS mutants of these species when grown on solid media. Cells fully competent in LPS have a smooth appearance, whilst mutations in LPS structure can lead to rough, or semi -rough colony appearance (Smit *et al.*, 1975; Klena, *et al.*, 1992).

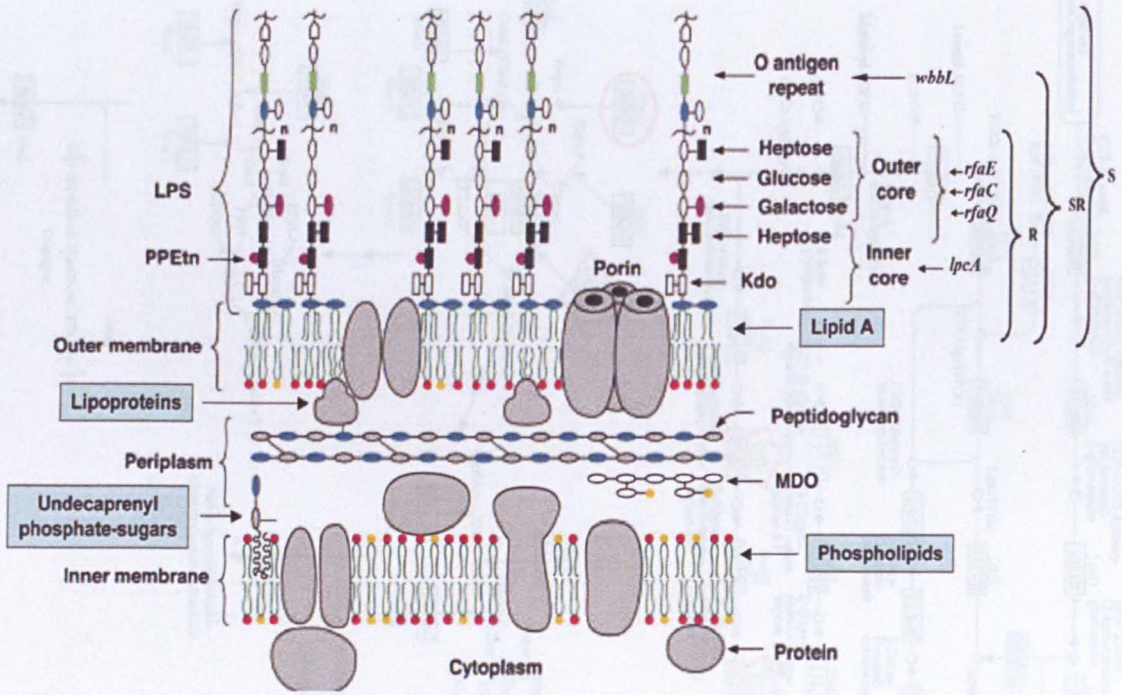

Figure 4.1 Schematic diagram of the inner and outer membrane of *E. coli* K-12. The Kdo and Lipid A of LPS structure are the only regions which are essential for the growth of *E. coli* and most other Gram negative strains. However, a few Gram negative strains can survive even without the lipid A (Modified from Raetz and Whitfield, 2002). . R=Rough-type; SR= Semi-Rough type and S= Smooth-type (Caroff and Karibian, 2003).

Researchers have identified and characterized a large number of genes in *E. coli* and *Salmonella* strains that are involved in the biosynthesis of LPS (Raetz, 1996). Figure 4.2 illustrates the LPS biosynthesis pathway (KEGG pathway database, 2012).

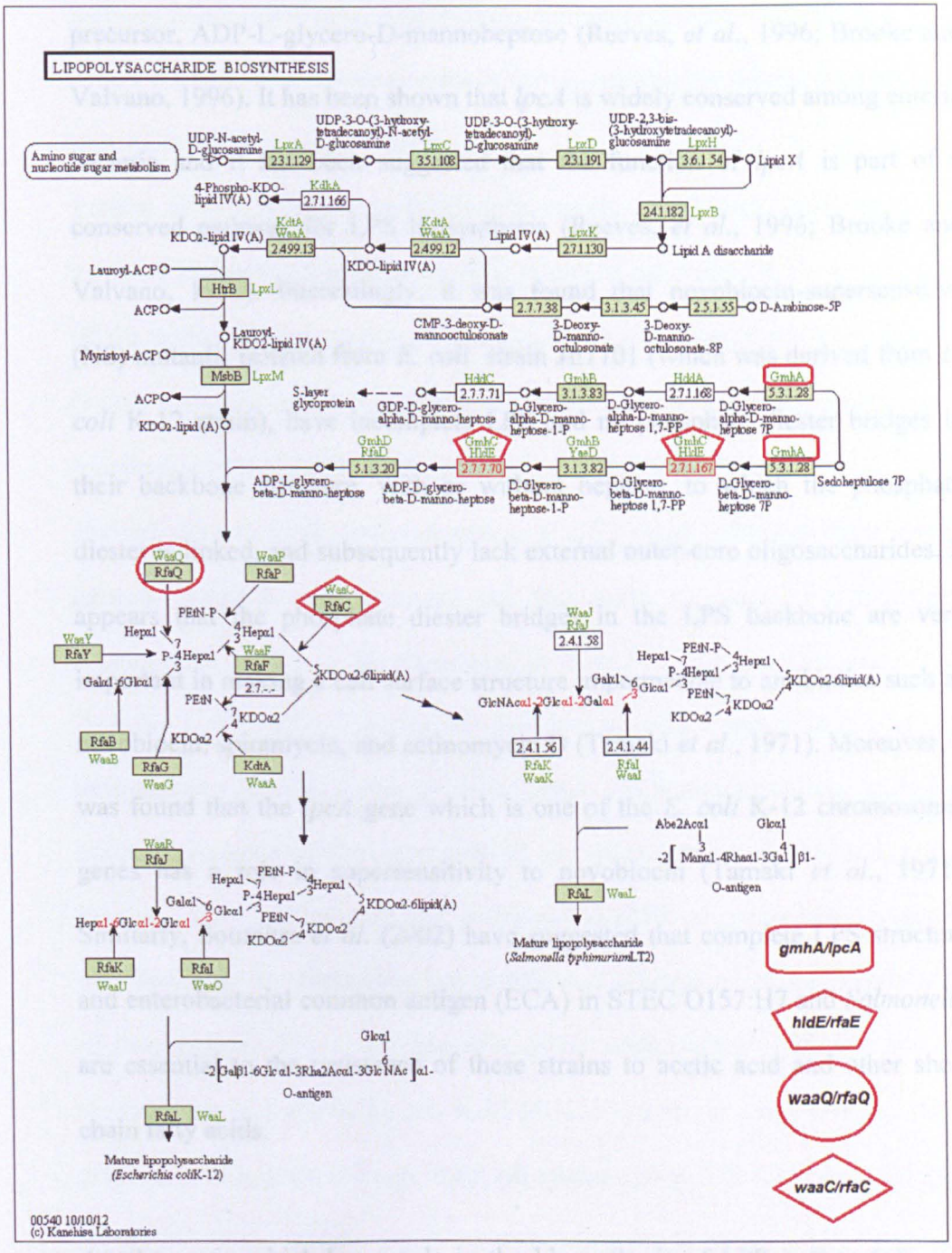

Figure 4.2: Lipopolysaccharide biosynthesis pathway (KEGG pathway database, 2012)

One of the genes involved in LPS biosynthesis is the *lpcA* gene (recently renamed *gmhA*) which was found to encode a phosphoheptose isomerase enzyme, which is necessary for the biosynthesis of the inner core LPS precursor, ADP-L-glycero-D-mannoheptose (Reeves, *et al.*, 1996; Brooke and Valvano, 1996). It has been shown that *lpcA* is widely conserved among enteric bacteria and it has been suggested that the function of *lpcA* is part of a conserved pathway for LPS biosynthesis (Reeves, *et al.*, 1996; Brooke and Valvano, 1996). Interestingly, it was found that novobiocin-supersensitive (NS) mutants, isolated from *E. coli* strain JE1101 (which was derived from *E. coli* K-12 strain), have incomplete LPS and no phosphate diester bridges in their backbone structure, with or without heptose, to which the phosphate diester is linked, and subsequently lack external outer-core oligosaccharides. It appears that the phosphate diester bridges in the LPS backbone are very important in making a cell surface structure impermeable to antibiotics such as novobiocin, spiramycin, and actinomycin D (Tamaki *et al.*, 1971). Moreover, it was found that the *lpcA* gene which is one of the *E. coli* K-12 chromosomal genes has a role in supersensitivity to novobiocin (Tamaki *et al.*, 1971). Similarly, Soumitra *et al.* (2002) have suggested that complete LPS structure and enterobacterial common antigen (ECA) in STEC O157:H7 and *Salmonella* are essential to the resistance of these strains to acetic acid and other short chain fatty acids.

Another gene which has a role in the biosynthesis of LPS is the *rfaE* gene (recently renamed *hldE*). The *rfaE* gene of *E. coli* encodes a bifunctinal protein, as it has two domains. The first domain is implicated in the synthesis

of D-glycero-D-manno-heptose 1-phosphate. The second catalyzes the ADP transfer to form ADP-D-*glycero*-D-*manno*-heptose (Valvano *et al.*, 2000). Similarly, Sirisena *et al.* (1992) reported that the *rfaD* and *rfaE* genes of *Salmonella* are involved in ADP-heptose formation and the *rfaC* gene (recently renamed *waaC*) encodes an enzyme which is essential for linking the proximal heptose to the lipopolysaccharide.

Yethon, *et al.*, (1998) reported that the *waaY*, *waaQ*, and *waaP* genes are involved in modification of the heptose region of the core LPS, the structure of which is vital to outer membrane stability. They confirmed that the *waaQ* gene (which was called *rfaQ*) encodes the HepIII transferase enzyme; however the functioning of *waaP* is a prerequisite for *waaQ* functionality.

Escherichia coli K-12 strains are phenotypically rough. Although these strains have a complete core structure and they have the genes for O-antigen biosynthesis, they lack the O-antigen in their LPS (Liu and Reeves, 1994; Hobman *et al.*, 2007). Lacking the O-antigen is a consequence of one of two independent mutations in the *rfb* gene cluster. These mutations have led to the rough appearance of the *E. coli* K-12 strains (Klena *et al.*, 1992; Liu and Reeves, 1994). The inability of *E. coli* K-12 MG1655 to produce the O-antigen was found to be exclusively as a result of disruption of the *wbbL* gene (Browning *et al.*, 2013). Insertion of an intact version of the *wbbL* gene into the chromosome of *Escherichia coli* K-12 MG1655 strain, in a way that has led to generation of a strain with an intact *rfb* cluster (DFB1655 L9), resulted in the restoration of the strain's capability to produce the O-antigen. The O-antigen ultimately increased the strain resistance to many environmentally adverse conditions such as human serum and mechanical shear (Browning *et al.*, 2013).

Colanic acid (CA) is an exopolysaccharide produced by many Enterobacteria, including the majority of *Escherichia coli* strains. Colonic acid forms a mesh that coats the bacteria, often within biofilms (Meredith *et al.*, 2007). It has been reported that it plays a role in protecting *E. coli* O157: H7 from acid stress in set yogurt (Lee, 2002). Similarly, Chen *et al.* (2004) found that colanic acid protects *E. coli* O157:H7 from osmotic and oxidative stress.

This chapter describes experiments to investigate the role of lipopolysaccharides in bacterial resistance to cold atmospheric plasma (CAP), using derivatives of *E. coli* K-12 (*E. coli* K-12 MG1655, *E. coli* K-12 BW25113 and *E. coli* K-12 W3110) - see Figure 4.3 for the relationship of *E. coli* K-12 with these strains - and their isogenic mutants which include *E. coli* K-12 MG1655 (Δ*waaQ*), *E. coli* K-12 BW25113 (Δ *rfaE*), *E. coli* K-12 BW25113 (Δ*lpcA*), and *E. coli* K-12 W3110 (Δ*rfaC*). In addition to these strains and mutants, partially and fully restored LPS derivatives of *E. coli* K-12 MG1655 where the *wbbL* gene had been repaired, were used. Furthermore, *Salmonella* Typhimurium TA98 which is a mutant of *Salmonella* Tphimurium LT2 with a defect in the *rfa* gene cluster was also tested for its sensitivity to CAP treatments (see Table 2.2 for more details of these strains and mutants).

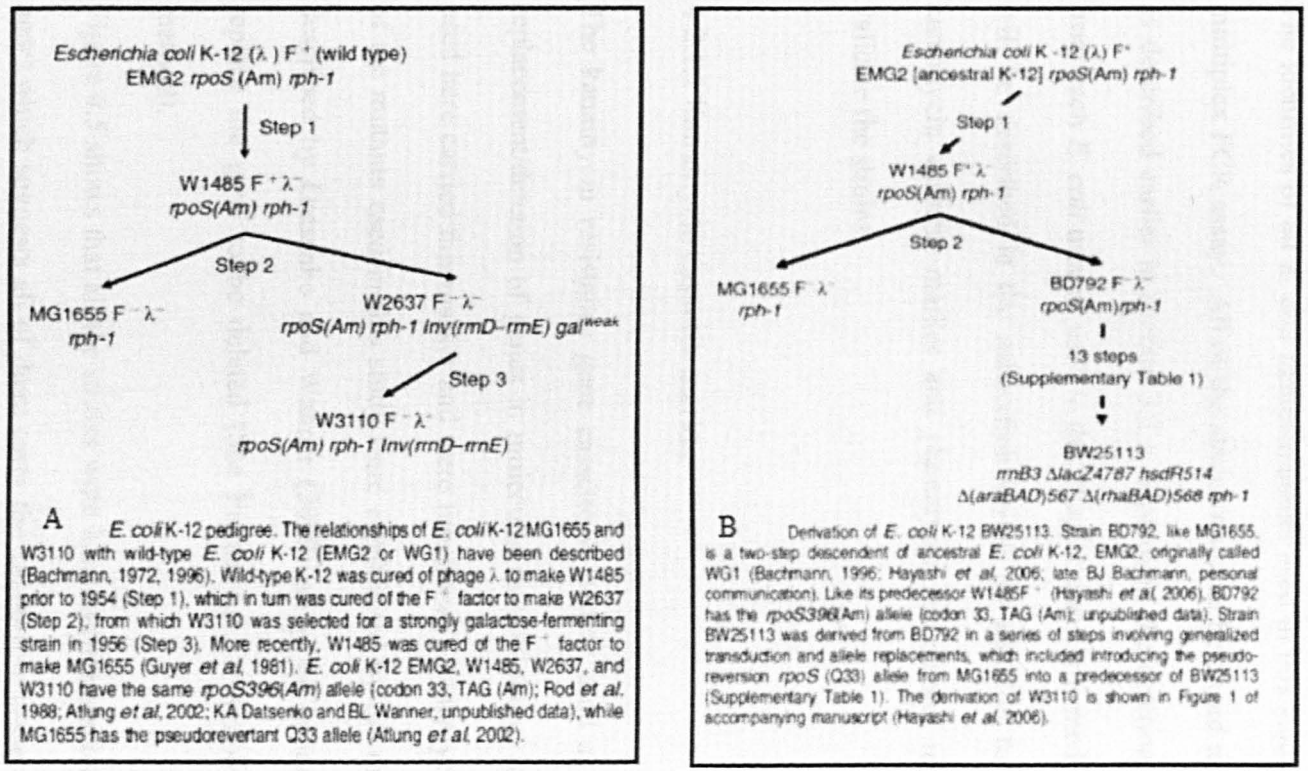

Figure 4.3: Relationship of *E. coli* K-12 strains with its derivatives, *E. coli* K-12 MG1655, *E. coli* K-12 BW25113 and *E. coli* K-12 W3110). A was taken from Hayashi *et al.* (2006) and B was taken from Baba *et al.* (2006).

4.2. RESULTS

4.2.1. Confirmation of the *E. coli* strains/mutants' identities

The identities of all *E. coli* strains/mutants used in this study were verified by multiplex PCR assay. All of the strains/mutants belonged to the genus *E. coli* as described earlier in section 3.2.1. Moreover, the deletion of specific genes from each *E. coli* mutant used in this chapter was confirmed by PCR assays as will be described in the subsection below. In addition to the PCR assays, kanamycin cassette marker and phenotypic *rfa* marker tests were used to validate the strains.

4.2.1.1 Kanamycin cassette marker

The kanamycin resistance gene cassette is widely used as a marker in the replacement/deletion of genes in molecular microbiology. All of the mutants used here carried this marker and were functionally kanamycin resistant. Most of the mutants used in this study were constructed by the method which was described by Datsenko and Wanner (2000) using the kanamycin cassette to replace the gene to be deleted (See Figure 4.4 for simplified steps of the method).

Figure 4.5 shows that all the strains were kanamycin resistant (50 μg/ml in LB agar) which suggests all of them carry the kanamycin cassette. Although this test is not enough to verify whether the gene of interest has been deleted, when it is combined with results of specific PCR tests it gives additional confirmation of the deletion mutation.

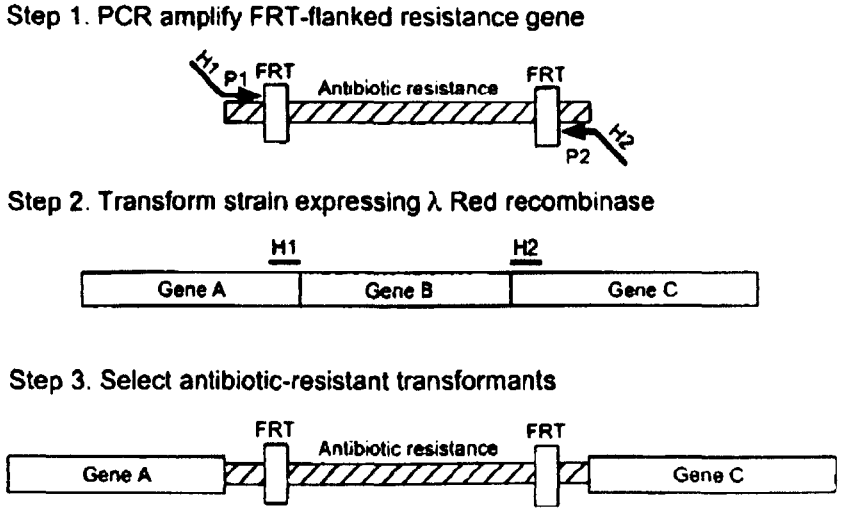

Figure 4.4: Datsenko and Wanner gene deletion/disruption strategy. H1 and H2 refer to the homology extensions region and P1 and P2 refer to priming sites. FRT is a (Flippase Recognition Target). Taken from Datsenko and Wanner (2000).

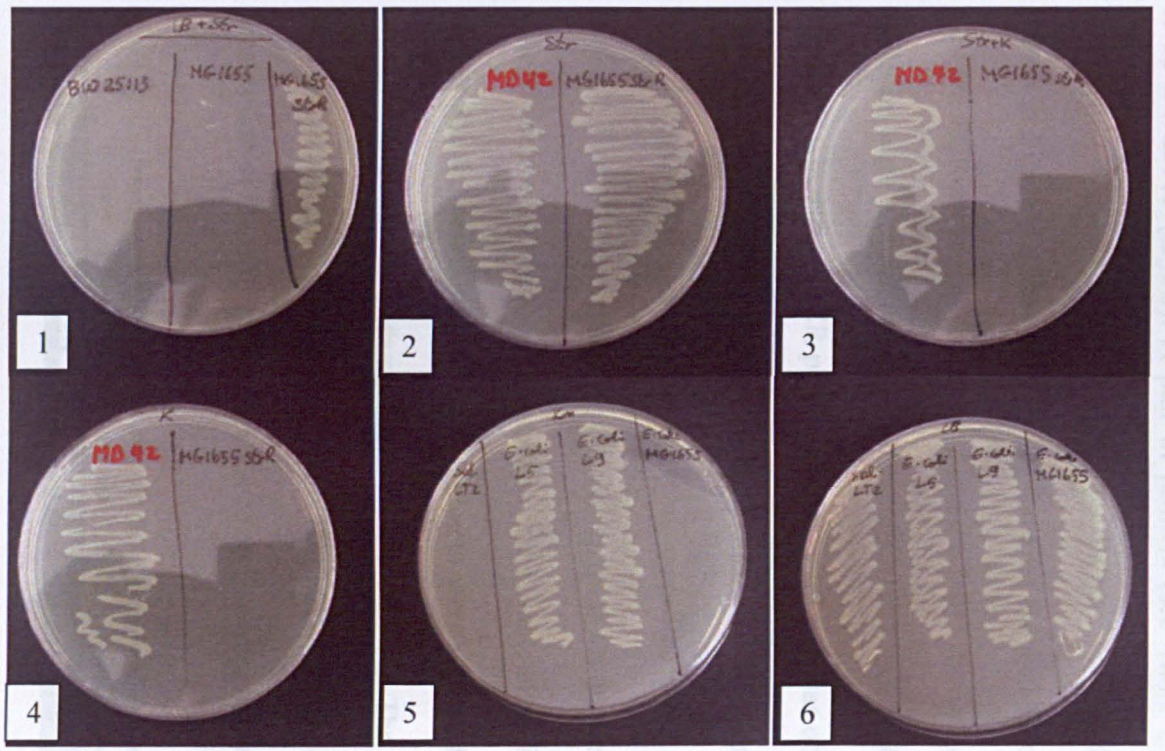

Figure 4.5: Confirmation of antibiotic resistance. Plate 1 and 2, strptomycine resistance (*E. coli* MG 1655 Str^R and *E. coli* MG 1655 Str^R Km^R); plate 3, strptomycine and kanamycin resistance (*E. coli* MG 1655 Str^R Km^R); plate 4 and 5, kanamycin resistance (*E. coli* MG 1655 Str^R Km^R *E. coli* DFB1655 L5 and plate 8, *E. coli* DFB1655 L9; plate 6, non resistant strains (*Salmonella* Typhimurium LT2 and *E. coli* MG1655) along side kanamycin resistant strains (*E. coli* DFB1655 L5 and plate 8, *E. coli* DFB1655 L9).

4.2.1.2. Confirmation of the *rfaE* gene deletion from *E. coli* K-12 BW25113 (Δ*rfaE*)

A single PCR assay was used to confirm deletion of the *rfaE* gene from *E. coli* K-12 BW25113 (Δ*rfaE*). A pair of primers (see Table 2.3) was specifically designed to target the sequences flanking the *rfaE* gene of the *E. coli* strains as shown in Figure 4.6 The agarose gel showing the PCR products amplified by the *rfaE* PCR assay is presented in Figure 4.7 and shows that PCR products of 1,526 bp were amplified from the *rfaE* gene of *E. coli* BW25113 and *E. coli* MG1655, whereas no PCR product of the same size was obtained from *E. coli* BW25113 (Δ*rfaE*). However, a PCR product of ~ 1,450 bp which is smaller than the expected size (1,526 bp) was amplified from *E. coli* BW25113 (Δ*rfaE*). This PCR product was amplified from sequences flanking the *rfaE* gene, which amplified the kanamycin gene cassette which was used as a marker for the deletion. No PCR products were obtained from the negative control (PCR mixture with no added template DNA). The result confirms the deletion of the *rfaE* gene from *E. coli* K-12 BW25113 (Δ*rfaE*) mutant, and substitution of the *rfaE* gene by the 1,400 bp Kanamycin gene cassette.

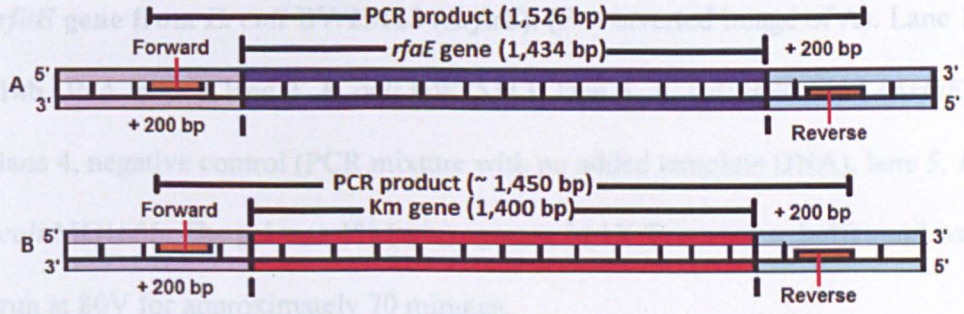

Figure 4.6: Diagram showing the forward (For-*rfaE*) and reverse (Rev-*rfaE*) PCR primer binding sites flanking the *rfaE* gene of *E. coli* K-12 MG1655 (A) and flanking the Km gene of *E. coli* K-12 BW25113 (Δ*rfaE*) mutant (B)

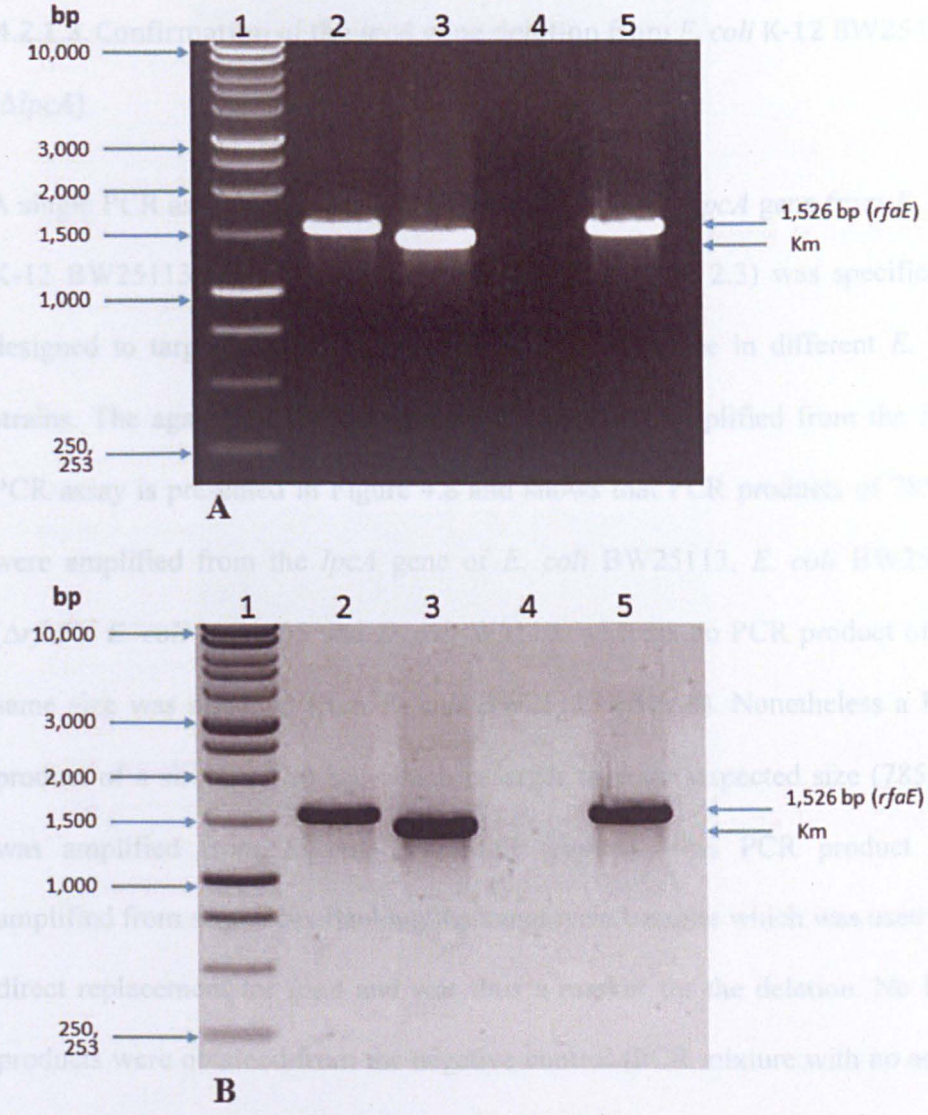

Figure 4.7: Agarose gel of PCR products confirming the deletion of the *rfaE* gene from *E. coli* BW25113 (Δ*rfaE*). (B = inverted image of A). Lane 1, 1kb DNA ladder; lane 2, *E. coli* BW25113; lane 3, *E. coli* BW25113 (Δ*rfaE*); lane 4, negative control (PCR mixture with no added template DNA); lane 5, *E. coli* MG1655. The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 70 minutes.

4.2.1.3. Confirmation of the *lpcA* gene deletion from *E. coli* K-12 BW25113 (Δ*lpcA*)

A single PCR assay was used to confirm deletion of the *lpcA* gene from *E. coli* K-12 BW25113 (Δ*lpcA*). A pair of primers (see Table 2.3) was specifically designed to target the sequences flanking the *lpcA* gene in different *E. coli* strains. The agarose gel showing the PCR products amplified from the *lpcA* PCR assay is presented in Figure 4.8 and shows that PCR products of 785 bp were amplified from the *lpcA* gene of *E. coli* BW25113, *E. coli* BW25113 (Δ*rfaE*), *E. coli* MG1655 and *E. coli* W3110, whereas no PCR product of the same size was obtained from *E. coli* BW25113 (Δ*lpcA*). Nonetheless a PCR product of a size (~1,500 bp) which is larger than the expected size (785 bp) was amplified from *E. coli* BW25113 (Δ*lpcA*). This PCR product was amplified from sequences flanking the kanamycin cassette which was used as a direct replacement for *lpcA* and was thus a marker for the deletion. No PCR products were obtained from the negative control (PCR mixture with no added template DNA).

The result confirms deletion of the *lpcA* gene from *E. coli* BW25113 (Δ*lpcA*) mutant, and substitution of the *lpcA* gene by the 1,400 bp Kanamycin cassette.

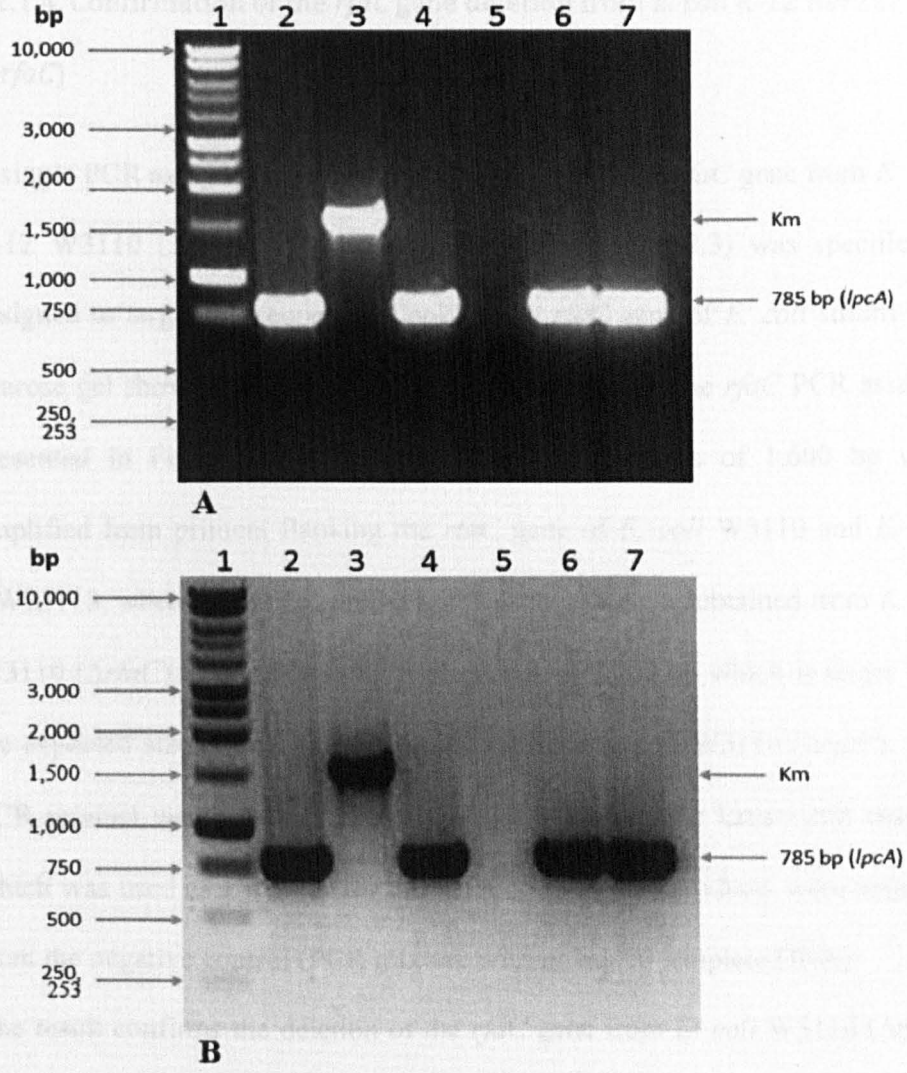

Figure 4.8: Agarose gel of PCR products confirming the deletion of the *lpcA* gene from *E. coli* BW25113 (Δ*lpcA*). (B = inverted image of A). Lane 1, 1kb DNA ladder; lane 2, *E. coli* BW25113; lane 3, *E. coli* BW25113 (Δ*lpcA*); lane 4, *E. coli* BW25113 (Δ*rfaE*); lane 5, negative control (PCR mixture with no added template DNA); lane 6, *E. coli* MG1655; lane 7, *E. coli* W3110. The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 70 minutes.

4.2.1.4. Confirmation of the *rfaC* gene deletion from *E. coli* K-12 BW25113 (Δ*rfaC*)

A single PCR assay was used to confirm deletion of the *rfaC* gene from *E. coli* K-12 W3110 (Δ*rfaC*). A pair of primers (see Table 2.3) was specifically designed to target the sequences flanking the *rfaC* gene of *E. coli* strains The agarose gel showing the PCR products amplified using the *rfaC* PCR assay is presented in Figure 4.9 and shows that PCR products of 1,600 bp were amplified from primers flanking the *rfaC* gene of *E. coli* W3110 and *E. coli* BW25113, whereas no PCR product of the same size was obtained from *E. coli* W3110 (Δ*rfaC*). Nevertheless a PCR product of 2,000 bp which is larger than the expected size (1,600 bp) was amplified from *E. coli* W3110 (Δ*rfaC*). This PCR product was amplified from sequences flanking the kanamycin cassette which was used as a marker for the deletion. No PCR products were obtained from the negative control (PCR mixture with no added template DNA).

The result confirms the deletion of the *rfaC* gene from *E. coli* W3110 (Δ*rfaC*) (Heurlier, K., personal comunication) mutant, and substitution of the *rfaC* gene by the 1,400bp Kanamycin cassette.

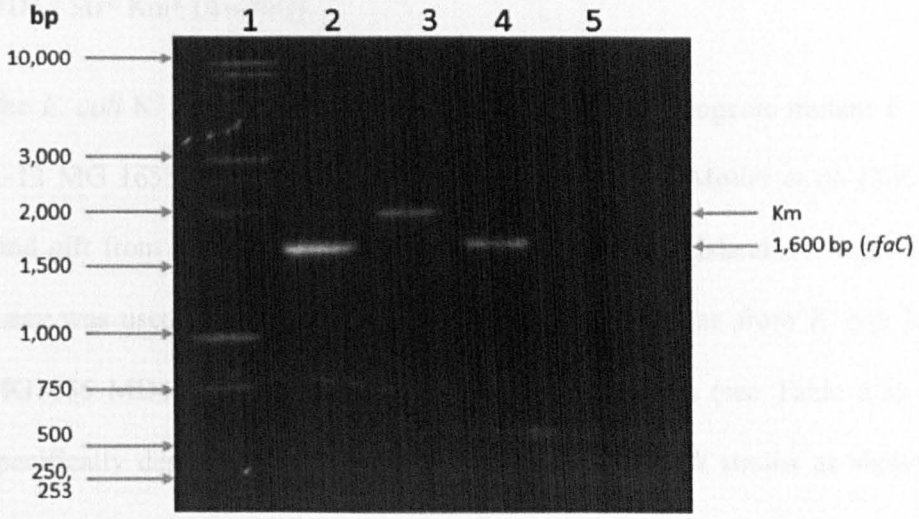

Figure 4.9: Agarose gel of PCR products confirming the deletion of the *rfaC* gene from *E. coli* W3110 (Δ*rfaC*). Lane 1, 1kb DNA ladder; lane 2, *E. coli* W3110; lane 3, *E. coli* W3110 (Δ*rfaC*); lane 4, *E. coli* BW25113; lane 5, negative control (PCR mixture with no added template DNA). The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 60 minutes.

4.2.1.5. Confirmation of the *waaQ* gene deletion from *E. coli* K-12 MG 1655 MD42 Str^R Km^R (Δ*waaQ*).

The *E. coli* K-12 MG 1655 MD42 Str^R strain and its' isogenic mutant *E. coli* K-12 MG 1655 MD42 Str^R Km^R (Δ*waaQ*) were from Møller *et al.* (2003) a kind gift from Dr. Paul S. Cohen, University of Rhode Island. A single PCR assay was used to confirm the deletion of the *waaQ* gene from *E. coli* K-12 MG1655 MD42 Str^R Km^R (Δ*waaQ*). A pair of primers (see Table 2.3) was specifically designed to target the *waaQ* gene in *E. coli* strains as shown in figure 4.10. The agarose gel showing the PCR products amplified from the *waaQ* PCR assay is presented in Figure 4.11 and shows that a PCR product of 1,348 bp was amplified from the *waaQ* gene of *E. coli* MG1655 Str^R and *E. coli* MG1655. In contrast no PCR product was obtained from the negative control (PCR mixture with no added template DNA) and from *E. coli* MG 1655 MD42 Str^R Km^R (Δ*waaQ*) mutant as its entire *waa* operon was inactivated by the mini-Tn*5* Km insertion (Møller *et al.* (2003).

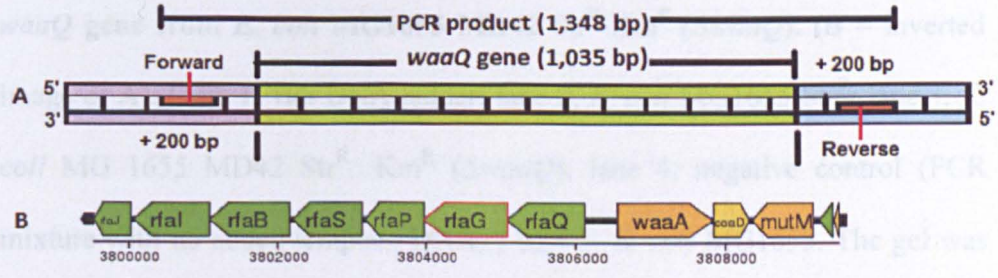

Figure 4.10: Diagram showing the forward (For-OE-*waaQ*) and reverse (Rev-OE-*waaQ*) PCR primer binding sites flanking the *waaQ* gene of *E. coli* K-12 MG1655. The PCR product is 1,348 bp (image A), and the *rfaQ* gene with the other genes of *rfa* operon which was recently renamed *waa* operon (image B, taken from *coli*BASE). Inactivation of the entire *waa* operon in *E .coli* MD42 Str^R Km^R (Δ*waaQ*) led to no PCR product was amplified from this mutant.

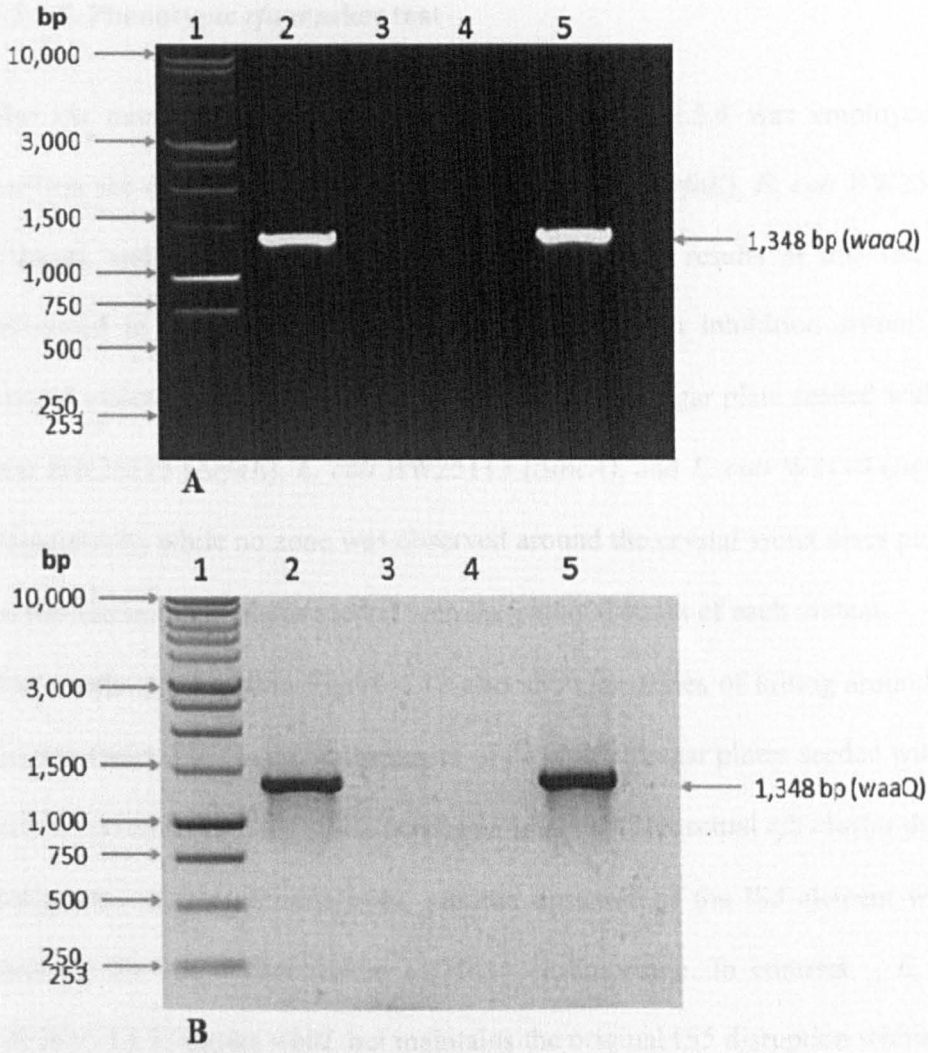

Figure 4.11: Agarose gel of PCR products confirming the deletion of the *waaQ* gene from *E. coli* MG1655 MD42 Str^R Km^R (Δ*waaQ*). (B = inverted image of A). Lane 1, 1kb DNA ladder; lane 2, *E. coli* MG1655 Str^R; lane 3, *E. coli* MG 1655 MD42 Str^R Km^R (Δ*waaQ*); lane 4, negative control (PCR mixture with no added template DNA) ; lane 5, *E. coli* MG1655. The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 70 minutes.

4.2.1.6. Phenotypic *rfa* marker test

The *rfa* marker test described earlier in section 2.2.5.4 was employed to confirm the deletion mutation in *E. coli* BW25113 (Δ*rfaE*), *E. coli* BW25113 (Δ*lpcA*), and *E. coli* W3110 (Δ*rfaC*) mutants. The results of this test are presented in Figure 4.12 and show zones of growth inhibition around the crystal violet disc placed in the centre of the nutrient agar plate seeded with *E. coli* BW25113 (Δ*rfaE*), *E. coli* BW25113 (Δ*lpcA*), and *E. coli* W3110 (Δ*rfaC*) respectively, while no zone was observed around the crystal violet discs placed in the nutrient agar plates seeded with the parental strain of each mutant.

The results presented in Figure 4.12 also show no zones of killing around the crystal violet disc placed in the centre of the nutrient agar plates seeded with *E. coli* DFB1655 L9. This strain carries an intact chromosomal *rfb* cluster due to integration of the pJP5603/*wbbL* plasmid upstream of the IS*5* element which disrupts the *rfb* cluster in the MG1655 chromosome. In contrast *E. coli* DFB1655 L5, carries *wbbL* but maintains the original IS*5* disruption within the *rfb* cluster due to integration of pJP5603/*wbbL* plasmid downstream of the IS*5* element into its chromosomal (Browning *et al.*, 2013). However, a very small zone of growth inhibition was observed around the crystal violet disc placed in the centre of the nutrient agar plate seeded with the parental strain *E. coli* MG1655.

The result confirms that the *rfaE* gene of *E. coli* BW25113 (Δ*rfaE*), the *lpcA* gene of *E. coli* BW25113 (Δ*lpcA*), and the *rfaC* gene of *E. coli* W3110 (Δ*rfaC*) had been deleted (deep rough phenotype). In contrast the *rfaE*, *lpcA* and *rfaC* genes of their parental strain (*E. coli* BW25113 and *E. coli* W3110), were

intact and functioning properly (smooth phenotype). This result complements phenotypic tests obtained by PCR assay.

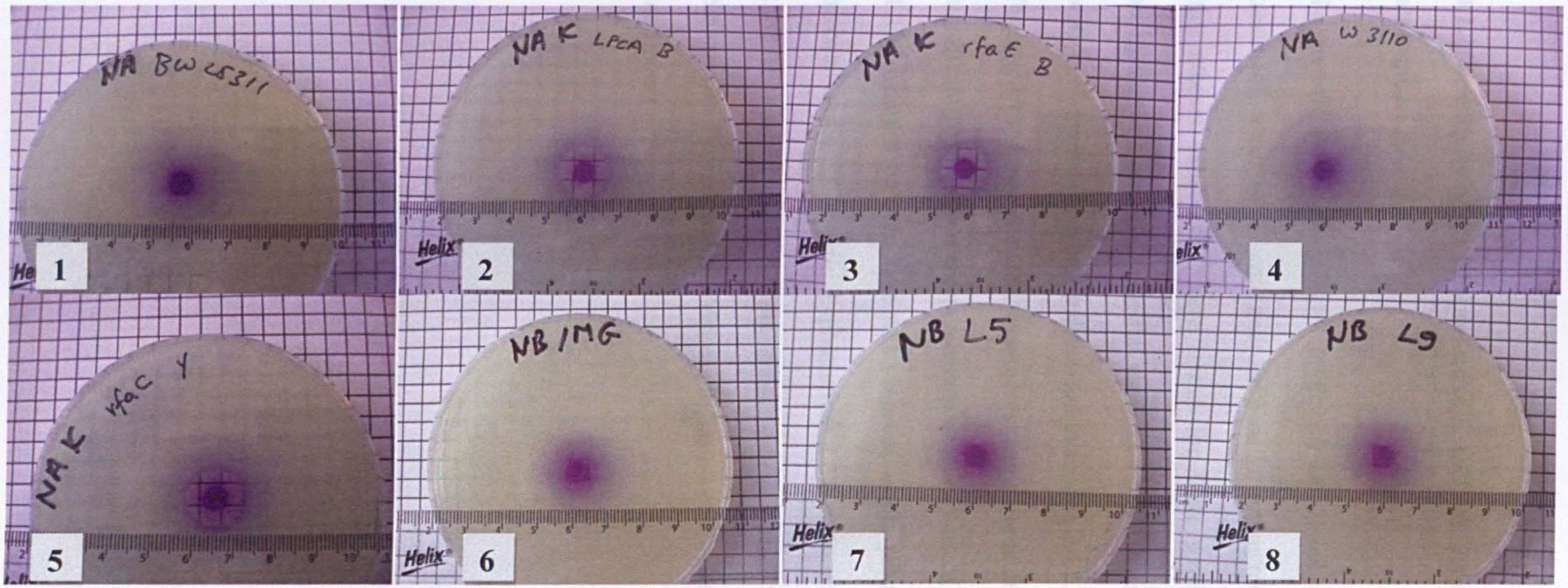

Figure 4.12: Nutrient agar plates seeded with mutants/parental strains and containing a crystal violet impregnated disc in the centre of each plate. Plates were incubated for 12 h at 37 °C. Plate 1, *E. coli* BW25113; plate 2, *E. coli* BW25113 (Δ*rfaE*); plate 3, *E. coli* BW25113 (Δ*lpcA*); plate 4, and *E. coli* W3110; plate 5, *E. coli* W3110 (Δ*rfaC*); plate 6, *E. coli* MG1655, plate 7, *Escherichia coli* DFB1655 L5 and plate 8, *Escherichia coli* DFB1655 L9.

4.2.2. Sensitivity of the LPS defective *E. coli* and *Salmonella* mutants to cold atmospheric plasma

To investigate the role of LPS in *E. coli* and *Salmonella* in resistance to CAP treatment, a set of *E. coli* and *Salmonella* strains/mutants with different LPS biosynthesis capabilities were tested for their sensitivity to CAP treatment alongside the appropriate parental strain as a positive control. The sensitivity tests were performed as previously described in section 2.6.1.

Figures 4.13 shows that CAP treatments for longer than one minute have caused a significant reduction in the percentage of survivors of *Salmonella* Typhimurium TA98 whereas no reduction was observed in the percentage of survivors of its parental strain *Salmonella* Typhimurium LT2, even with 4 minutes of exposure time to CAP treatment. This result indicates that the deletion of *rfa* from *Salmonella* Typhimurium TA98 might have a role in its higher sensitivity to CAP treatments

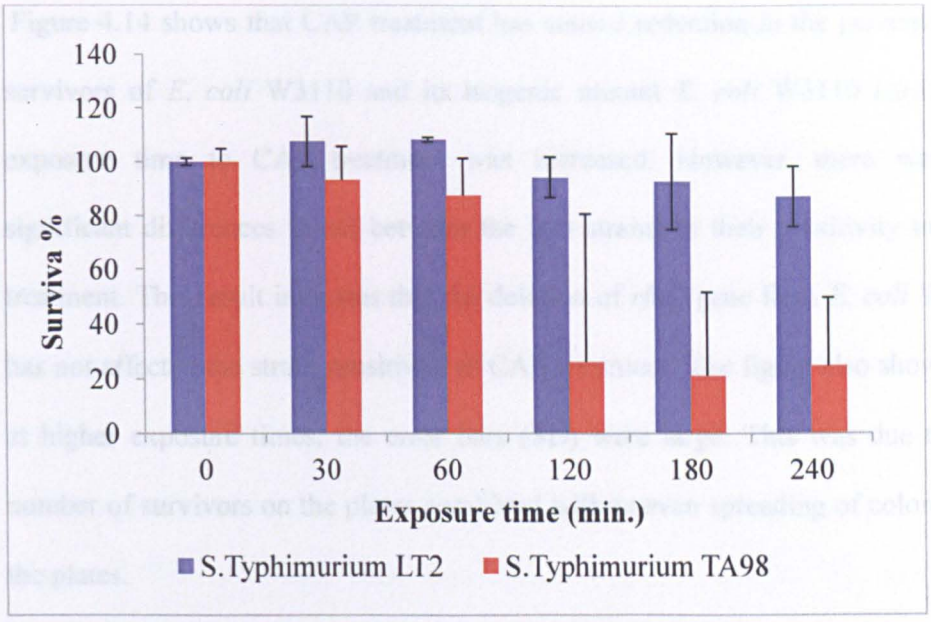

Figure 4.13: Effect of different exposure times to CAP treatment on *Salmonella* Typhimurium LT2 and its isogenic mutant, *Salmonella* Typhimurium TA98. (Mean survival % ± 1 SD, n = 3 replicates).

Figure 4.14 shows that CAP treatment has caused reduction in the percentage of survivors of *E. coli* W3110 and its isogenic mutant *E. coli* W3110 (Δ*rfaC*) as exposure time to CAP treatment was increased. However, there were no significant differences found between the two strains in their sensitivity to CAP treatment. This result indicates that the deletion of *rfaC* gene from *E. coli* W3110 has not affected the strain sensitivity to CAP treatment. The figure also shows that at higher exposure times, the error bars (SD) were large. This was due to low number of survivors on the plates combined with uneven spreading of colonies on the plates.

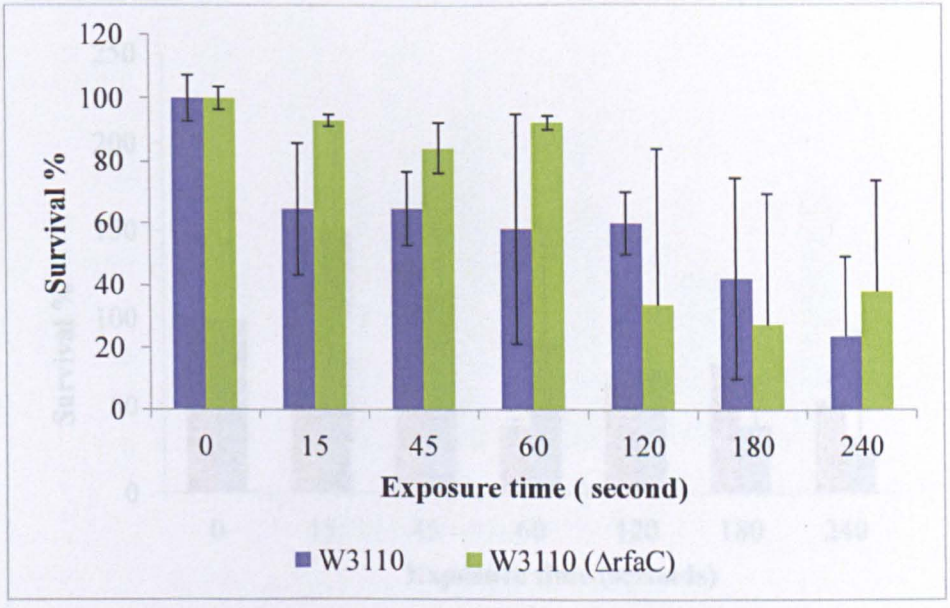

Figure 4.14: Effect of different exposure times to CAP treatment on *E. coli* W3110 and its isogenic mutant (Δ*rfaC*). (Mean survival % ± 1 SD, n = 3 replicates).

Figure 4.15 shows that CAP treatment has caused a reduction in the percentage of survivors of *E. coli* BW25113 and its isogenic mutant *E. coli* BW25113 (Δ*rfaE*) as exposure time to CAP treatment was increased. However, no significant differences were found between them in the sensitivity to CAP treatment. This result indicates that the deletion of the *rfaE* gene from *E. coli* BW25113 has not affected the strain sensitivity to CAP treatment. The figure also shows that at low exspoure times, CAP has caused a non signigicant increase in the survival percentage of both strains. This increase might be due hormetic effect of CAP treatment. The error bars (SD) were large. This was due to low number of survivors on the plates combined with uneven spreading of colonies on the plates.

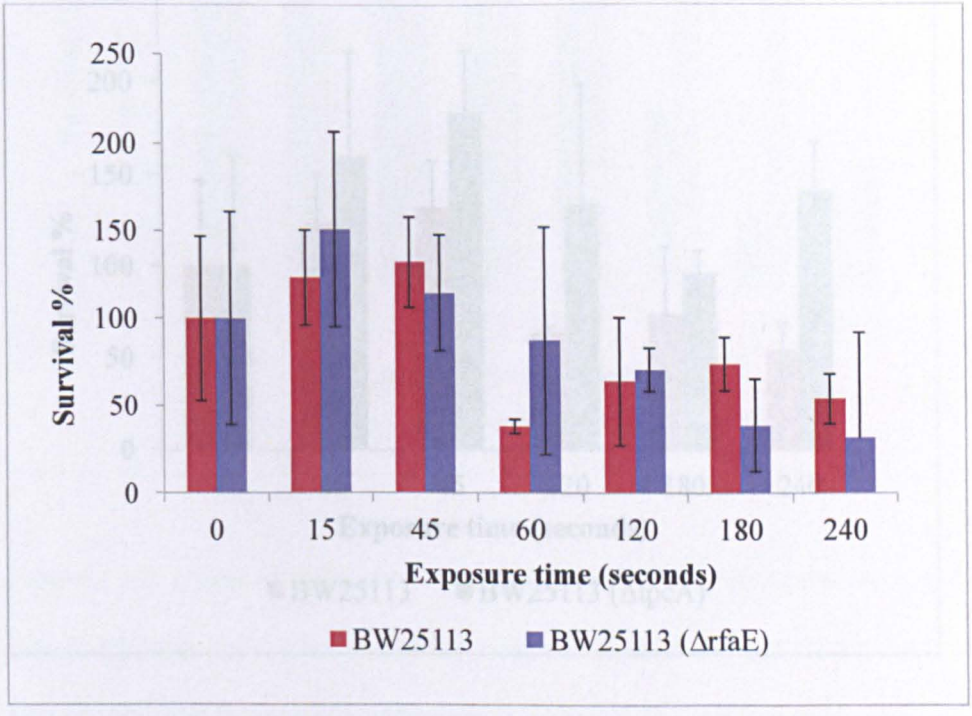

Figure 4.15: Effect of different exposure times to CAP treatment on *E. coli* BW25113 and its isogenic mutant (Δ*rfaE*). (Mean survival % ± 1 SD, n = 3 replicates).

Figure 4.16 shows that there were no significant differences between the survivors of *E. coli* BW25113 and its isogenic mutant *E. coli* BW25113 (Δ*lpcA*) due to CAP treatment. This result indicates that the deletion of *lpcA* gene from *E. coli* BW24113 has not affected the strain sensitivity to CAP treatment. The figure also shows that at low exposure times, CAP has caused a non significant increase in the survival percentage of both strains. This increase might be due hormetic effect of CAP treatment. The error bars (SD) were large. This was due to low number of survivors on the plates combined with uneven spreading of colonies on the plates.

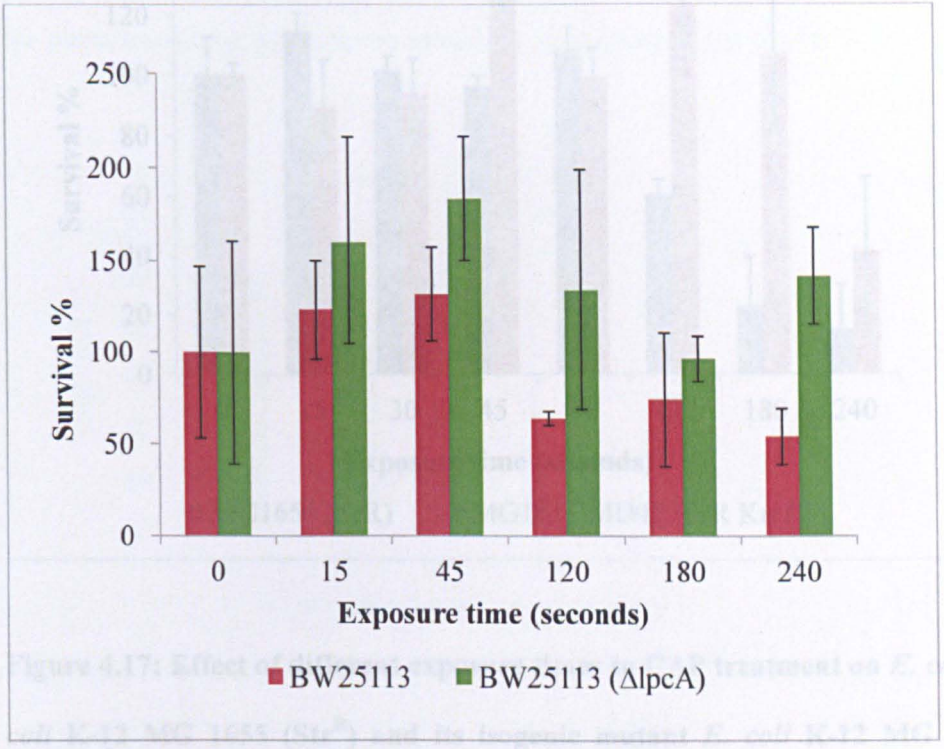

Figure 4.16: Effect of different exposure times to CAP treatment on *E. coli* BW25113 and its isogenic mutant (Δ*lpcA*). (Mean survival % ± 1 SD, n = 3 replicates).

Figure 4.17 shows that CAP treatment for 2 minutes has caused a reduction in the percentage of survivors of *E. coli* K-12 MG1655 (Str^R) as the exposure time was increased. In contrast a significant reduction in the percentage of survivors of *E. coli* K-12 MG 1655 MD42 (Str^R Km^R) was observed only at the highest exposure time. This result indicates that the deletion of the *waaQ* gene from *E. coli* K-12 MG 1655 (Str^R) has not increased the strain sensitivity to CAP treatment.

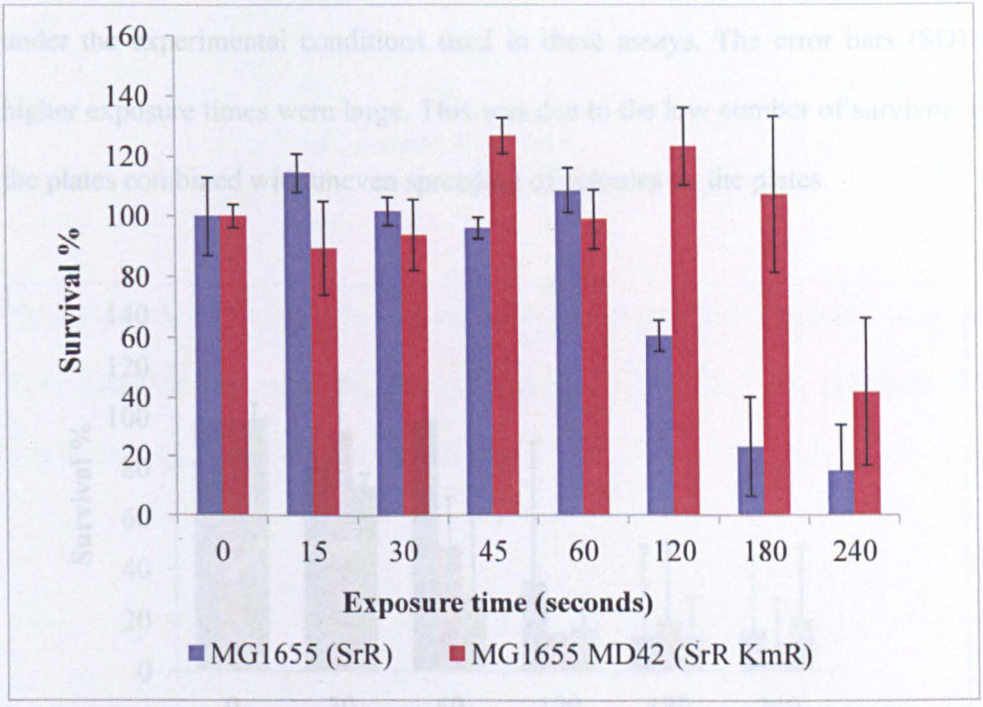

Figure 4.17: Effect of different exposure times to CAP treatment on *E. coli* *E. coli* K-12 MG 1655 (Str^R) and its isogenic mutant *E. coli* K-12 MG 1655 MD42 (Str^R Km^R) (Δ*waaQ*). (Mean survival % ± 1 SD, n = 3 replicates).

Figures 4.18.a and 4.18.b show that CAP treatment has caused an exponential reduction in the percentage of survivors of *E. coli* K-12 MG1655 and its derivatives (DFB1655 L5 and DFB1655 L9) as exposure time to CAP treatment was increased. However, there were no significant differences between them in the sensitivity to CAP treatment found. This result indicates that the insertion of an intact version of the *wbbL* gene into the chromosome of *Escherichia coli* K12 MG1655 strain has not improved the resistance of the strains to CAP treatment under the experimental conditions used in these assays. The error bars (SD) at higher exposure times were large. This was due to the low number of survivors on the plates combined with uneven spreading of colonies on the plates.

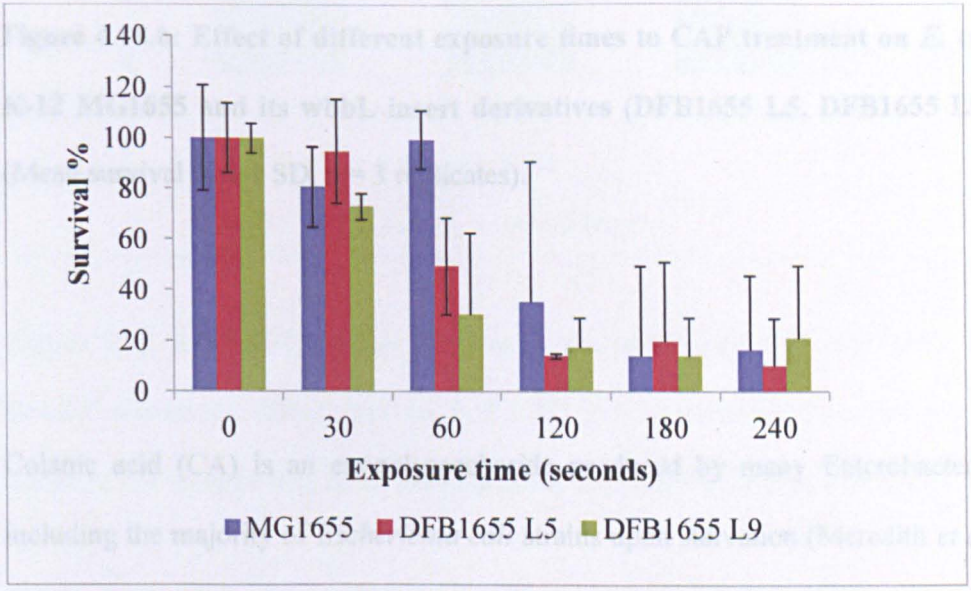

Figure 4.18.a: Effect of different exposure times to CAP treatment on *E. coli* K-12 MG1655 and its *wbbL* insert derivatives (DFB1655 L5, DFB1655 L9). (Mean survival % ± 1 SD, n = 3 replicates).

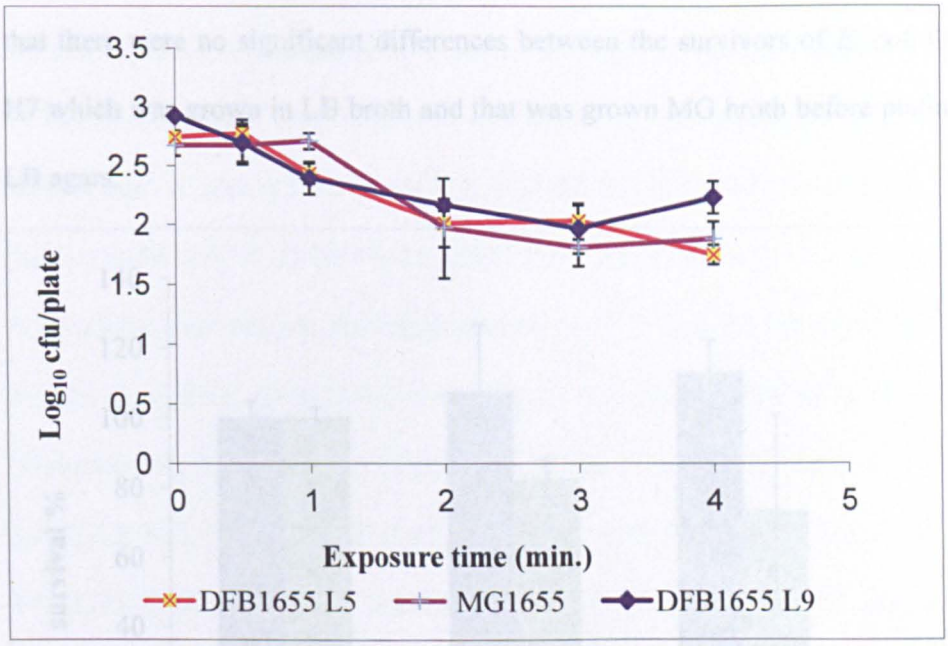

Figure 4.18.b: Effect of different exposure times to CAP treatment on *E. coli* K-12 MG1655 and its wbbL insert derivatives (DFB1655 L5, DFB1655 L9). (Mean survival % ± 1 SD, n = 3 replicates).

Colanic acid (CA) is an exopolysaccharide produced by many Enterobacteria, including the majority of *Escherichia coli* strains upon starvation (Meredith *et al.*, 2007). Chen and co workers found that colanic acid protects *E. coli* O157:H7 from osmotic and oxidative stress (Chen *et al.*, 2004). To investigate the possible effect of colanic acid production on the resistance of *E. coli* O157: H7 to CAP treatment, overnight cultures of *E. coli* O157: H7 were prepared in LB and MG broths and

incubated at 20 °C then plated on LB agar for CAP treatments. Figures 4.19 shows that there were no significant differences between the survivors of *E. coli* O157: H7 which was grown in LB broth and that was grown MG broth before plating on LB agars.

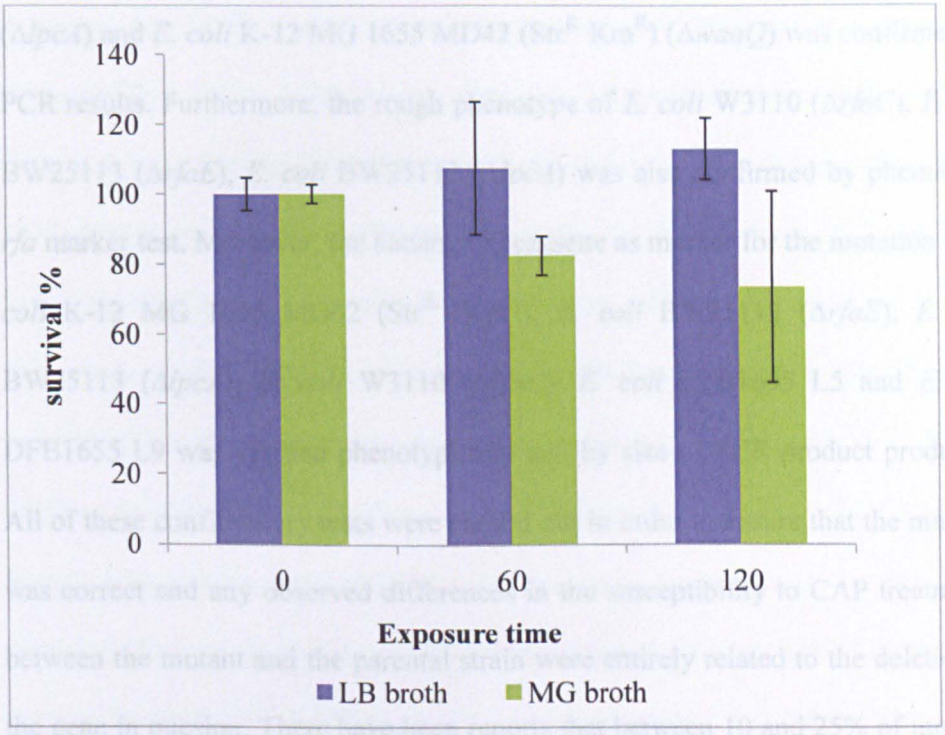

Figure 4.19: Effect of growing medium on the sensitivity of *E. coli* O157: H7 to CAP treatment. (Mean survival % ± 1 SD, n = 3 replicates).Overnight culture grown in LB broth or MG broth then plated on LB agar for CAP treatments.

4.3. DISCUSSION

In addition to the confirmation of identities of all *E. coli* strains/mutants as shown by the results which were presented in Chapter 3, the deletion of the gene of interest from *E. coli* W3110 (Δ*rfaC*), *E. coli* BW25113 (Δ*rfaE*), *E. coli* BW25113 (Δ*lpcA*) and *E. coli* K-12 MG 1655 MD42 (Str^R Km^R) (Δ*waaQ*) was confirmed by PCR results. Furthermore, the rough phenotype of *E. coli* W3110 (Δ*rfaC*), *E. coli* BW25113 (Δ*rfaE*), *E. coli* BW25113 (Δ*lpcA*) was also confirmed by phenotypic *rfa* marker test. Moreover, the kanamycin cassette as marker for the mutation in *E. coli* K-12 MG 1655 MD42 (Str^R Km^R); *E. coli* BW25113 (Δ*rfaE*); *E. coli* BW25113 (Δ*lpcA*); *E. coli* W3110 (Δ*rfaC*); *E. coli* DFB1655 L5 and *E. coli* DFB1655 L9 was verified phenotypically and by size of PCR product produced. All of these confirmatory tests were carried out in order to ensure that the mutants was correct and any observed differences in the susceptibility to CAP treatments between the mutant and the parental strain were entirely related to the deletion of the gene in quesion. There have been reports that between 10 and 25% of mutants from the Keio collection from which the BW25113 mutants were obtained are incorrect (Browning, D.F., personal communication)

To test the hypothesis that LPS might have a role in tolerance/ increased resistance to CAP treatments, the sensitivity of *S. enterica* serovar Typhimurium LT2 and an Ames strain mutant *S. enterica* serovar Typhimurium TA98 were tested. The result obtained using these strains has shown that *S. enterica* serovar Typhimurium TA98 was more susceptible to CAP treatments than the parental strain, *S. enterica* serovar Typhimurium LT2. Since the latter has a complete lipopolysaccharide

(McClelland *et al.*, 2001) unlike *S. enterica* serovar Typhimurium TA98 which is a deep rough mutant (McCann *et al.*, 1975), therefore, the susceptibility of *S. enterica* serovar Typhimurium TA98 could be attributed to the possible involvement of the lipopolysaccharide. However; this strain also has more deletions (in DNA repair genes) which may contribute to this increased susceptibility. These deletions involved the galactose operon, biotin operon, nucleotide-excision-repair *uvrB* gene and chlorate-resistance genes (Ames *et al.*, 1973; Maron and Ames 1983; Porwollika *et al.*, 2001). The strain was also found to be missing additional genes, which include *mfdA* (encoding a multi-drug translocase), *mdaA* (a major nitroreductase) and *dps* (encoding a DNA-binding protein), genes involved in molybdenum cofactor biosynthesis and a number of ORFs of unknown functions (Porwollika *et al.*, 2001). The *dps* gene in *E. coli* was found to be involved in oxidative stress resistance and regulated by the OxyR regulon (Zheng *et al.*, 2001a). This suggests that the susceptibly of *S. enterica* serovar Typhimurium TA98 might be a result of oxidative stress or most likely due to the synergetic effect of lacking the LPS structure and of loss of oxidative stress defence. Therefore, the result obtained with this strain led to more investigations to verify whether this finding is related to oxidative stress or to defective LPS structure and to find out which region of the LPS may have the most important role in the resistance to CAP treatment. The result of investigations into the role of oxidative stress will be addressed in the next chapter, and that addressing the role of LPS will discussed.

To investigate the role of the LPS in resistance of bacteria to CAP treatments derivatives of *E. coli* K-12 strain with a single mutation were tested for their sensitivities to CAP treatments.

The result of the sensitivity test obtained with *E. coli* W3110 and its isogenic mutant, *E. coli* W3110 (Δ*rfaC*) have shown no differences in the sensitivity to CAP treatment between the parental strain and its isogenic mutant. This finding indicates that deletion of the *rfaC* gene from *E. coli* W3110 (Δ*rfaC*) has not increased its susceptibility to CAP treatment. Similar results were also obtained with *E. coli* BW25113 and its isogenic mutants, *E. coli* BW25113 (Δ*rfaE*) and *E. coli* BW25113 (Δ*lpcA*). This also indicates that deletion of the *rfaE* gene from *E. coli* BW25113 (Δ*rfaE*) and *lpcA* from *E. coli* BW25113 (Δ*lpcA*) has no significant effect on their susceptibility to CAP treatments. *E. coli* W3110 and *E. coli* BW25113 are derivatives of *Escherichia coli* K-12 (Hayashi, *et al.*, 2006; Baba *et al.*, 2006) which means that they have a complete core structure but they lack the O-antigen in their LPS and have the same phenotypic rough appearance (Klena *et al.*, 1992; Liu and Reeves, 1994; Hobman *et al.*, 2007; Browning *et al.*, 2013) and each of their isogenic mutants, *E. coli* W3110 (Δ*rfaC*), *E. coli* BW25113 (Δ*rfaE*) and *E. coli* BW25113 (Δ*lpcA*) has a deletion mutation in one of the genes involved in the biosynthesis of either the inner core or the outer core parts of the core polysaccharides region (Heurlier, K., personal communication; Baba *et al.*, 2006). This suggests that losing the capability to synthesise the core polysaccharide region, partially or completely, has not increased the strain's susceptibility to CAP treatment.

The result of the sensitivity test obtained with *E. coli* MG1655 (Str^R) and its isogenic mutant, *E. coli* MG1655 (Str^R Km^R) has shown no differences in the sensitivity to CAP treatment between the parental strain and its isogenic mutant at low exposure times to CAP treatment however, at higher exposure times *E. coli* MG1655 (Str^R Km^R) has shown some resistance to CAP treatment. This finding indicates that the deletion of the *waaQ* gene from *E. coli* MG1655 (Str^R Km^R) has decreased its susceptibility to CAP treatment. This finding was not expected because *E. coli* MG1655 (Str^R Km^R) was defective in the *waaQ* gene which encodes the HepIII transferase enzyme - the enzyme involved in the biosynthesis of the core polysaccharide region (Yethon *et al.*, (1998; Møller *et al.*, 2003). However, this resistance is possibly a result of clump formation since this mutant has the tendency to form clumps (Møller *et al.*, 2003). The cells on the outside of the clumps might have prevented the CAP species from reaching the internal cells of the clumps- a shielding effect. It has also been reported by Yethon *et al.* (2000) that a *waaO* mutant of *E. coli* F470, had wild-type levels of resistance to sodium dodecyl sulfate (SDS).

Further results obtained using *E. coli* K-12 MG1655 and its derivatives (DFB1655 L5 and DFB1655 L9) showed no significant differences in the sensitivity to CAP treatments between these strains, though *E. coli* K-12 DFB1655 L5 and *E. coli* K-12 DFB1655 L9 have complete lipopolysaccharide (Browning *et al.*, 2013) unlike *E. coli* K-12 MG1655 which lacks the O-antigen (Klena, *et al.*, 1992; Liu and Reeves, 1994; Hobman *et al.*, 2007; Browning *et al.*, 2013).

To investigate whether colanic acid plays a role in resistance to CAP treatments, *E. coli* O157: H7 cultures grown on minimal medium and incubated under

conditions ideal for colanic acid production were compared with those grown in normal medium and conditions which were tested for their sensitivity to CAP treatments. No differences were found in the sensitivity between the two cultures. This indicates that growing *E. coli* O157: H7 under conditions suitable for colonic acid production has no effect on strain resistance to CAP treatments.

It can be concluded that LPS structures of Gram negative bacteria might have some role in the resistance to CAP treatment that was shown by *E. coli* O157: H7 and *E. coli* H10407 in comparison to the *E. coli* K-12 strain used in the assays, however, these results suggest that it might be other genes in these two strains that play important roles in CAP resistance especially since the complete genome sequences of *E. coli* O157: H7 has shown many differences between this strain and *E. coli* K-12 strains (Hayashi *et al.*, 200; Hobman *et al.*, 2007; Browning *et al.*, 2013). Similarly, the complete genome sequence of *E. coli* H10407 has shown that this strain, although it is similar to *E. coli* K-12, has a larger genome and four extra plasmids (Crossman *et al.*, 2010). It was also concluded that oxidative stress might have a role in the inactivation of bacteria since the *S. enterica* serovar Typhimurium TA98 has shown high susceptibility to CAP treatments.

CHAPTER 5

THE ROLE OF OXIDATIVE STRESS IN BACTERIAL INACTIVATION BY COLD ATMOSPHERIC PLASMA

5. THE ROLE OF OXIDATIVE STRESS IN BACTERIAL INACTIVATION BY COLD ATMOSPHERIC PLASMA

5.1. INTRODUCTION

The cells of aerobically grown organisms use molecular oxygen (O_2) for respiration or oxidation of nutrients, in order to meet their energy requirements. As a result of this process, cells continually produce reactive by-products such as hydrogen peroxide (H_2O_2), the superoxide anion radical (O_2^-) and highly reactive hydroxyl radicals (•OH). It has been reported that about 87% of total H_2O_2 production in *Escherichia coli* is due to the respiration process (Cabiscol *et al.*, 2000). In healthy cells, production of these reactive oxygen species (ROS) occurs at a controlled rate, and cellular defences mitigate the effects of ROS. However, under oxidative stress conditions, ROS production increases dramatically and when the concentration of these species exceeds the defence capability of the cells, subsequent alteration of membrane lipids, proteins, and nucleic acids occurs (Cabiscol *et al.*, 2000; Scandalios, 2005). Environmental conditions such as exposure to ionizing, near-UV radiation, or exposure of cells to compounds which produce intracellular O_2^- (redox-cycling agents such as menadione and paraquat) are capable of causing oxidative stress (Cabiscol *et al.*, 2000). *E. coli* possesses two regulons activated by agents that cause oxidative stress, the *soxRS* and *oxyR* regulons. The *soxRS* regulon is activated by superoxide-generating agents. It is positively controlled by a single locus and responds to agents that transfer electrons from NADH or NADPH to molecular oxygen in order to generate superoxide (Nunoshiba *et al.*, 1992). However, Krapp and co-workers have found

that the response of SoxRS in *E. coli* can be activated by modulation of NADPH even in the absence of oxidative stress and oxygen. The functional *soxRS* locus includes the *soxR* and *soxS* genes (Nunoshiba *et al.*, 1992). The *soxRS* was found to be involved in functions of at least 12 genes in response to superoxide or nitric oxide stress (Demple, 1996).

OxyR is activated by hydrogen peroxide by formation of disulfide bonds. The oxidized OxyR then activates transcription of many genes involved in oxidative stress resistance which include *dps* (encoding a nonspecific DNA binding protein), *katG* (encoding hydroperoxidase I), *ahpCF* (encoding an alkyl hydroperoxide reductase), *grxA* (encoding glutaredoxin I), *gorA* (encoding glutathione reductase), and *oxyS* (encoding a small regulatory RNA) (reviewed by Zheng *et al.*, 2001a; Krapp *et al.*, 2011).

Yoon *et al.* (2002) have found that *E. coli* K-12 TA4112 with a deletion mutation in the *oxyR* gene was more sensitive to agents that induce lipid peroxidation than *oxyR*-constitutive *E. coli* K-12 TA4110, which indicated the involvement of the OxyR protein in cell protection against lipid-peroxidation-mediated oxidative stress. Similarly, Patil *et al.* (2011) have also found that deletion of oxidative stress response genes (*oxyR* and *soxRS*) from *E. coli* K-12 BW25113 increased its susceptibility to ozone treatment, which means that these genes play an important role in the strain's resistance to free radicals produced by ozone.

In a classic study, the genome-wide transcriptional profile of *E. coli* cells treated with hydrogen peroxide (H_2O_2) was studied by Zheng and co-workers (Zheng *et al.*, 2001a). The study identified several novel genes in *E. coli* MG1655 that were

regulated by OxyR and confirmed the already known OxyR regulated genes. This study showed that there are a total of 22 genes in the OxyR regulon (see Table 5.1). Interestingly, the study has also shown that many genes in *E. coli* were H_2O_2 inducible but their regulation was independent of OxyR showing that the cellular response to oxidative stress is a complex process.

These findings clearly indicate the involvement of the *oxyR* gene and OxyR-regulated genes of *E. coli* strains in resistance to some forms of oxidative stress. Therefore, in this chapter, *E. coli* mutants with a deletion of the *oxyR* gene or deletion of one of the OxyR-regulated genes were employed to investigate the role of oxidative stress in bacterial inactivation by cold atmospheric plasma (CAP) generated at atmospheric pressure using helium and oxygen gases. This approach was chosen as it was found that the reactive species of CAP generated at atmospheric pressure using a helium oxygen mixture or ambient air are chiefly composed of reactive oxygen species (ROS), including ozone and hydroxide radicals, and reactive nitrogen species (RNS), including nitrogen oxides (Perni *et al.*, 2007; Klämpfl *et al.*, 2012).

Table 5.1: OxyR regulated genes of *E. coli* MG1655 *

Gene	Length (bp) [e]	Induction ratio in strain by H_2O_2		Function description
		Wild type	Δ*oxyR*	
ahpC	564	20	2.2	Alkyl hydroperoxide reductase small subunit [a]
ahpF	1566	22	1.2	Alkyl hydroperoxide reductase large subunit
dps	504	180	2.0	Stress response DNA binding protein; starvation induced resistance to H_2O_2 phase [a]
dsbG	747	0.7	0.7	Thiol-disulphide oxidase; multicopy resistance to DTT; mutants accumulate reduced proteins, corrected by DsbA/B overexpression [a]
fhuF	789	0.4	5.7	Ferric hydroxamate transport [a]
flu	3120	1.0	1.9	Antigen 43, phase-variable bipartite outer membrane protein; affects surface properties, piliation, colonial morphology; unstable gene [a]
fur	447	2.9	1.1	Ferric uptake regulation, negative regulatory gene [a]
gor	1353	2.1	0.9	Glutathione oxidoreductase [a]
grxA	258	37	1.2	Glutaredoxin I [a]
hemH	963	11	1.1	Ferrochelatase [a]
katG	2181	44	2.1	Catalase hydrogen peroxidise I [a]
sufA	369	21	3.6	Homology with IscA[a]
sufB	1488	16	4.0	May participate in Fe-S cluster assembly or repair

Table 5.1. Continued

sufC	747	12	3.4	Putative ABC transporter [a]
sufD	1272	8.3	3.0	May participate in Fe-S cluster assembly or repair [d]
sufE	417	8.2	3.5	transfers sulphur from sufS to sufB for iron-sulfur cluster assembly [c]
sufS	1221	3.5	1.5	Selenocysteine lyase, PLP-dependent [e]
trxC	420	21	2.1	Thioredoxin 2 [a]
yaaA	777	18	4.2	Suppress intracellular iron levels [b]
yaiA	192	56	16	predicted protein [e]
ybjM	378	15	1.1	predicted inner membrane protein [e]
yljA	321[a]	11	4.6	Function unknown

* All information in the table was taken from Zheng *et al.* (2001a) except, [a] taken from Colibri (1999), [b] taken from Liu *et al.* (2011), [c] taken from Layer *et al.* (2007), [d] taken from Bolstad and Wood (2010) and [e] *coli*BASE.

5.2 RESULTS

5.2.1. Confirmation of the *E. coli* strains/mutants' identities

The *E. coli* K-12 BW25113 strain/and all of its isogenic mutants (see Table 2.2) used in this study were from the Keio collection (Baba *et al.*, 2006) and were a kind gift from Dr. Douglas Browning of the University of Birmingham. Each mutant was obtained as up to two independent samples from stocks stored at -70°C. The *E. coli* K-12 MG1655 strain and *E. coli* O157: H7 were from the University of Nottingham. In addition *E. coli* K-12 MG1655 (Δ*oxyR*) and *E. coli* O157: H7 (Δ*oxyR*) were from Dr. Neil Doherty and Dr. Karin Heurlier both of the University of Nottingham.

The identities of all of the *E. coli* strains/mutants used in this study were verified by multiplex PCR assay. It was confirmed that all of them belonged to the species *E. coli* as described in section 3.2.1. Moreover, deletion of the specific gene in seven of *E. coli* K-12 BW25113 mutants was confirmed by PCR assays as will be described in the subsections below.

5.2.1.1. Confirmation of the *oxyR* gene deletion from *E. coli* K-12 MG1655 (Δ*oxyR*) and *E. coli* O157: H7 (Δ*oxyR*).

A multiplex PCR assay was used to confirm deletion of the *oxyR* gene from *E. coli* K-12 MG1655 (Δ*oxyR*) and *E. coli* O157: H7 (Δ*oxyR*). Three sets of primers (see Table 2.3) were specifically designed to target the *uspA* gene in all *E. coli* strains

(Chen and Griffiths, 1998), the *oxyR* gene in all *E. coli* strains (this study) and the *eaeA* gene in *E. coli* O157: H7 (China, *et al.*, 1996). The agarose gel showing the PCR products amplified from the *uspA*, *oxyR*, *eaeA* multiplex PCR assay is presented in Figure 5.1 and shows that PCR products of 884 bp were amplified from the *uspA* gene of *E. coli* MG1655, *E. coli* O157: H7 and their isogenic Δ*oxyR* mutants. In contrast, no PCR products were obtained from *S.* Typhimurium LT2 and the negative control (PCR mixture with no added template DNA). The gel also shows PCR products of 570 bp were amplified from the *eaeA* gene of *E. coli* O157: H7 strain and its isogenic Δ*oxyR* mutant, whereas no PCR products were obtained from *E. coli* MG1655, *E. coli* MG1655 (Δ*oxyR*), *S.* Typhimurium LT2 and the negative control (PCR mixture with no added template DNA). Within the same PCR, products of 353 bp were amplified from the *oxyR* gene of *E. coli* O157: H7 and *E. coli* MG1655. No PCR products were obtained from *E. coli* MG1655 (Δ*oxyR*), *E. coli* O157: H7 (Δ*oxyR*), *S.* Typhimurium LT2 and the negative control (PCR mixture with no added template DNA).

The results confirm that all *E. coli* strains/mutants used in this study belonged to the species *E. coli* (Chen and Griffiths, 1998). It also confirmed that the *E. coli* O157: H7 strain and its isogenic Δ*oxyR* mutant used in this study belonged to EPEC/EHEC group (China, *et al.*, 1996), and confirmed deletion of the *oxy*R genes of *E. coli* O157: H7 (Δ*oxyR*) and *E. coli* MG1655 (Δ*oxyR*).

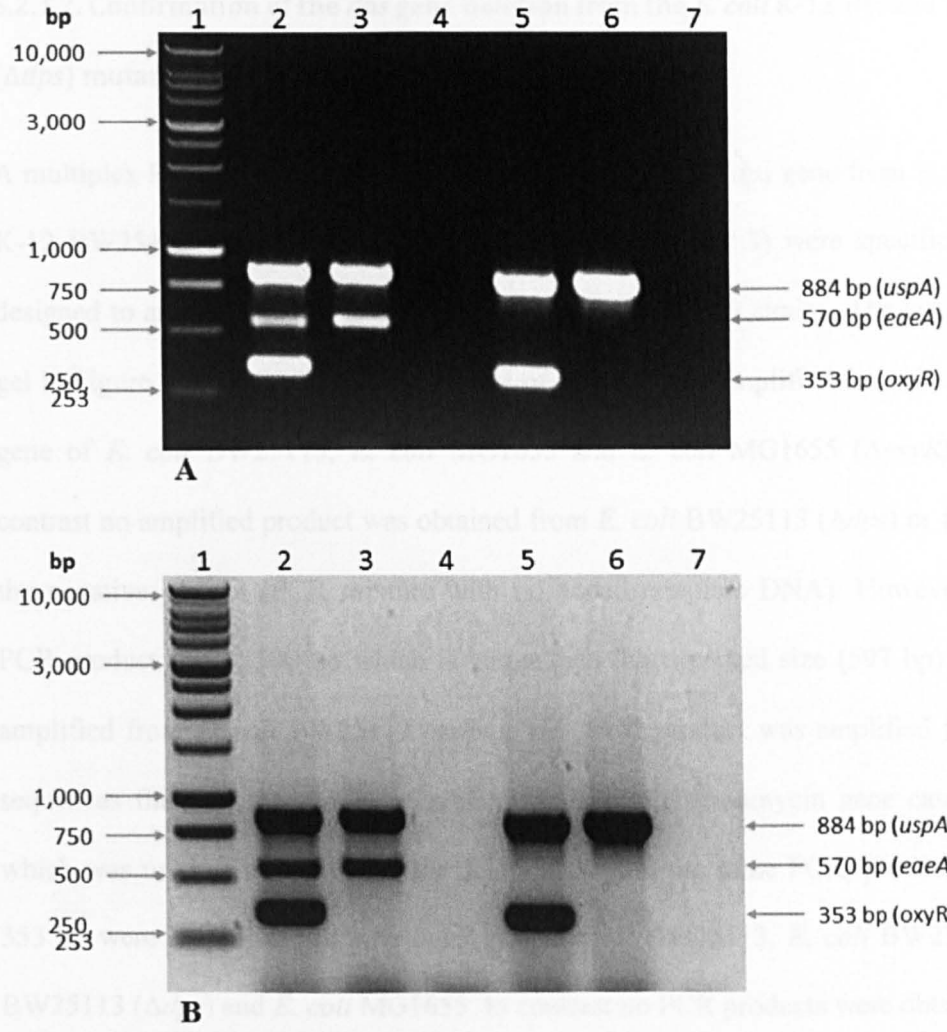

Figure 5.1: Agarose gel of PCR products confirming deletion of *oxyR* gene from *E. coli* O157: H7 (Δ*oxyR*) and *E. coli* MG1655 (Δ*oxyR*). (B = inverted image of A). Lane 1, 1 kb DNA ladder; lane 2, *E. coli* O157: H7; lane 3, *E. coli* O157: H7 (Δ*oxyR*); lane 4, *S.* Typhimurium LT2; lane 5, *E. coli* MG1655; lane 6, *E. coli* MG1655 (Δ*oxyR*); lane 7, negative control (PCR mixture with no added template DNA). The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 90 minutes.

5.2.1.2. Confirmation of the *dps* gene deletion from the *E. coli* K-12 BW25113 (Δ*dps*) mutant

A multiplex PCR assay was used to confirm deletion of the *dps* gene from *E. coli* K-12 BW25113 (Δ*dps*). Two sets of primers (see Table 2.3) were specifically designed to amplify the *dps* and *oxyR* genes from the *E. coli* strains. The agarose gel in Figure 5.2 shows that PCR products of 597 bp were amplified from the *dps* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* MG1655 (Δ*oxyR*). In contrast no amplified product was obtained from *E. coli* BW25113 (Δ*dps*) or from the negative control (PCR mixture with no added template DNA). However, a PCR product of ~ 1,500 bp which is larger than the expected size (597 bp) was amplified from *E. coli* BW25113 (Δ*dps*). This PCR product was amplified from sequences flanking the *dps* gene, which amplified the kanamycin gene cassette which was used as a marker for the deletion. Within the same PCR, products of 353 bp were amplified from the *oxyR* gene *E. coli* BW25113, *E. coli* BW25113 BW25113 (Δ*dps*) and *E. coli* MG1655. In contrast no PCR products were obtained from *E. coli* MG1655 (Δ*oxyR*), and the negative control (PCR mixture with no added template DNA).

The results confirmed deletion of the *dps* gene in BW25113 (Δ*dps*).

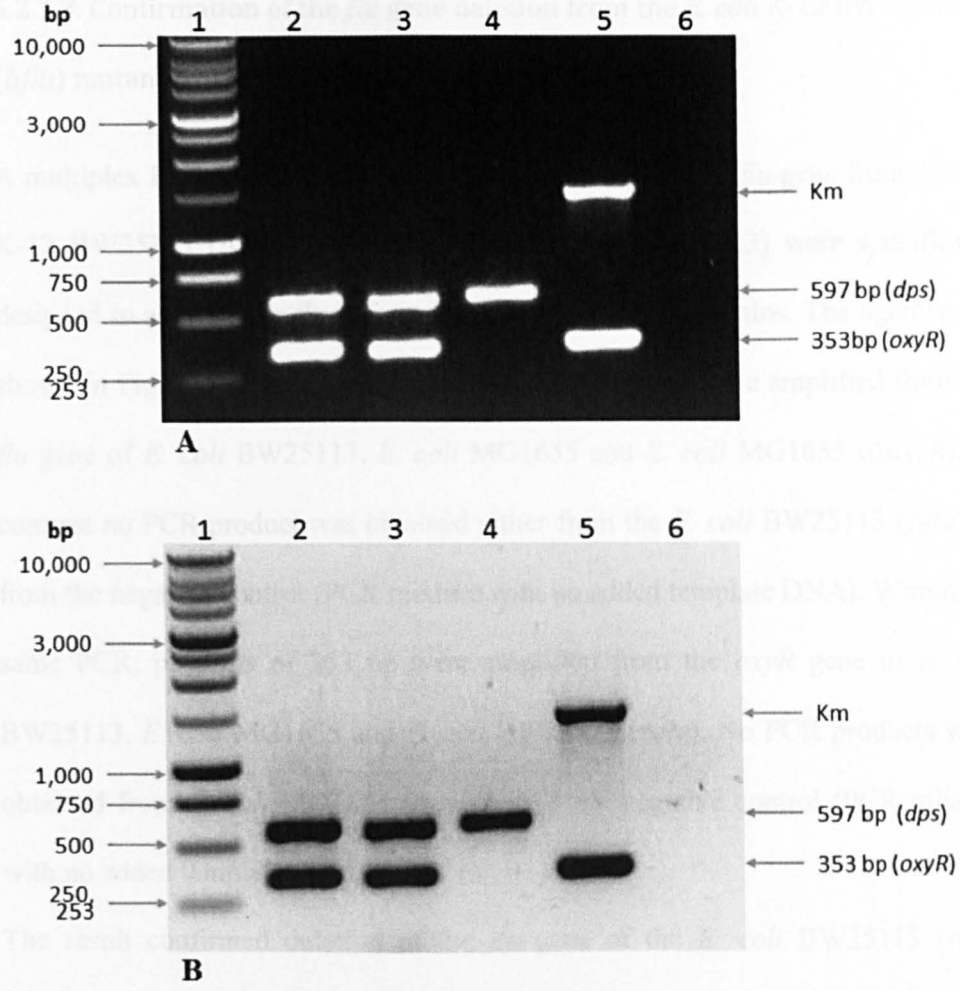

Figure 5.2: Agarose gel of PCR products confirming deletion of the *dps* gene from *E. coli* BW25113 (Δ*dps*). (B = inverted image of A). Lane 1, 1 kb DNA ladder; lane 2, *E. coli* BW25113; lane 3, *E. coli* MG1655; lane 4, *E. coli* MG1655 (Δ*oxyR*); lane 5, *E. coli* BW25113 (Δ*dps*) MG1655; lane 6, negative control (PCR mixture with no added template DNA). The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 85 minutes.

5.2.1.3. Confirmation of the *flu* gene deletion from the *E. coli* K-12 BW25113 (Δ*flu*) mutant

A multiplex PCR assay was used to confirm deletion of the *flu* gene from *E. coli* K-12 BW25113 (Δ*flu*). Two sets of primers (see Table 2.3) were specifically designed to amplify the *flu* and *oxyR* genes of the *E. coli* strains. The agarose gel shown in Figure 5.3 shows that PCR products of 985 bp were amplified from the *flu* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* MG1655 (Δ*oxyR*). In contrast no PCR product was obtained either from the *E. coli* BW25113 (Δ*flu*) or from the negative control (PCR mixture with no added template DNA). Within the same PCR, products of 353 bp were amplified from the *oxyR* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* BW25113 (Δ*flu*). No PCR products were obtained from *E. coli* MG1655 (Δ*oxyR*), and the negative control (PCR mixture with no added template DNA).

The result confirmed deletion of the *flu* gene of the *E. coli* BW25113 (Δ*flu*) mutant.

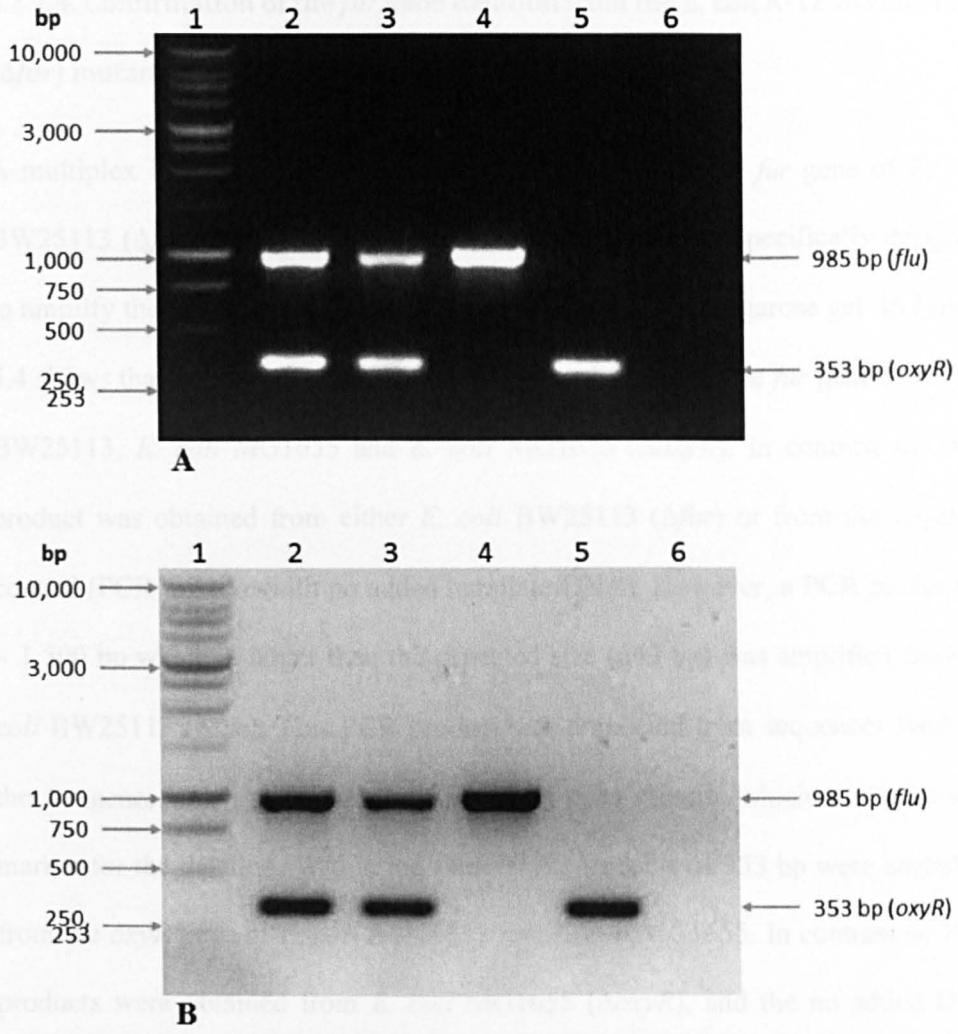

Figure 5.3: Agarose gel of PCR products confirming the deletion of the *flu* gene from *E. coli* BW25113 (Δ*flu*). (B = inverted image of A). Lane 1, 1kb DNA ladder; lane 2, *E. coli* BW25113; lane 3, *E. coli* MG1655; lane 4, *E. coli* MG1655 (Δ*oxyR*); lane 5, *E. coli* BW25113 (Δ*flu*); lane 6, negative control (PCR mixture with no added template DNA). The gel was preparted at a concentration of 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 85 minutes.

5.2.1.4. Confirmation of the *fur* gene deletion from the *E. coli* K-12 BW25113 (Δ*fur*) mutant

A multiplex PCR assay was used to confirm deletion of the *fur* gene of *E. coli* BW25113 (Δ*fur*). Two sets of primers (see Table 2.3) were specifically designed to amplify the *fur* and *oxyR* genes of the *E. coli* strains. The agarose gel in Figure 5.4 shows that PCR products of 669 bp were amplified from the *fur* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* MG1655 (Δ*oxyR*). In contrast no PCR product was obtained from either *E. coli* BW25113 (Δ*fur*) or from the negative control (PCR mixture with no added template DNA). However, a PCR product of ~ 1,500 bp which is larger than the expected size (669 bp) was amplified from *E. coli* BW25113 (Δ*fur*). This PCR product was amplified from sequences flanking the *fur* gene, which amplified the kanamycin gene cassette which was used as a marker for the deletion. Within the same PCR, products of 353 bp were amplified from the *oxyR* gene of *E. coli* BW25113 and *E. coli* MG1655. In contrast no PCR products were obtained from *E. coli* MG1655 (Δ*oxyR*), and the no added DNA control (PCR mixture with no added template DNA).

The result confirmed deletion of the *fur* gene of the *E. coli* BW25113 (Δ*fur*) mutant.

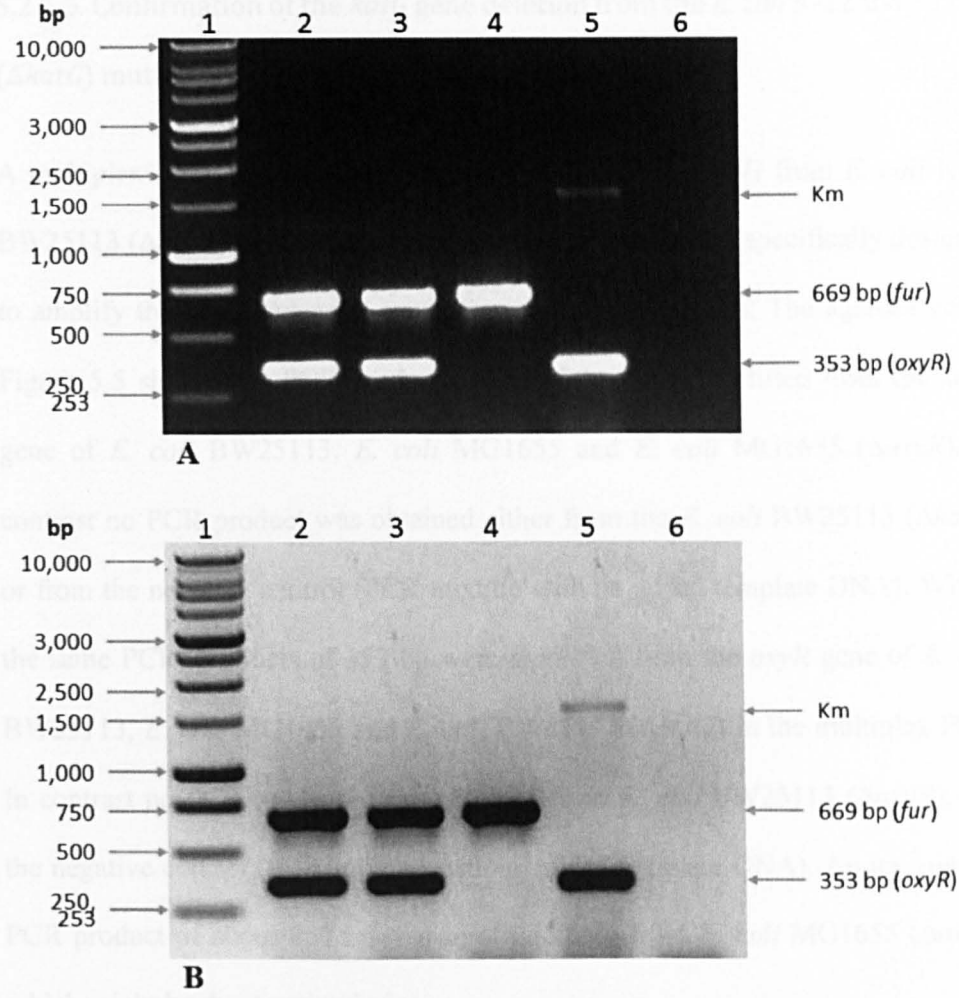

Figure 5.4: Agarose gel of PCR products confirming deletion of the *fur* gene from *E. coli* BW25113 (Δ*fur*). (B = inverted image of A). Lane 1, 1kb DNA ladder; lane 2, *E. coli* BW25113; lane 3, *E. coli* MG1655; lane 4, *E. coli* MG1655 (Δ*oxyR*); lane 5, *E. coli* BW25113 (Δ*fur*); lane 6, negative control (PCR mixture with no added template DNA). The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 85 minutes.

5.2.1.5. Confirmation of the *katG* gene deletion from the *E. coli* K-12 BW25113 (Δ*katG*) mutant

A multiplex PCR assay was used to confirm deletion of *katG* from *E. coli* K-12 BW25113 (Δ*katG*). Two sets of primers (see Table 2.3) were specifically designed to amplify the *katG* and *oxyR* genes from the *E. coli* strains. The agarose gel in Figure 5.5 shows that PCR products of 1,055 bp were amplified from the *katG* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* MG1655 (Δ*oxyR*). In contrast no PCR product was obtained either from the *E. coli* BW25113 (Δ*katG*) or from the negative control (PCR mixture with no added template DNA). Within the same PCR, products of 353 bp were amplified from the *oxyR* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* BW25113 (Δ*katG*) in the multiplex PCR. In contrast no PCR products were obtained from *E. coli* BW25113 (Δ*oxyR*), and the negative control (PCR mixture with no added template DNA). An unexpected PCR product of about 850 bp was amplified only from *E. coli* MG1655 (Δ*oxyR*) which might be due to mispriming.

The result confirmed deletion of the *katG* gene in the *E. coli* MG1655 (Δ*katG*) mutant.

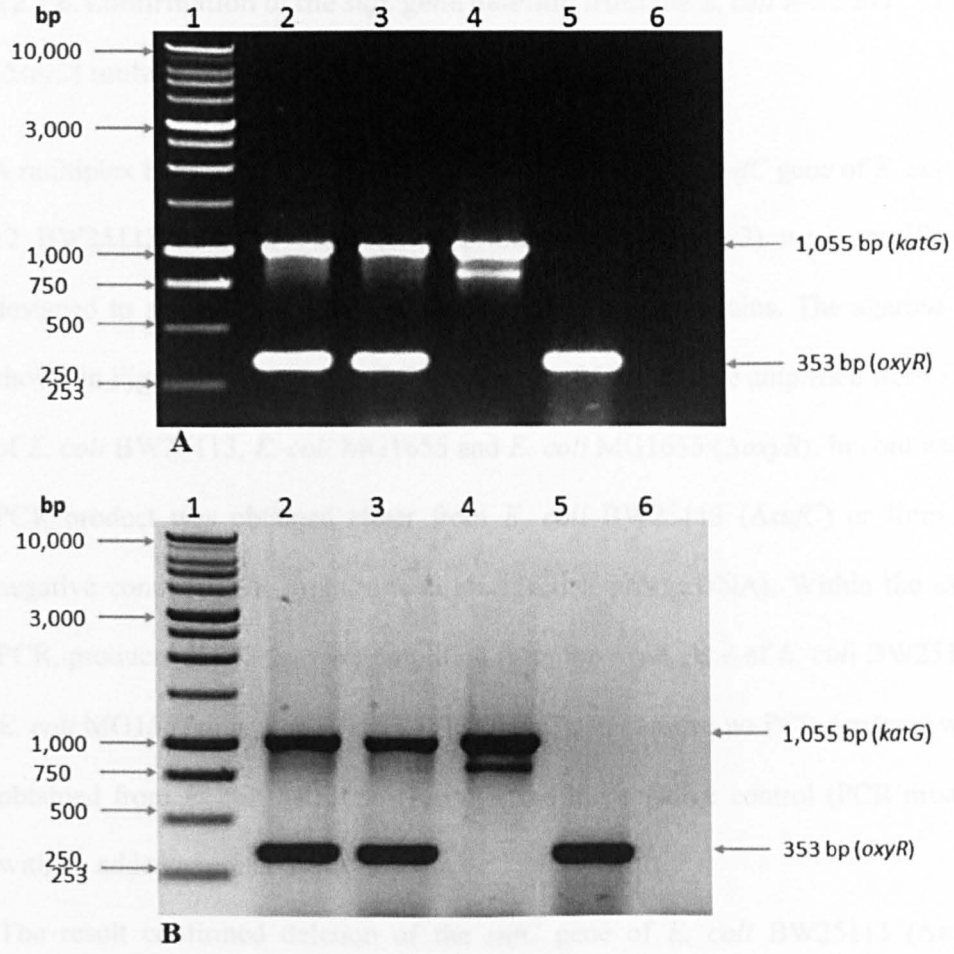

Figure 5.5: Agarose gel of PCR products confirming the deletion of the *katG* gene from *E. coli* BW25113 (Δ*katG*). (B = inverted image of A). Lane 1, 1kb DNA ladder; lane 2, *E. coli* BW25113; lane 3, *E. coli* MG1655; lane 4, *E. coli* MG1655 (Δ*oxyR*); lane 5, *E. coli* BW25113 (Δ*katG*); lane 6, negative control (PCR mixture with no added template DNA). The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 85 minutes.

5.2.1.6. Confirmation of the *sufC* gene deletion from the *E. coli* K-12 BW25113 (Δ*sufC*) mutant

A multiplex PCR assay was used to confirm deletion of the *sufC* gene of *E. coli* K-12 BW25113 (Δ*sufC*). Two sets of primers (see Table 2.3) were specifically designed to amplify the *sufC* and *oxyR* genes of *E. coli* strains. The agarose gel shown in Figure 5.6 shows that PCR products of 502 bp were amplified from *sufC* of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* MG1655 (Δ*oxyR*). In contrast no PCR product was obtained either from *E. coli* BW25113 (Δ*sufC*) or from the negative control (PCR mixture with no added template DNA). Within the same PCR, products of 353 bp were amplified from the *oxyR* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* BW25113 (Δ*sufC*). In contrast no PCR products were obtained from *E. coli* MG1655 (Δ*oxyR*), and the negative control (PCR mixture with no added template DNA).

The result confirmed deletion of the *sufC* gene of *E. coli* BW25113 (Δ*sufC*) mutant.

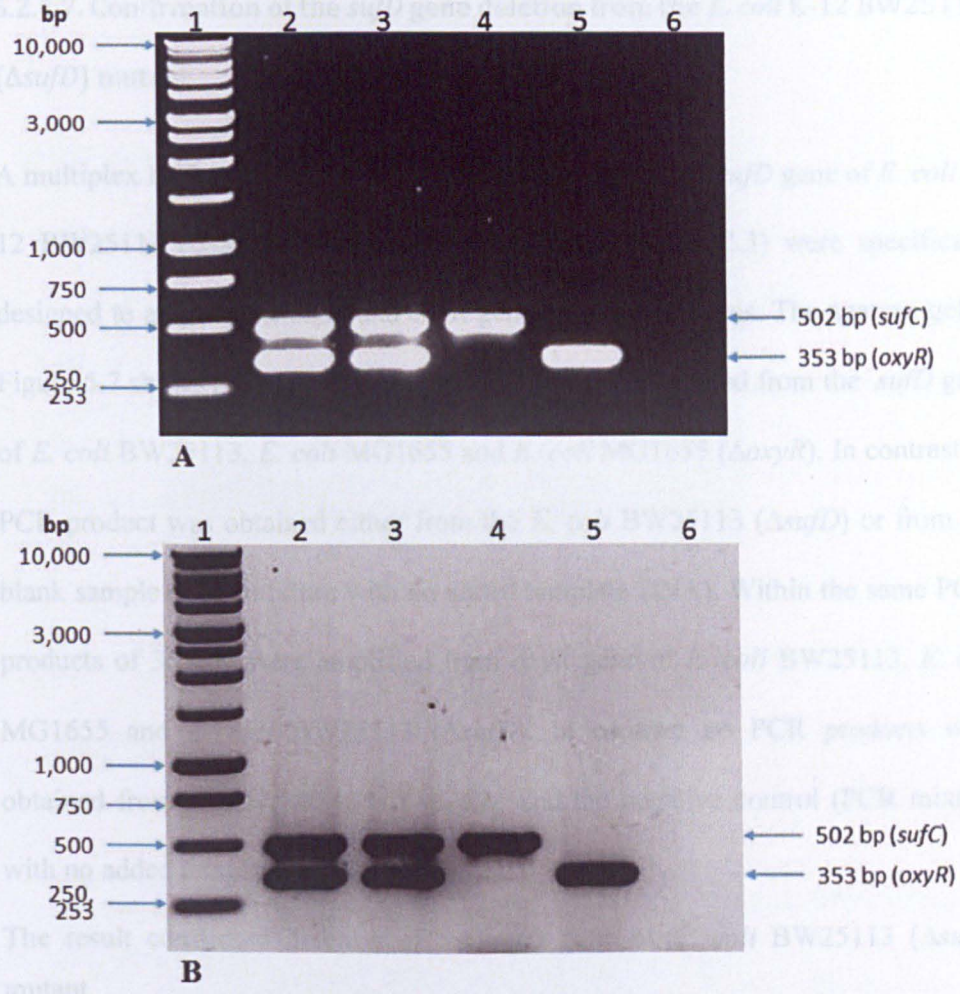

Figure 5.6: Agarose gel of PCR products confirming the deletion of the *sufC* gene from *E. coli* BW25113 (Δ*sufC*). (B = inverted image of A). Lane 1, 1kb DNA ladder; lane 2, *E. coli* BW25113; lane 3, *E. coli* MG1655; lane 4, *E. coli* MG1655 (Δ*oxyR*); lane 5, *E. coli* BW25113 (Δ*sufC*); lane 6, negative control (PCR mixture with no added template DNA). The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for about 85 minutes.

5.2.1.7. Confirmation of the *sufD* gene deletion from the *E. coli* K-12 BW25113 (Δ*sufD*) mutant

A multiplex PCR assay was used to confirm deletion of the *sufD* gene of *E. coli* K-12 BW25113 (Δ*sufD*). Two sets of primers (see Table 2.3) were specifically designed to amplify the *sufD* and *oxyR* genes of *E. coli* strains. The agarose gel in Figure 5.7 shows that PCR products of 703 bp were amplified from the *sufD* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* MG1655 (Δ*oxyR*). In contrast no PCR product was obtained either from the *E. coli* BW25113 (Δ*sufD*) or from the blank sample (PCR mixture with no added template DNA). Within the same PCR, products of 353 bp were amplified from *oxyR* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* BW25113 (Δ*sufD*). In contrast no PCR products were obtained from *E. coli* MG1655 (Δ*oxyR*), and the negative control (PCR mixture with no added template DNA).

The result confirmed deletion of the *sufD* gene of *E. coli* BW25113 (Δ*sufD*) mutant.

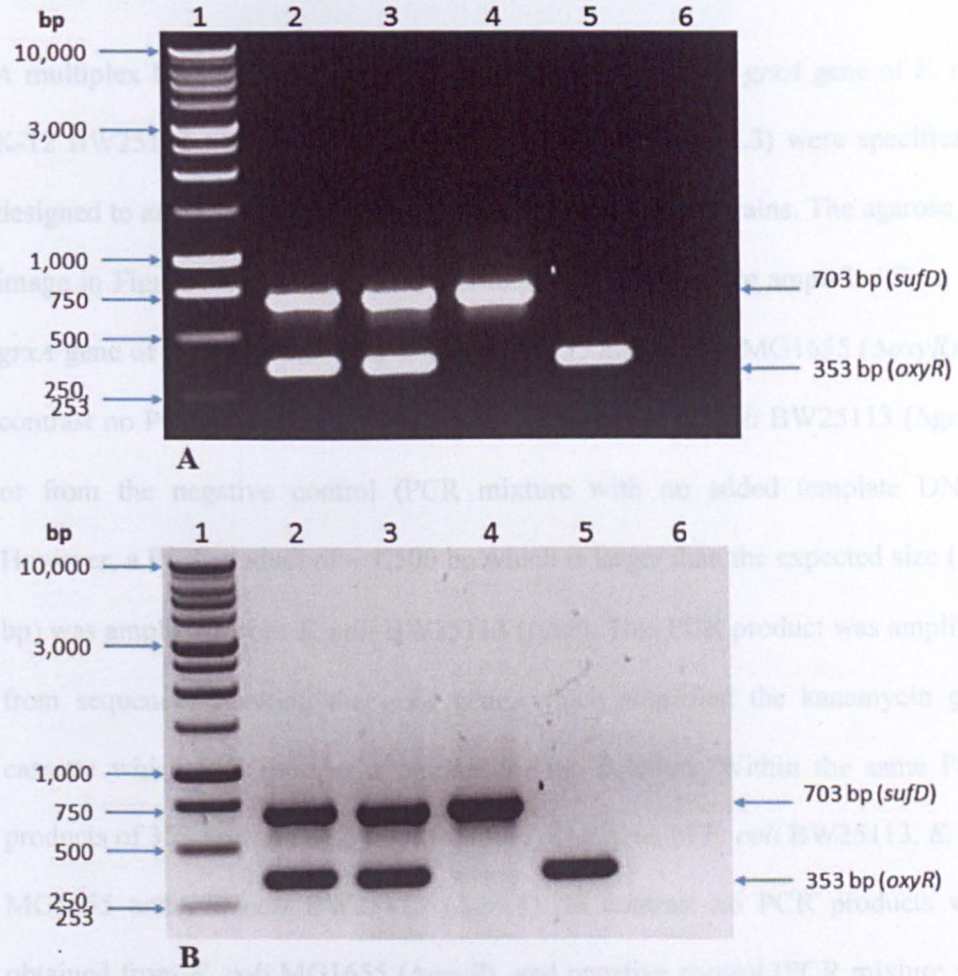

Figure 5.7: Agarose gel of PCR products confirming the deletion of the *sufD* gene from *E. coli* BW25113 (Δ*sufD*). (B = inverted image of A). Lane 1, 1kb DNA ladder; lane 2, *E. coli* BW25113; lane 3, *E. coli* MG1655; lane 4, *E. coli* MG1655 (Δ*oxyR*); lane 5, *E. coli* BW25113 (Δ*sufD*); lane 6, negative control (PCR mixture with no added template DNA). The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 85 minutes.

5.2.1.8. Confirmation of the *grxA* gene deletion from the *E. coli* K-12 BW25113 (Δ*grxA*) mutant

A multiplex PCR assay was used to confirm deletion of the *grxA* gene of *E. coli* K-12 BW25113 (Δ*grxA*). Two sets of primers (see Table 2.3) were specifically designed to amplify the *grxA* and *oxyR* genes of the *E. coli* strains. The agarose gel image in Figure 5.8 shows that PCR products of 534 bp were amplified from the *grxA* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* MG1655 (Δ*oxyR*). In contrast no PCR product was obtained either from the *E. coli* BW25113 (Δ*grxA*) or from the negative control (PCR mixture with no added template DNA). However, a PCR product of ~ 1,500 bp which is larger than the expected size (534 bp) was amplified from *E. coli* BW25113 (Δ*fur*). This PCR product was amplified from sequences flanking the *grxA* gene, which amplified the kanamycin gene cassette which was used as a marker for the deletion. Within the same PCR, products of 353 bp were amplified from the *oxyR* gene of *E. coli* BW25113, *E. coli* MG1655 and *E. coli* BW25113 (Δ*grxA*). In contrast no PCR products were obtained from *E. coli* MG1655 (Δ*oxyR*), and negative control (PCR mixture with no added template DNA).

The result confirmed deletion of the *grxA* gene of *E. coli* BW25113 (Δ*grxA*) mutant.

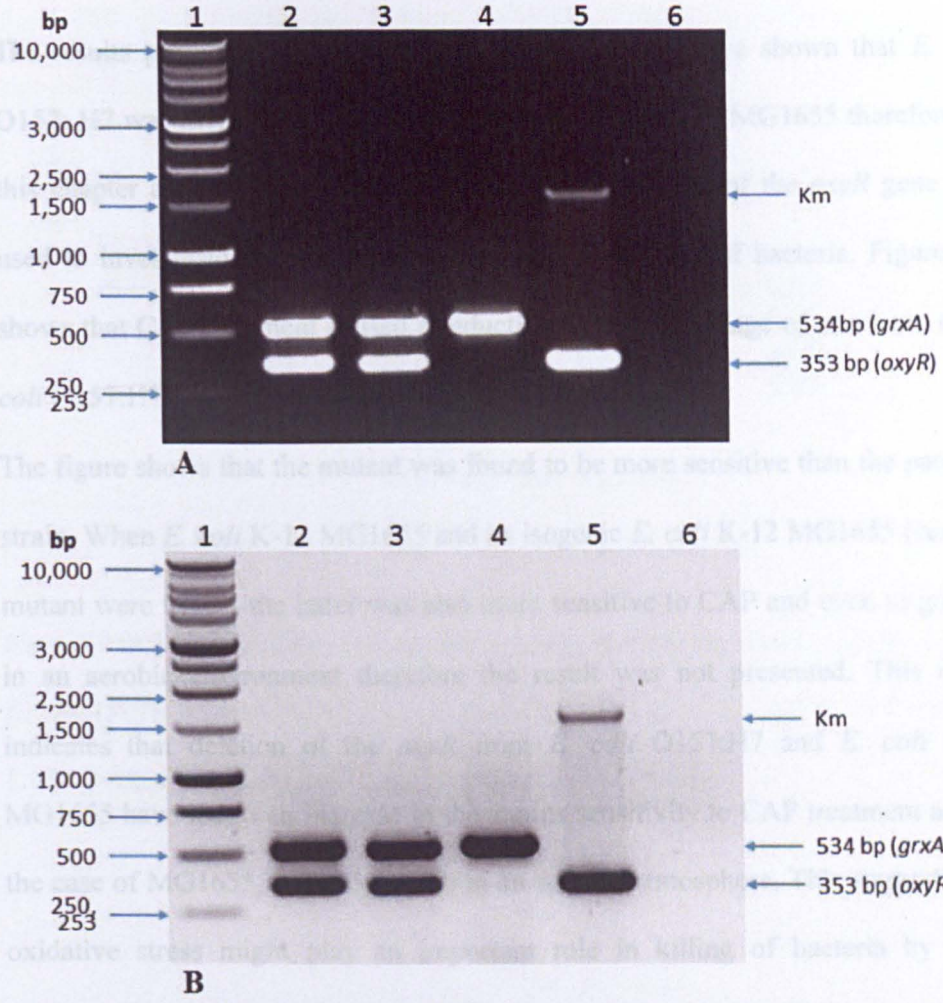

Figure 5.8: Agarose gel of PCR products confirming deletion of the *grxA* gene from *E. coli* BW25113 (Δ*grxA*). (B = inverted image of A). Lane 1, 1kb DNA ladder; lane 2, *E. coli* BW25113; lane 3, *E. coli* MG1655; lane 4, *E. coli* MG1655 (Δ*oxyR*); lane 5, *E. coli* BW25113 (Δ*grxA*); lane 6, negative control (PCR mixture with no added template DNA). The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 85 minutes.

5.2.2. Sensitivity of *E. coli* strains and mutants to cold atmospheric plasma

The results presented earlier in chapter 3 (Figure 3.4) have shown that *E. coli* O157: H7 was more resistant to CAP treatment than *E. coli* MG1655 therefore, in this chapter a mutant of *E. coli* O157:H7 with a deletion of the *oxyR* gene was used to investigate the role of oxidative stress in killing of bacteria. Figure 5.9 shows that CAP treatment caused a reduction in the percentage of survivors of *E. coli* O157:H7 (Δ*oxyR*) compared to the wild-type strain.

The figure shows that the mutant was found to be more sensitive than the parental strain. When *E. coli* K-12 MG1655 and its isogenic *E. coli* K-12 MG1655 (Δ*oxyR*) mutant were tested, the latter was also more sensitive to CAP and even to growth in an aerobic environment therefore the result was not presented. This result indicates that deletion of the *oxyR* from *E. coli* O157:H7 and *E. coli* K-12 MG1655 have led to an increase in the strains sensitivity to CAP treatment and in the case of MG1655, even to growth in an aerobic atmosphere. This suggests that oxidative stress might play an important role in killing of bacteria by CAP treatment. Therefore, further investigations into the role of oxidative stress were conducted using mutants of *E. coli* BW25113 with a deletion of one of the genes in the OxyR regulon.

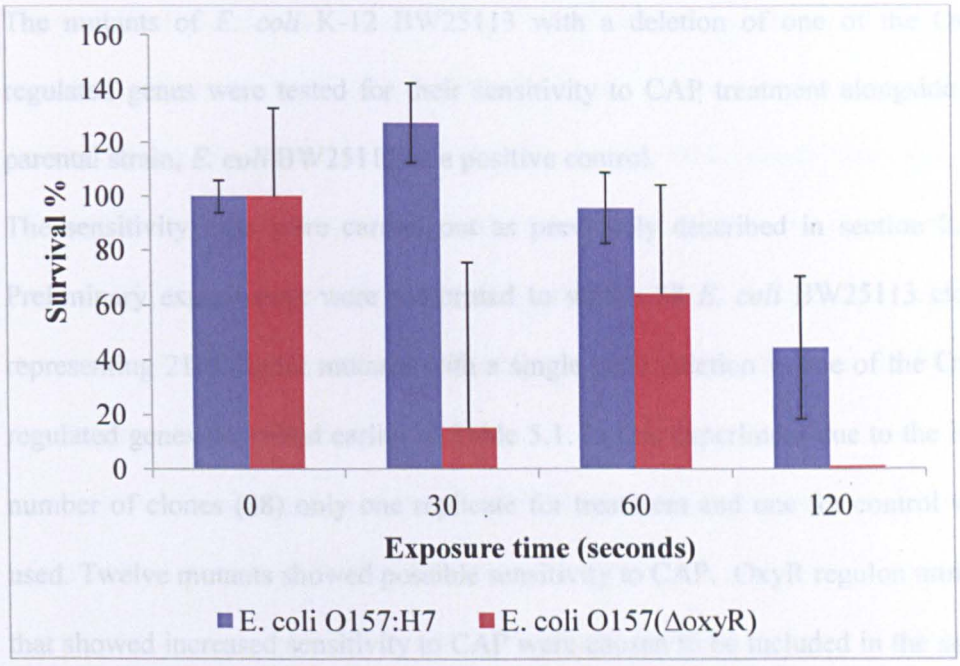

Figure 5.9: Effect of different exposure times to CAP treatment on survival of *E. coli* O157:H7 and its isogenic *E. coli* O157:H7 (Δ*oxyR*). (Mean survival % ± 1 SD, n = 3 replicates).

The mutants of *E. coli* K-12 BW25113 with a deletion of one of the OxyR regulated genes were tested for their sensitivity to CAP treatment alongside the parental strain, *E. coli* BW25113 as a positive control.

The sensitivity tests were carried out as previously described in section 2.6.1. Preliminary experiments were performed to screen 38 *E. coli* BW25113 clones representing 21 different mutants with a single gene deletion in one of the OxyR regulated genes described earlier in Table 5.1. In this experiment due to the large number of clones (38) only one replicate for treatment and one for control were used. Twelve mutants showed possible sensitivity to CAP. OxyR regulon mutants that showed increased sensitivity to CAP were chosen to be included in the study. To confirm the sensitivity of these mutants to CAP treatment, plates of each mutant were prepared and were exposed to CAP treatment for 0, 1 and 2 minutes. Four replicates were used for each treatment. After incubation at 30°C for 24 hours, colonies on the entire plate were counted and the percentage of survivors calculated. The percentage of survivors was used to compare the sensitivity of each mutant to the parent strain *E. coli* BW25113. Practically, it was not possible to test all mutants at the same time (due to the large number of plates) therefore; four separate experiments were conducted. In each experiment three mutants only were tested together in one experiment. In addition to the mutants, the parental strain (*E. coli* BW25113) was included as a control in each experiment, in order to avoid variations which may occur due to CAP instability from one day to another. The results of these experiments are presented in Figures 5.10 - 5.12.

Figure 5.10 shows that there were no significant differences between the means of percentage survivors of *E. coli* BW25113 strains carried out at different times (weeks) using the same experimental conditions. This result indicates that although there was some variation between the means of the percentage survivors between different experiments, which is difficult to avoid, results of CAP treatments were reproducible. The average percentage of survivors of the four experiments was used as a control for all experiments and the results were combined into two graphs in order to make it easier to compare between the means of percentage survivors of different mutants.

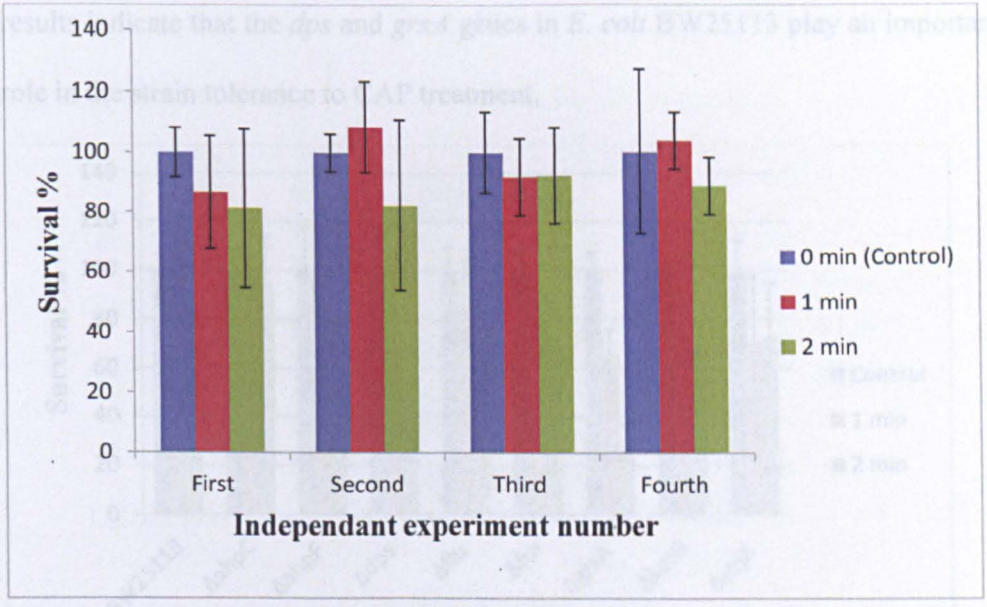

Figure 5.10: Reproducibility of CAP treatments of *E. coli* BW25113. (Mean survival % ± 1 SD, n = 4 replicates).

The result presented in Figure 5.11 show that CAP treatment for up to 2 minutes has not significantly reduced the percentage survivors of *E. coli* BW25113, *E. coli* BW25113 (Δ*ahpC*), *E. coli* BW25113 (Δ*ahpF*), and *E. coli* BW25113 (Δ*yajA*). In contrast, CAP has significantly ($P < 0.05$) reduced the percentage survivors of *E. coli* BW25113 (Δ*dps*), *E. coli* BW25113 (Δ*flu*), *E. coli* BW25113 (Δ*fur*), *E. coli* BW25113 (Δ*grxA*) and *E. coli* BW25113 (Δ*katG*). However, when the percentage survivors of these mutant were compared with the control treated with CAP for the same time (2 minutes) only *E. coli* BW25113 (Δ*dps*) and *E. coli* BW25113 (Δ*grxA*) were found to be significantly ($P < 0.05$) more sensitive to CAP treatment than other mutants and the parental strain *E. coli* BW25113. These results indicate that the *dps* and *grxA* genes in *E. coli* BW25113 play an important role in the strain tolerance to CAP treatment.

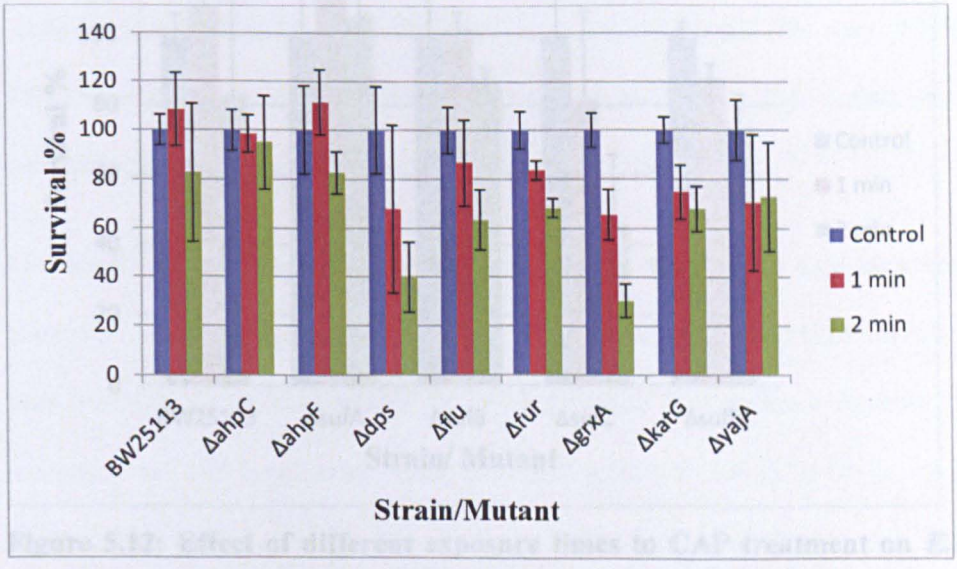

Figure 5.11: Effect of different exposure times to CAP treatment on *E. coli* BW25113 and its isogenic mutants (Δ*ahpC*, Δ*ahpF*, Δ*dps*, Δ*flu*, Δ*fur*, Δ*grxA*, Δ*katG* and Δ*yajA*). (Mean survival % ± 1 SD, n = 4 replicates).

The results shown in Figure 5.12 shows that CAP treatment for up to 2 minutes had not significantly reduced the survival percentage of *E. coli* BW25113, *E. coli* BW25113 (Δ*sufA*) and *E. coli* BW25113 (Δ*sufB*). Whereas CAP has significantly ($P < 0.05$) reduced the survival percentage of *E. coli* BW25113 (Δ*sufD*) and *E. coli* BW25113 (Δ*sufC*). Nevertheless, the results have shown that only *E. coli* BW25113 (Δ*sufC*) was significantly ($P < 0.05$) more sensitive to CAP treatment than other mutants and the parental strain *E. coli* BW25113. This result indicates that the *sufC* in *E. coli* BW25113 plays an important role in the strain's resistance to CAP treatment.

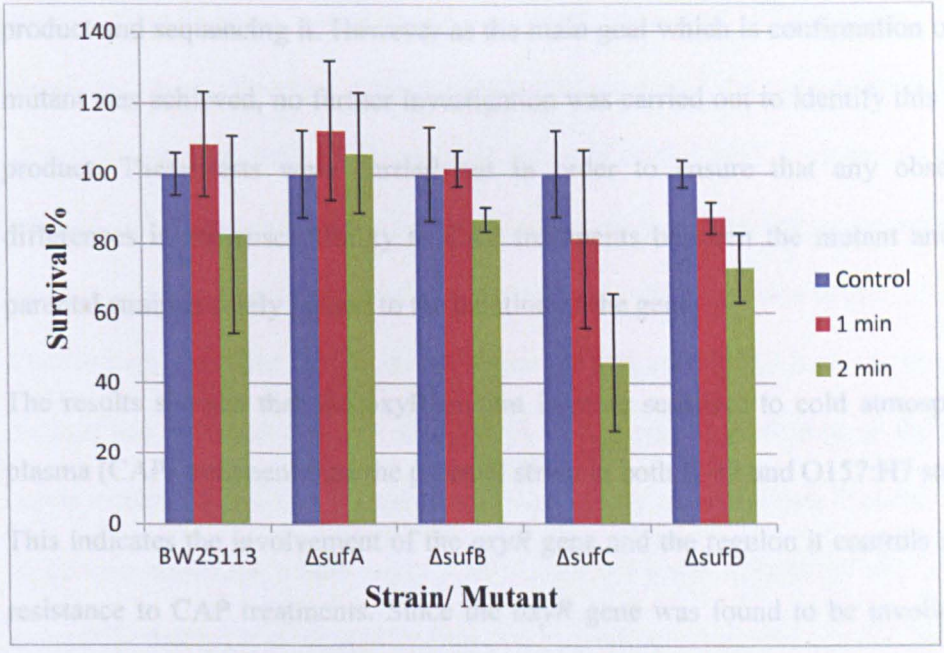

Figure 5.12: Effect of different exposure times to CAP treatment on *E. coli* BW25113 and its isogenic mutants (Δ*sufA*, Δ*sufB*, Δ*sufC* and Δ*sufD*). (Mean survival % ± 1 SD, n = 4 replicates).

5.3 DISCUSSION

The identities of all of the strains/mutants were confirmed to belong to the *E. coli* species as shown in chapter 3.The deletion of the gene of interest from *E. coli* K-12 MG1655 (Δ*oxyR*), *E. coli* O157: H7 (Δ*oxyR*), *E. coli* K-12 BW25113 (Δ*dps*), *E. coli* K-12 BW25113 (Δ*flu*), *E. coli* K-12 BW25113 (Δ*fur*), *E. coli* K-12 BW25113 (Δ*katG*), *E. coli* K-12 BW25113 (Δ*sufC*), *E. coli* K-12 BW25113 (Δ*sufD*) and *E. coli* K-12 BW25113 (Δ*grxA*) were all confirmed by PCR assays. The unexpected PCR product of about 850 bp amplified from *E. coli* MG1655 (Δ*oxyR*) using primers designed to amplify the *oxyR* and *katG* genes might be due to mispriming. Identification of this product can be achived by isolating the PCR product and sequencing it. However as the main goal which is confirmation of the mutant was achieved, no further investigation was carried out to identify this PCR product. These tests were carried out in order to ensure that any observed differences in the susceptibility to CAP treatments between the mutant and the parental strain is solely related to the deletion of the gene.

The results showed that the oxyR mutant is more sensitive to cold atmospheric plasma (CAP) treatment than the parental strain in both K-12 and O157:H7 strains. This indicates the involvement of the *oxyR* gene and the regulon it controls in the resistance to CAP treatments. Since the *oxyR* gene was found to be involved in resistance to oxidative stress (Yoon *et al.*, 2002; Patil *et al.*, 2011), this finding suggests that the inactivation of the bacterial cells might be due to an oxidizing agent existing in the gas mixture, or that plasma causes the generation of ROS species within the bacterial cell. This has led to more investigations, in which *E.*

coli BW25113 mutants (Baba *et al.*, 2006) with single gene deletions in one of the genes that have been found to be regulated by OxyR were exposed to CAP (Zheng *et al.*, 2001a). These investigations showed that *E. coli* BW25113 (Δ*dps*), *E. coli* BW25113 (Δ*grxA*) and *E. coli* BW25113 (Δ*sufC*), were significantly ($P < 0.05$) more sensitive to CAP treatment than other mutants and the parental strain. The *dps* gene is regulated by *oxy*R and encodes a DNA binding protein (Zheng *et al.* (2001a). Dps is a nonspecific DNA-binding protein first isolated from starved cells and has an important role in gene expression and also protects DNA during stationary phase from oxidative stress (Almiron *et al.*, 1992) and many other adverse conditions which include UV and gamma irradiation, iron and copper toxicity, thermal stress, and acid and base shock (Nair and Finkel, 2004). It was found that Dps reduces the number of DNA single-strand breaks and the deletion of the *dps* gene has led to an increase in the level of G.C to T.A mutations in *dps* mutants, when the mutant was treated with hydrogen peroxide. Transversion mutations in DNA are features of oxidative damage (Martinez and Kolter, 1997). Chodavarapu and co-workers have suggested that during oxidative stress, Dps reduces the initiation of DNA replication which gives opportunity to the DNA repair mechanisms to repair oxidative DNA damage and increases the probability of genetic mutations which might lead to survival under conditions of oxidative stress (Chodavarapu *et al.*, 2008). Dps was found to protect cells against oxidative stress by binding to the Fe^{2+}, which can act as a Fenton reagent leading to generation of free radicals (Yamamoto *et al.*, 2011).

The *sufB*, *sufC*, and *sufD* genes are regulated by OxyR and encode components of an ATP binding cassette (ABC) transporter (Zheng *et al.*, 2001a). It was found

that SufC and SufD are required for formation of an iron-sulfur [Fe-S] cluster on SufB and the ATPase activity of SufC is essential for iron acquisition through [Fe-S] cluster assembly under oxidative stress and iron limitation (Nachin *et al.*, 2003; Saini *et al.*, 2010). [Fe-S] clusters are required as cofactors in may critical cellular pathways and are involved in many reactions which include electron transfer and substrate binding and activation (Nachin *et al.*, 2003; Saini *et al.*, 2010).

The *grx*A gene is an OxyR regulated gene which encodes glutaredoxin 1 and is inducible by hydrogen peroxide (Tao, 1997; Zheng *et al.*, 2001a; Zheng *et al.*, 2001b). Glutaredoxin 1 is one of the three Glutaredoxins found in *E. coli*. Glutaredoxin and thioredoxin systems have a role in reducing disulfide bonds in cytoplasmic proteins and they have the ability to partially substitute for each other (Prinz *et al.*,1997).

Interestingly, all the mutants that showed higher sensitivity to CAP were in genes that have important roles in protecting cells aginst the damage caused by oxidative stress, and since the UV and thermal effects can be neglected as was discussed earlier, this finding suggests that the inactivation of bacteria outside the direct effect of plasma etching and damage to cells is mainly a result of oxidative stress by reactive oxygen species possibly by damaging the DNA of the cells as the deletion of the *dps* gene has led to an increase in the level of G.C to T.A mutations in the *dps* mutants, when they were treated with hydrogen peroxide which produces oxidative damage (Martinez and Kolter, 1997). Therefore further investigation in order to find out whether CAP might induce such mutations is essential. This will be addressed using Ames strains test which are used worldwide

as an initial screening test for the assessment of the likely mutagenic effects of new chemicals and drugs (ISO, 2012).

CHAPTER 6

ASSESSMENT OF COLD ATMOSPHERIC PLASMA MUTAGENICITY USING THE AMES *SALMONELLA* MUTAGENICITY ASSAY

6. ASSESSMENT OF COLD ATMOSPHERIC PLASMA MUTAGENICITY USING THE AMES *SALMONELLA* MUTAGENICITY ASSAY

6.1. INTRODUCTION

The Ames/*Salmonella* assay is a test designed to detect genetic alterations in the deoxyribonucleic acid (DNA) of a bacterial cell which lead to gene mutations. The test uses a set of *Salmonella* strains. These strains have different gene mutations in their histidine synthesis operon. As a result of these mutations, the functionality of the histidine synthesis genes in these strains has been lost and consequently they have become histidine dependant. Therefore, these strains cannot grow and form colonies on media that lacks the amino acid histidine. However, the occurrence of compensating or revertant mutations in the histidine synthesis genes of these strains can lead to the restoration of their functionality, allowing the strains to become histidine independent, which allows them to grow and form colonies on media that is deficient in histidine (Mortelmans and Zeiger, 2000). Each of the Ames strains was initially constructed by making a mutation in the histidine operon at different sites so that the strain can respond to mutagens that act by a different mechanism which could not be detected by the other strains (see Table 6.1).

Table 6.1 DNA sequence reversion target specificity for the *Salmonella* tester strains*

Strain	Mutation	DNA target	Reversion event	Reference
TA100 TA1535	*hisG46*	-G-G-G-	Base-pair substitution	Barnes *et al.* (1982) Cited in Mortelmans and Zeiger (2000)
TA98 TA1538	*hisD3052*	-C-G-C-G-C-G-C-G-	Frameshifts	Isono, and Yourno, (1974) Cited in Mortelmans and Zeiger (2000)
TA1537	*hisC3076*	+1 Frameshifts (near -C-C-C- run)	Frameshifts	Ames *et al.* (1973)
TA97	*hisD6610* *hisO1242*	-C-C-C-C-C-C- (+1 cytosine at run of C's)	Frameshifts	Levin *et al.* (1982) Cited in Mortelmans and Zeiger (2000)
TA102 TA104	*hisG428*	TAA(ochre)	Transition/transversion	Levin *et al.* (1982a)

(*): Table taken from Mortelmans and Zeiger (2000)

More mutations were constructed into these strains by Ames and co-workers (Mortelmans and Zeiger, 2000) in order to increase their sensitivities to a wide range of mutagenic substances. These mutations included: 1) a deletion mutation in the *uvrB-bio* genes that has been constructed in all of the strains, except the TA102 strain. Deletion of *uvrB* inactivates the UvrABC highly accurate DNA damage detection and excision repair mechanism, consequently allowing the error-prone DNA repair mechanism to repair more DNA lesions. The method used to delete the *uvrB* gene also caused the deletion of the *bio* genes (which makes the strains biotin dependent), and deleted the galactose operon (*gal*), and the nitrate reductase gene (*chl*) (Ames *et al.*, 1973); 2) a deletion in the *rfa* genes. This mutation has disabled the strains' capability to synthesize the lipolysaccharide (LPS) layer on the outside of the cell. Thus, the strains have become more permeable to bulky chemicals (Wilkinson *et al.*, 1972; Ames *et al.*, 1973).

Furthermore the pKM101 plasmid, is a 35.4 kb plasmid derived from plasmid R 46. It was constructed by deletion of 13.8 kb region which carries the marker conferring resistance to streptomycin, sulfonamide and tetracycline of plasmid R46 (Mortelmans, 2006). Plasmid pKM101 boosts the error-prone recombinational DNA repair pathway, consequently improving chemical and UV-induced mutagenesis (Langer, Shanabruch and Walker, 1981; Mortelmans and Zeiger, 2000), was introduced into some of the Ames strains. This process resulted in new Ames strains which include TA97, TA98, TA100, TA102 and TA104. Plasmid pKM101 is ampicillin resistant thus its presence in the strains can be easily verified (Levin *et al.*, 1982; Mortelmans and Zeiger, 2000; Mortelmans, 2006). Additionally, a multicopy plasmid, pAQ1, was

introduced into the TA102 strain in order to increase the number of potential target sites for mutations. This plasmid carries the *hisG428* mutation and confers teteracycline resistance, so its existence in the strain can be easily verified. This strain, TA102, has retained an intact copy of its *uvrB* gene, which has made the strain proficient in DNA repair and improves its capability to detect DNA cross-linking agents (Levin *et al.*, 1982; Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

A summary of the genotypes of the most commonly used *Salmonella* tester strains are shown in Table 6.2.

Table 6.2. Genotype of the most commonly used *Salmonella* tester strains*

Mutation (strain)	*bio chlD uvrB gal*	LPS defect	Plasmid
hisG46			
TA1535	Deletion mutation	*rfa*	No plasmid
TA100	Deletion mutation	*rfa*	pKM101
hisD3052			
TA1538	Deletion mutation	*rfa*	No plasmid
TA98	Deletion mutation	*rfa*	pKM101
hisC3076			
TA1537	Deletion mutation	*rfa*	No plasmid
hisD6610			
hisO1242			
TA97	Deletion mutation	*rfa*	pKM101
hisG428			
TA104	Deletion mutation	*rfa*	No plasmid
TA102	Wild type	*rfa*	pKM101, pAQ1

(*): Table taken from Mortelmans and Zeiger (2000)

The Ames test was specifically designed to detect chemicals that cause mutagenic effects. Over time, the Ames test has become recognized by many scientists, governmental and nongovernmental organizations as a standard test, and has been adopted as an ISO test (ISO, 2012). The test is used widely all over the world as an initial screening test for assessment of the likely mutagenic effects of new chemicals and drugs because there is a greater likelihood that a chemical or drug is carcinogenic to animals when it is positive as a mutagen in the Ames *Salmonella* strains (Marton and Ames, 1983; Mortelmans and Zeiger, 2000). International guidelines have also been

established in order to ensure uniformity of testing procedures e.g. Organisation for Economic Co-operation and Development (OECD, 1997) and International Commission on Harmonization (ICH, 2012) International Organization for Standardization, (ISO, 2012) and European Medicines Agency (EMA, 2013).

Therefore, and in view of the results obtained in the previous chapter which indicate that the killing of bacteria by CAP was due to oxidative stress caused by reactive species in the CAP, in this chapter a set of Ames strains which have been recommended by many international organizations (OECD, 1997; ISO, 2012 and EMA, 2013) to detect mutagenic effects caused by chemicals, were employed to investigate the hypothesis that CAP causes DNA alteration/damage due to generation of Reactive Oxygen Species (ROS) in the plasma. The Ames strains set included the *S* Typhimurium TA102 strain, which was specifically designed to detect a variety of oxidative mutagens (Levin *et al.*, 1982), in addition to *S.* Typhimurium TA98, *S.* Typhimurium TA100, S. Typhimurium TA1535 and *S.* Typhimurium TA1537.

6.2. RESULTS

The Ames strains were obtained from a commercial laboratory: Moltox Incorporated (Moltox Inc. Florida, USA). Before any mutagenicity tests were carried out, the strains were tested to confirm their identity, phenotypes and response to mutagens, in accordance with standard tests (OECD, 1997; ISO, 2012; EMA, 2013).

6.2.1. Confirmation of the identity of the Ames strains

The strains were tested to confirm that they were *S.* Typhimurium. The results presented in Fig. 6.1 shows that a PCR product of 571 bp was amplified from the *spvC* gene of each of the Ames strains used in this study (*S.* Typhimurium TA98, *S.* Typhimurium TA100, *S.* Typhimurium TA102, *S.* Typhimurium TA1535 and *S.* TyphimuriumTA1537). No PCR products were obtained from the negative controls (*S.* Typhimurium LT2 as this strain was attenuated (has no *spvC* gene); *E. coli* MG1655, and a no template negative control (PCR mixture containing *spvC* primers, but with no added template DNA).

The result confirms that all Ames strains used in this study belonged to the genus *Salmonella* (Chiu and Ou, 1996).

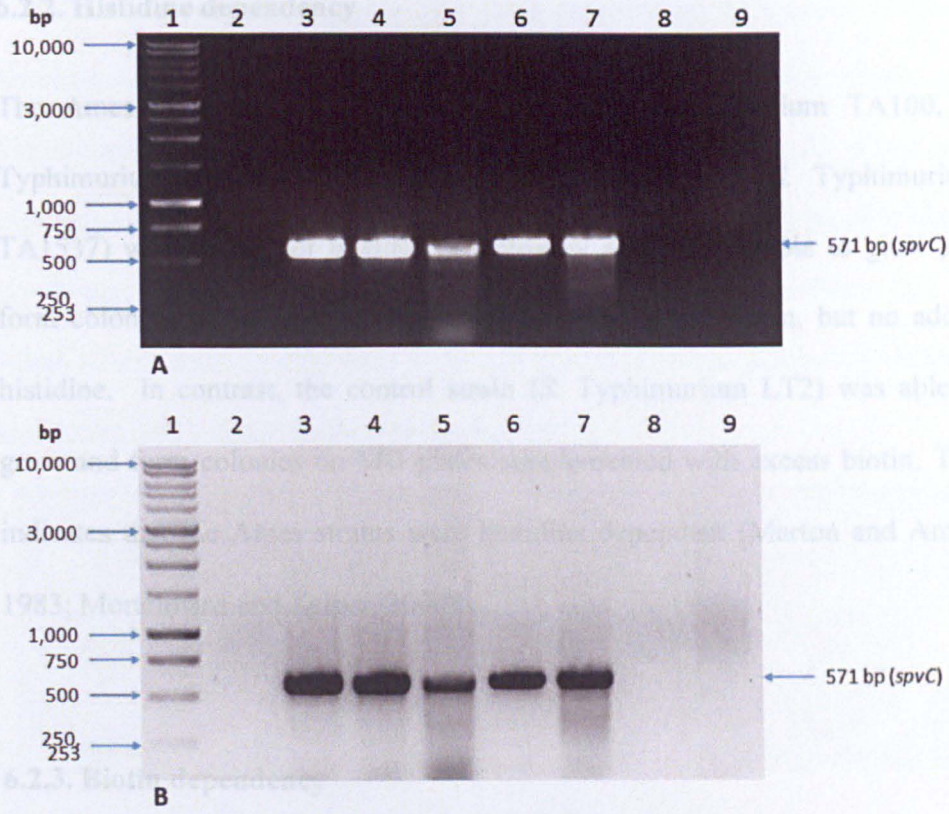

Figure 6.1: Agarose gel showing PCR products amplified from *Salmonella* strains using primers specific for the *spvC* gene. (B = inverted image of A). Lane 1, 1kb DNA ladder sizes marked in base pairs (bp); lane 2, *S.* Typhimurium LT2; lane 3, *S.* Typhimurium TA98; lane 4, *S.* Typhimurium TA100; lane 5, *S.* Typhimurium TA102; lane 6, *S.* Typhimurium TA1535; lane 7, *S.* Typhimurium TA1537; lane8, negative control (PCR mixture with no added template DNA); lane 9, *E. coli* MG1655. The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 75 minutes.

6.2.2. Histidine dependency

The Ames strains (*S.* Typhimurium TA 98, *S.* Typhimurium TA100, *S.* Typhimurium TA 102, *S.* Typhimurium TA 1535, and *S.* Typhimurium TA1537) were tested for histidine auxotrophy and were unable to grow and form colonies on MG plates supplemented with excess biotin, but no added histidine. In contrast, the control strain (*S.* Typhimurium LT2) was able to grow and form colonies on MG plates supplemented with excess biotin. This indicates that the Ames strains were histidine dependant (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

6.2.3. Biotin dependency

The Ames strains (*S.* Typhimurium TA 98, *S.* Typhimurium TA100, *S.* Typhimurium TA 102, *S.* Typhimurium TA 1535, and *S.* Typhimurium TA1537 were tested for biotin auxotrophy and were unable to grow and form colonies on MG plates supplemented with excess histidine, but no added biotin. In contrast, the control strain (*S.* Typhimurium LT2) was able to grow and form colonies on MG plates supplemented with excess histidine. This verifies that the Ames strains were biotin dependant (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

6.2.4. Histidine and biotin dependency

The Ames strains (*S.* Typhimurium TA 98, *S.* Typhimurium TA100, *S.* Typhimurium TA 102, *S.* Typhimurium TA 1535, and *S.* Typhimurium

TA1537) were unable to grow and form colonies on MG plates with no histidine or biotin supplementation, whereas the control strain (*S.* Typhimurium LT2) was able to grow and form colonies on MG plates (see Figure.6.2). All of the Ames strains tested were able to grow on MG plates supplemented with excess histidine and biotin. The result confirms that all Ames strains used in this study were histidine and biotin dependant (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

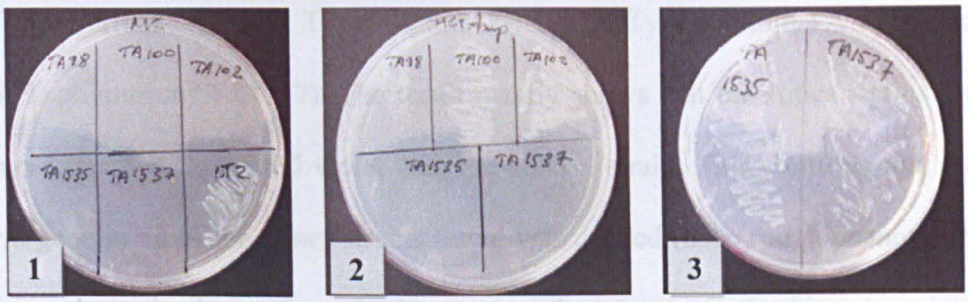

Figure 6.2: Confirmation of histidine/biotin auxotropy of the Ames strains. Different MG plates were streaked with the *Salmonella* strains and incubated overnight at 37 °C. Plate 1, MG (only *S.* Typhimurium LT2 has grown); plate 2, MG supplemented with histidine, biotin and ampicillin (only the ampicillin resistant strains grew) and plate 3, MG supplemented with histidine and biotin (*S.* Typhimurium TA1535 and *S.* Typhimurium TA1537 were able to grow in the absence of ampicillin).

6.2.5. Phenotypic *rfa* marker test

The Ames strains are also defective in LPS, due to deletion of the *rfa* genes. The result of the *rfa* phenotypic marker test presented in Figures 6.3.a and 6.3.b show a zone of growth inhibition around the crystal violet impregnated

Whatman (6mmØ) filter paper disc placed on the centre of the supplemented with excess histidine and biotin or nutrient agar plate inoculated with one of the Ames strains (*S.* Typhimurium TA 98), whereas no zone of growth inhibition was observed around the crystal violet disc placed on the nutrient agar plate seeded with the parental strain *S.* Typhimurium LT2. Similar zones of growth inhibition were also observed around the crystal violet discs placed on the centre of nutrient agar plates seeded with other Ames strains (*S.* Typhimurium TA100, *S.* Typhimurium TA 102, *S.* Typhimurium TA 1535, and *S.* Typhimurium TA1537). The result clearly shows that the Ames strains are more sensitive to crystal violet than the control strain. This confirms that the *rfa* gene in all of the Ames strains tested was deleted (deep rough phenotype). In contrast the lack of sensitivity to crystal violet in *S.* Typhimurium LT2 indicated that the *rfa* genes in the control strain were intact and functioning properly (smooth phenotype). LPS acts as a barrier to entrance of larger sized compounds (Ames *et al*, 1973).

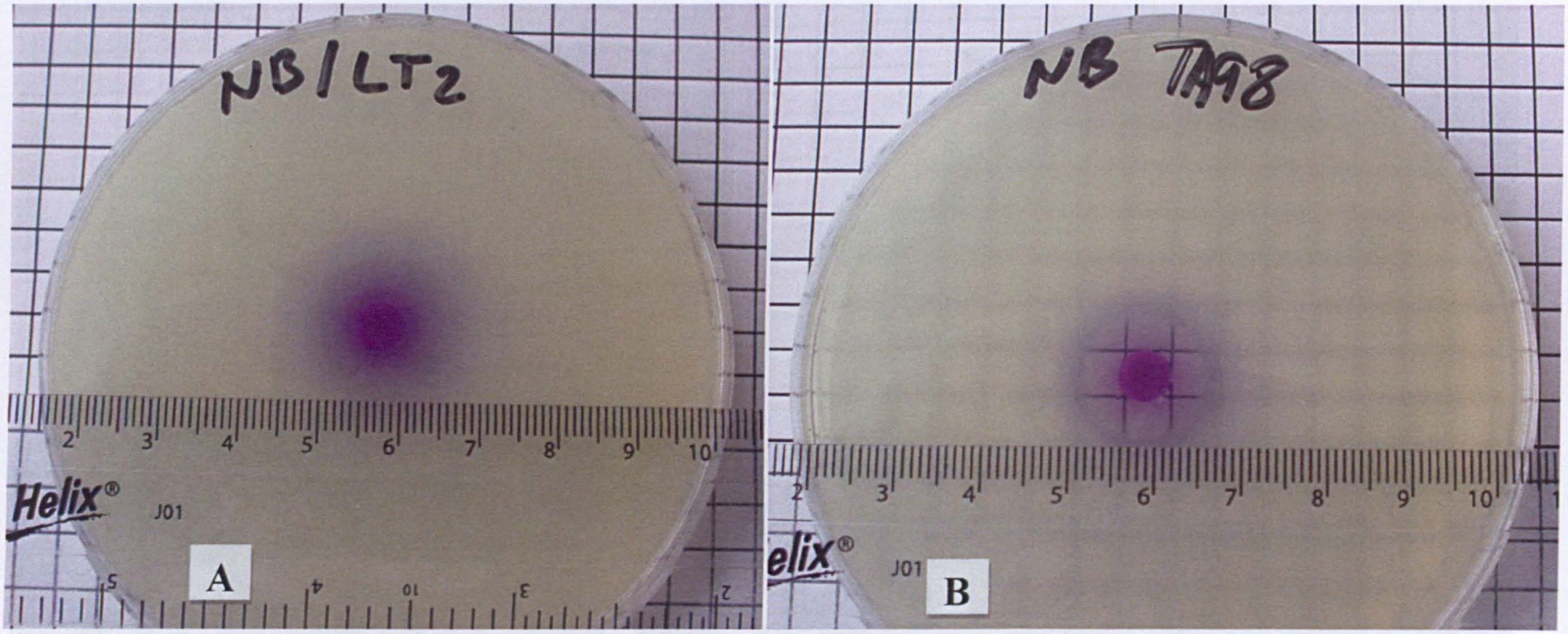

Figure 6.3.a: Crystal violet sensitivity tests of the Ames strains. Nutrient agar plates inoculated with *S.* Typhimurium LT2 (plate A) and *S* Typhimurium TA98 (plate B), containing a crystal violet impregnated filter paper disc placed on the centre of the agar each plate and incubated for 12 h at 37 °C.

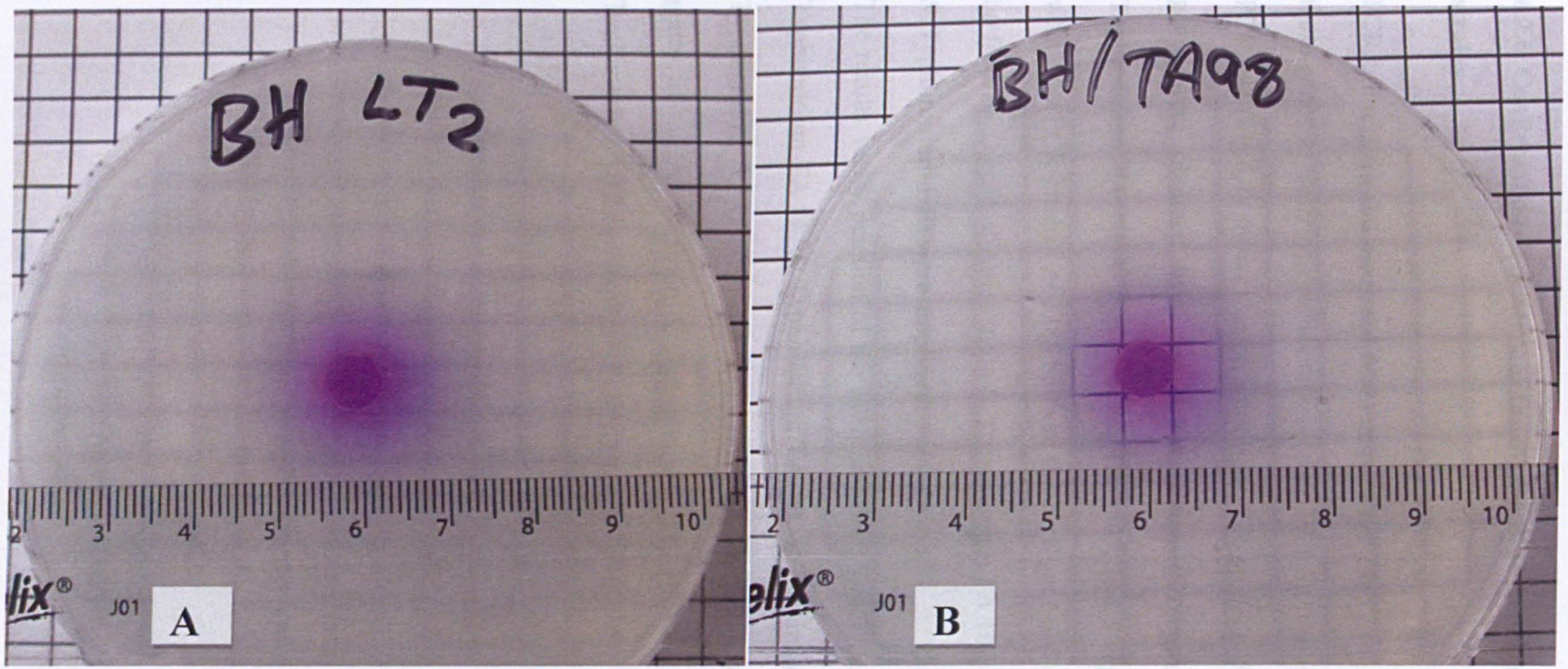

Figure 6.3.b: Crystal violet sensitivity tests of the Ames strains. MG plates supplemented with excess histidine and biotin inoculated with *S.* Typhimurium LT2 (plate A) and *S* Typhimurium TA98 (plate B), containing a crystal violet impregnated filter paper disc placed on the centre of the agar each plate and incubated for 12 h at 37 °C.

6.2.6. Confirmation of deletion of the *uvrB* and *bioD* genes in the Ames strains.

PCR primers for the *uvrB* and *bioD* genes were used to test for the presence of these genes as described earlier (see section 2.2.5.5). The result of the multiplex PCR test (Fig.6.4) shows that no amplified PCR products for the *uvrB* and *bioD* genes were obtained from the Ames strains (*S.* Typhimurium TA 98, *S.* Typhimurium TA100, *S.* Typhimurium TA 1535, and *S.* Typhimurium TA1537) whereas a PCR product of 1285 bp from the *uvrB* gene and a PCR product of 598 bp from *bioD* gene of Ames strain *S.* Typhimurium TA 102 and the parental strain *S.* Typhimurium LT2 were amplified.

The results verify deletion of the *uvrB* and *bioD* genes in the Ames strains: (*S.* Typhimurium TA 98, *S.* Typhimurium TA100, *S.* Typhimurium TA 1535, and *S.* Typhimurium TA1537) whilst it confirms no disruption to the *uvrB* and *bioD* genes in the Ames strain *S.* Typhimurium TA 102 and the control strain *S.* Typhimurium LT2.

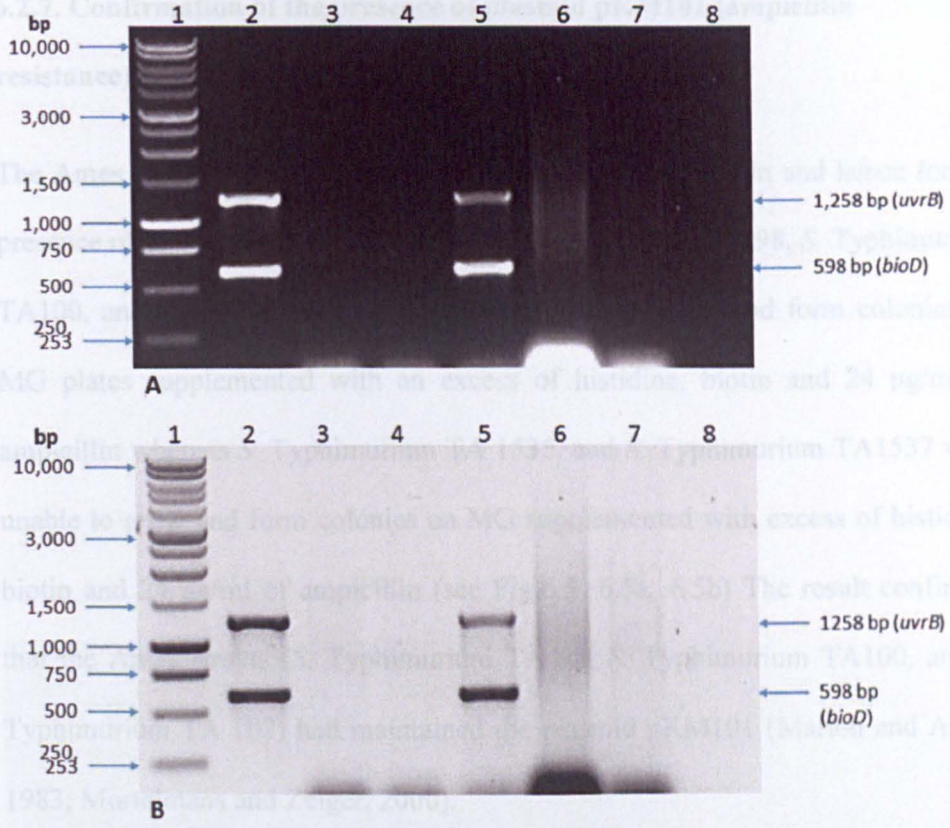

Figure 6.4: Agarose gel of PCR products confirming the deletion of the *uvrB* and *bioD* genes from the Ames strains. (B = inverted image of A). Lane 1, 1kb DNA Ladder; lane 2, *S.* Typhimurium LT2; lane 3, *S.* Typhimurium TA98; lane 4, *S.* Typhimurium TA100; lane 5, *S.* Typhimurium TA102; lane 6, *S.* Typhimurium TA1535; lane 7, *S.* Typhimurium TA1537; lane 8, negative control (PCR mixture with no added template DNA). The gel was 1% (w/v) agarose in 1X Tris-acetate buffer and was run at 80V for approximately 75 minutes.

6.2.7. Confirmation of the presence of plasmid pKM101 (ampicillin resistance)

The Ames strains were tested for their sensitivity to ampicillin and hence for the presence of pKM101. The Ames strains (*S.* Typhimurium TA 98, *S.* Typhimurium TA100, and *S.* Typhimurium TA 102) were able to grow and form colonies on MG plates supplemented with an excess of histidine, biotin and 24 µg/ml of ampicillin whereas *S.* Typhimurium TA 1535, and *S.* Typhimurium TA1537 were unable to grow and form colonies on MG supplemented with excess of histidine, biotin and 24 µg/ml of ampicillin (see Fig.6.5, 6.5a, 6.5b) The result confirmed that the Ames strains (*S.* Typhimurium TA 98, *S.* Typhimurium TA100, and *S.* Typhimurium TA 102) had maintained the plasmid pKM101 (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

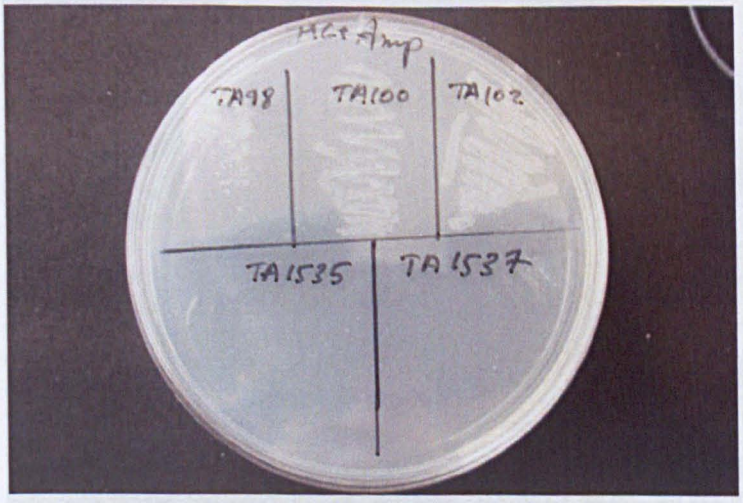

Figure 6.5: Testing for the presence of pKM101 in the Ames strains. Growth of Ames strains on MG plates supplemented with excess histidine, biotin and 24 µg/ml of ampicillin.

6.2.8. Confirmation of the presence of plasmid pAQ1 (tetracycline resistance)

The Ames strain *S.* Typhimurium TA 102 was able to grow and form colonies on MG plates supplemented with an excess of histidine, biotin and 24 µg/ml of ampicillin whereas the Ames strains *S.* Typhimurium TA98, *S.* Typhimurium TA 100, *S.* Typhimurium TA 1535 and *S.* Typhimurium TA1537 were unable to grow and form colonies on MG supplemented with an excess of histidine, biotin and 2 µg/ml of tetracycline (see Fig.6.5a and 6.5b) The result indicates that the Ames strain *S.* Typhimurium TA 102 had maintained the plasmid pAQ1 (Marton and Ames, 1983; Mortelmans and Zeiger, 2000).

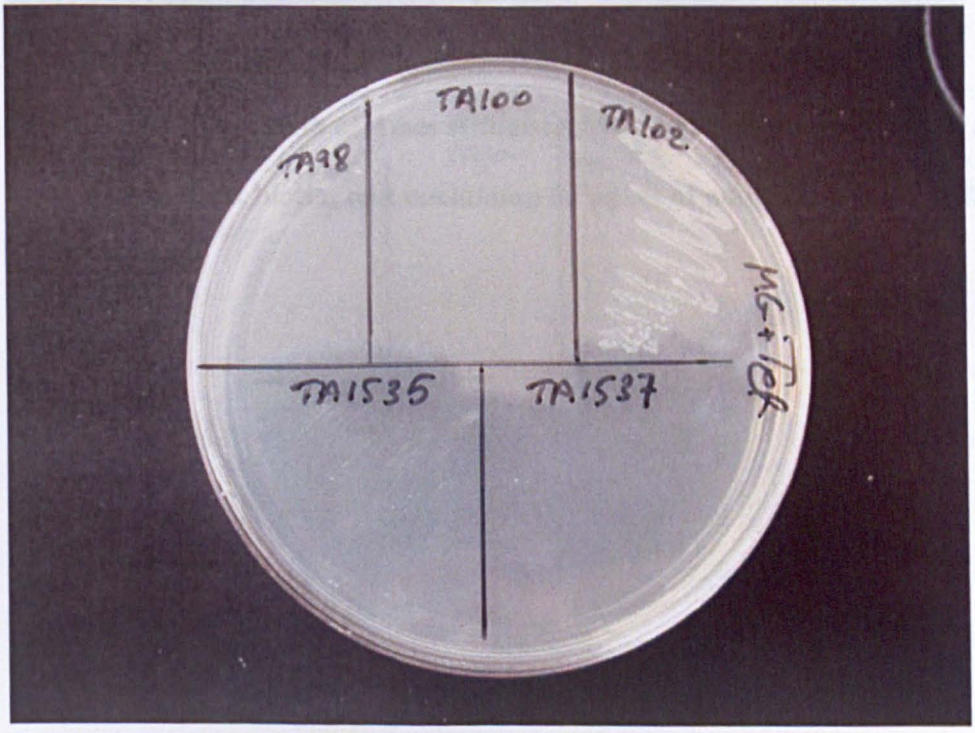

Figure 6.5.a: The growth of Ames strains on MG plates supplemented with excess of histidine, biotin and 2 µg/ml of tetracycline.

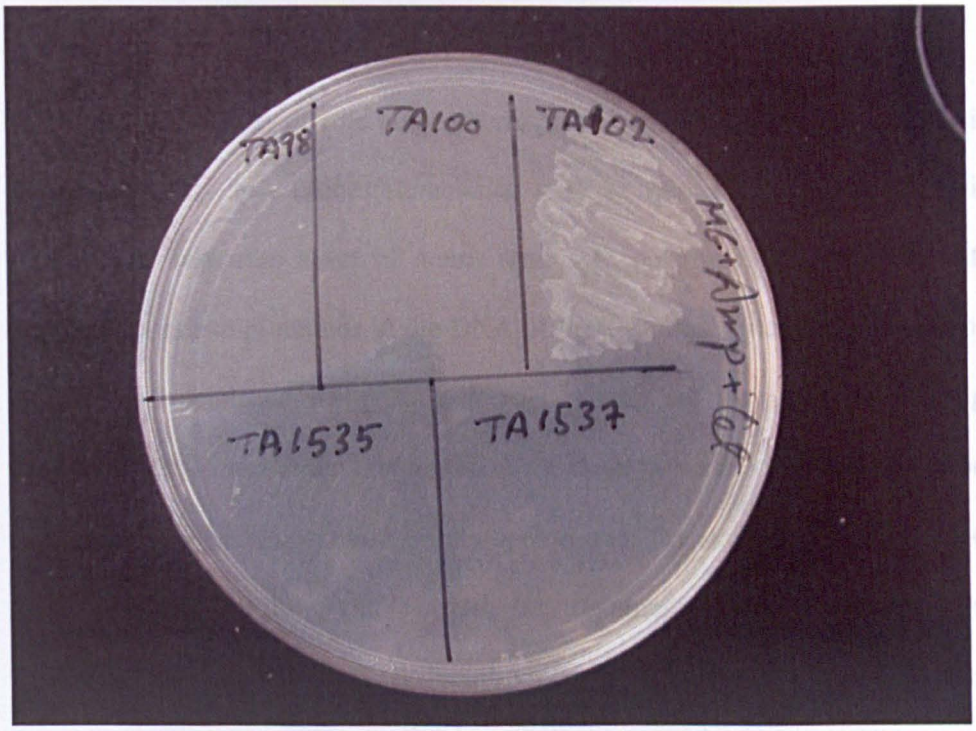

Figure 6.5.b: The growth of Ames strains on MG plates supplemented with an excess of histidine, biotin, and containing 24 µg/ml of ampicillin, and 2 µg/ml of tetracycline.

6.2.9. Mutagenic effects of cold atmospheric plasma (CAP)

The mutagenic effects of cold atmospheric plasma (CAP) were investigated using the mutagenicity test (Ames/*Salmonella* test) as described earlier, in section 2.2.6.2. The test uses a set of Ames strains to determine the capability of a chemical to induce mutations in the DNA of these strains, by causing reversion to histidine independence for growth. It was carried out by preparing overnight cultures of the Ames strains then, 100 µl of these cultures were inoculated onto each plate (MGLHB agar) and evenly spread over the surface of the agar. Four plates were used for the negative control (no treatment) and the rest were exposed to cold atmospheric plasma for different periods of time. Four replicates were used for each treatment. All plates were incubated at 37 °C for 48 h. The revertant colonies on each plate were counted and reversion curves were produced. The test results are presented in Figures 6.6-6.8. Figure 6.6 shows that the number of spontaneous revertant colonies (reversion background) of *S.* Typhimurium TA100 was within the range (75-200) reported by Marton and Ames (1983) and Mortelmans and Zeiger (2000). It also shows that treatment of *S.* Typhimurium TA100 with CAP for 5 and 10 seconds significantly ($P < 0.05$) increased the number of revertant colonies of *S.* Typhimurium TA100. Furthermore, the Figure shows that exposure of these strains to CAP treatment for 20 seconds or more has led to a reduction in the number of revertant colonies of the strain. i.e 20 seconds of exposure to CAP, is above the sub-lethal exposure time for *S.* Typhimurium TA100. This result indicates that CAP species has the capability to induce base-

pair substitution mutations in the DNA of this strain and potentially in the DNA of other organisms too.

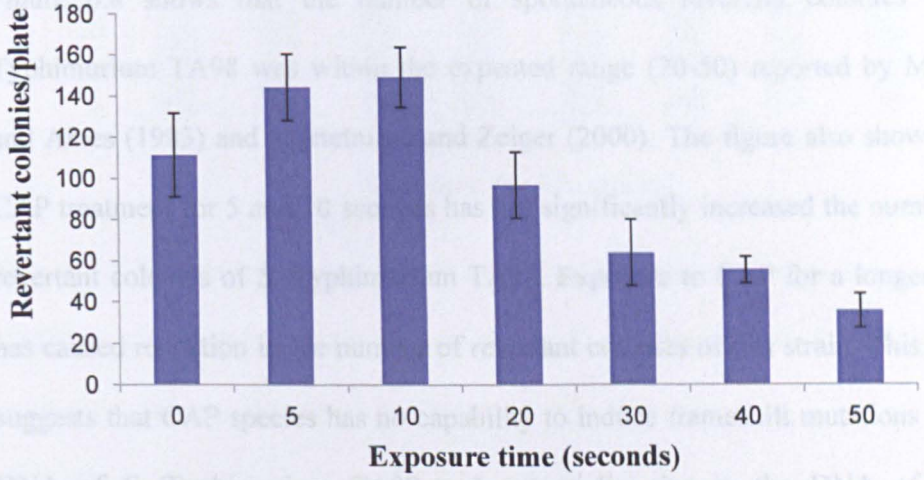

Figure 6.6: Effect of different exposure time to CAP treatment on reversion of histidine auxotrophy of the Ames strain *S.* Typhimurium TA100. (Mean number of revertant colonies ± 1 SD, n = 4 replicates).

Similar results were obtained using *S.* Typhimurium TA102. Figure 6.7 shows that the number of spontaneous revertant colonies of *S.* Typhimurium TA102 was within the range (250-500) reported by Snowball, (2002). The figure also shows that treatment of *S.* Typhimurium TA102 with CAP for 5 and 10 seconds has significantly ($P < 0.05$) increased the number of revertant colonies of *S.* Typhimurium TA102. Additionally, the figure shows that exposure of the strains

to CAP treatment for 20 seconds or more has caused a reduction in the number of revertant colonies of this strain. i.e 20 seconds of exposure to CAP, is the lethal exposure time for *S.* Typhimurium TA102. This result indicates that CAP species also have the capability to induce transition/transversion mutations in the DNA of this strain and potentially in the DNA of other organisms.

Figure 6.8 shows that the number of spontaneous revertant colonies of *S.* Typhimurium TA98 was within the expected range (20-50) reported by Marton and Ames (1983) and Mortelmans and Zeiger (2000). The figure also shows that CAP treatment for 5 and 10 seconds has not significantly increased the number of revertant colonies of *S.* Typhimurium TA98. Exposure to CAP for a longer time has caused reduction in the number of revertant colonies of this strain. This result suggests that CAP species has no capability to induce frameshift mutations in the DNA of *S.* Typhimurium TA98 and potentially also in the DNA of other organisms.

The results obtained using *S.* Typhimurium TA1535 and *S.* Typhimurium TA1537 showed that the number of spontaneous revertant colonies of these strains were also within the expected range (5-20) reported by Marton and Ames (1983) and Mortelmans and Zeiger (2000). Treatments of these strains with CAP for 5 and 10 seconds have not caused any increase in the number of revertant colonies. CAP treatment for a longer time has caused reduction in the number of revertant colonies of these strains. Because the number of revertant colonies were very low, it was not possible to present the results obtained using these strains graphically.

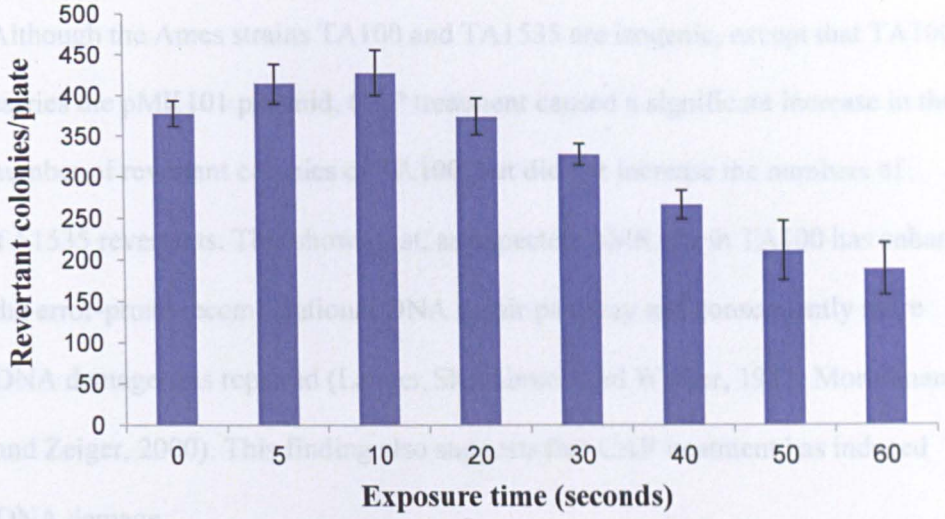

Figure 6.7 Effect of different exposure time to CAP treatment on reversion of histidine auxotrophy of the Ames strain *S.* Typhimurium TA102. (Mean number of revertant colonies ± 1 SD, n = 4 replicates).

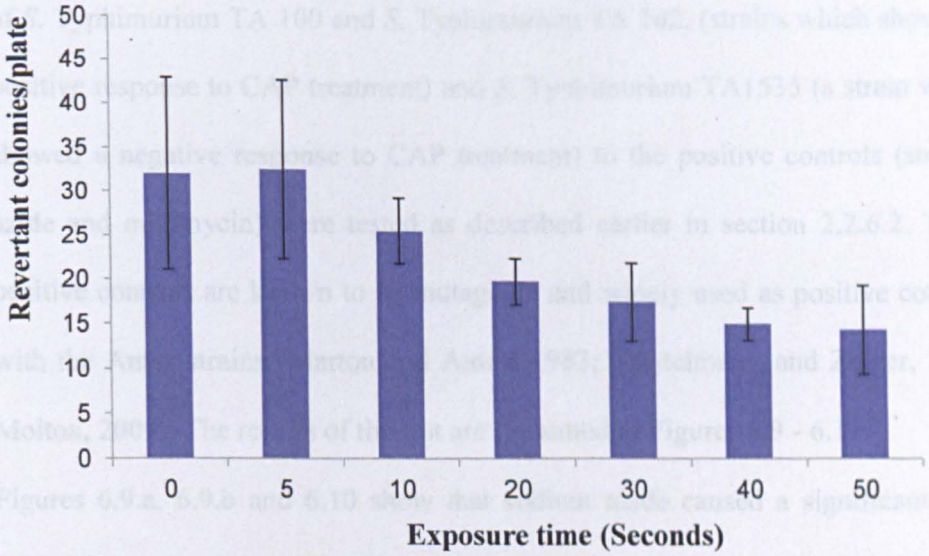

Figure 6.8 Effect of different exposure time to CAP treatment on reversion of histidine auxotrophy of the Ames strain *S.* Typhimurium TA98. (Mean number of revertant colonies ± 1 SD, n = 4 replicates).

Although the Ames strains TA100 and TA1535 are isogenic, except that TA100 carries the pMK101 plasmid, CAP treatment caused a significant increase in the number of revertant colonies of TA100, but did not increase the numbers of TA1535 revertants. This shows that, as expected, pMK101 in TA100 has enhanced the error-prone recombinational DNA repair pathway and consequently more DNA damage was repaired (Langer, Shanabruch and Walker, 1981; Mortelmans and Zeiger, 2000). This finding also suggests that CAP treatment has induced DNA damage.

To confirm that the Ames strains used were effective in detecting certain mutagenic chemicals the test was specifically designed for detecting, the response of *S.* Typhimurium TA 100 and *S.* Typhimurium TA 102, (strains which showed a positive response to CAP treatment) and *S.* Typhimurium TA1535 (a strain which showed a negative response to CAP treatment) to the positive controls (sodium azide and mitomycin) were tested as described earlier in section 2.2.6.2. These positive controls are known to be mutagenic and widely used as positive controls with the Ames strains (Marton and Ames, 1983; Mortelmans, and Zeiger, 2000; Moltox, 2009). The results of the test are presented in Figures 6.9 - 6.11.

Figures 6.9.a, 6.9.b and 6.10 show that sodium azide caused a significant ($P < 0.05$) increase in the number of revertant colonies of *S.* Typhimurium TA100 and *S.* Typhimurium TA 1535. On the other hand mitomycin has not caused any significant increase in the number of revertant colonies of these strains. This indicates that the strains were effective and has the specificity of detecting

mutagenic chemicals that causes base-pair substitution mutations, as sodium azide is a known cause of base-pair substitutions, whilst mitomycin C does not.

Figure 6.10 shows that mitomycin caused a significant ($P < 0.05$) increase in the number of revertant colonies of *S.* Typhimurium TA102. On the other hand sodium azide has not caused any significant increase in the number of revertant colonies of the strain. This indicates that the strain was effective at detecting the positive control mutagenic chemical, and has the specificity of detecting mutagenic chemicals that causes transition/transversion mutations.

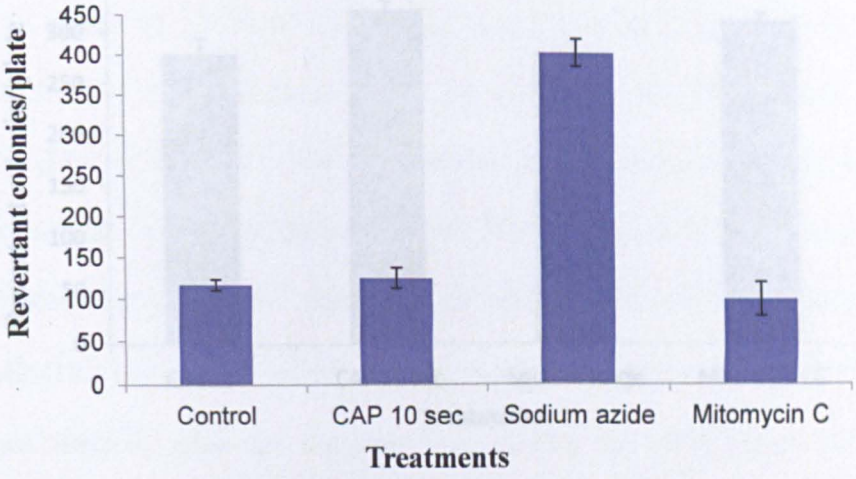

Figure 6.9.a Effect of CAP, sodium azide, and Mitomycin treatments on reversion of histidine auxotrophy of the Ames strain *S.* Typhimurium TA100. (Mean number of revertant colonies ± 1 SD, n = 4 replicates).

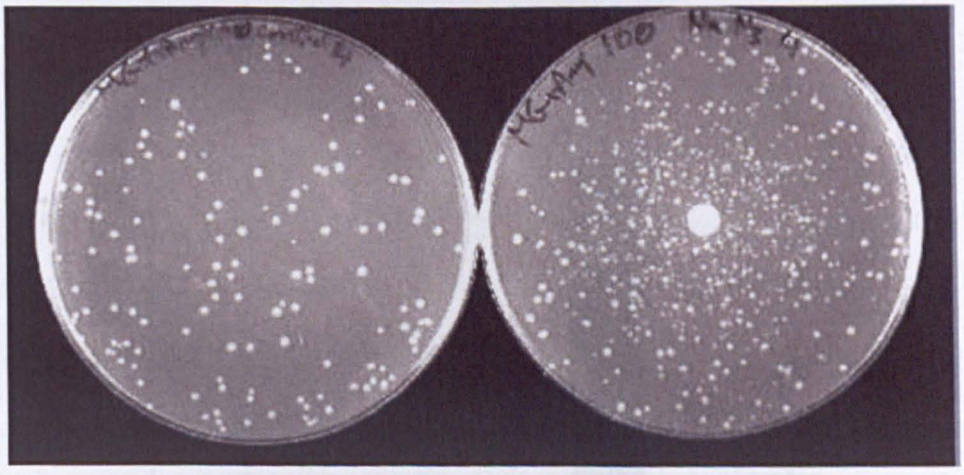

Figure 6.9.b Effect of sodium azide treatments on reversion of histidine auxotrophy of the Ames strain *S.* Typhimurium TA100.

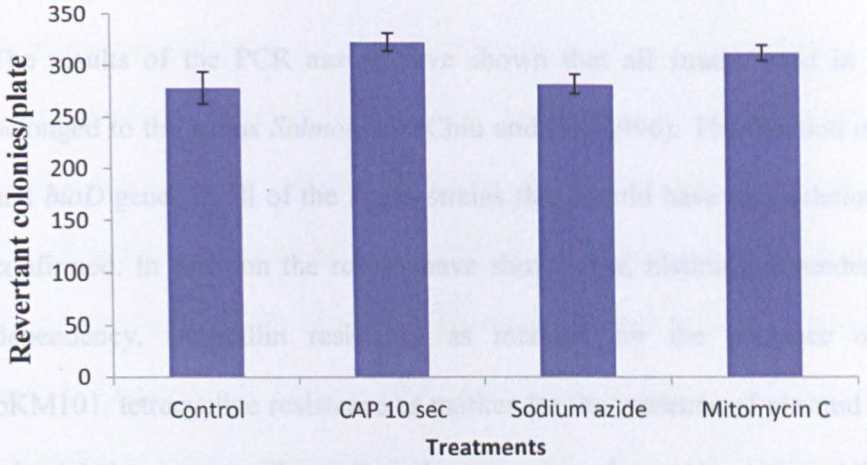

Figure 6.10 Effect of CAP, Sodium azide, and Mitomycin treatments on reversion of histidine auxotrophy of the Ames strain *S.* Typhimurium TA102. (Mean number of revertant colonies ± 1 SD, n = 4 replicates).

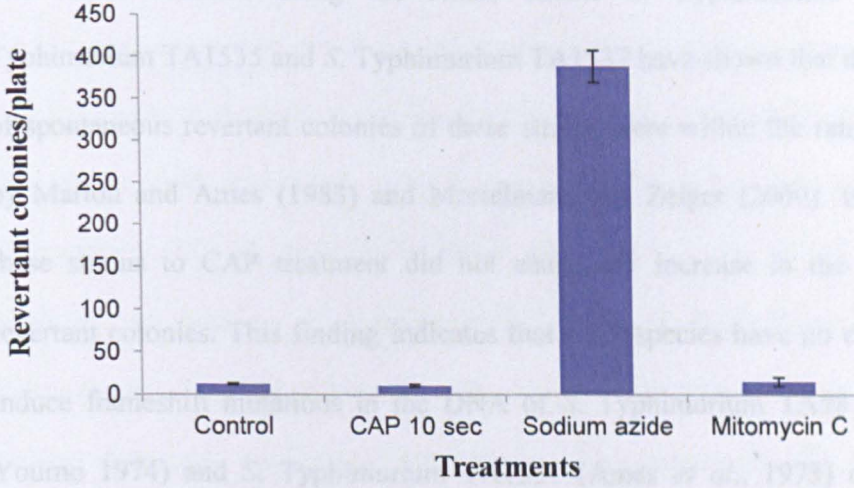

Figure 6.11 Effect of CAP, Sodium azide, and Mitomycin treatments on reversion of histidine auxotrophy of the Ames strain *S.* Typhimurium TA1535. (Mean number of revertant colonies ± 1 SD, n = 4 replicates).

6.3. DISCUSSION

The results of the PCR assays have shown that all strains used in this study belonged to the genus *Salmonella* (Chiu and Ou, 1996). The deletion of the *uvrB* and *bioD* genes in all of the Ames strains that should have this deletion was also confirmed. In addition the results have shown that, histidine dependency, biotin dependency, ampicillin resistance as markers for the presence of plasmid pKM101, tetracycline resistance as marker for the presence of plasmid pAQ1 and *rfa* deletion, were all confirmed as correct for each strain. All of these confirmatory tests were carried out in order to ensure the characteristics of each mutant was correct so that ultimately any observed differences in the number of revertant colonies between the control and the treatments was entirely related to the mutagenic effect of CAP treatment.

The result obtained using the Ames strains *S.* Typhimurium TA98, *S.* Typhimurium TA1535 and *S.* Typhimurium TA1537 have shown that the numbers of spontaneous revertant colonies of these strains were within the range reported by Marton and Ames (1983) and Mortelmans and Zeiger (2000). Exposure of these strains to CAP treatment did not cause any increase in the number of revertant colonies. This finding indicates that CAP species have no capability to induce frameshift mutations in the DNA of *S.* Typhimurium TA98 (Isono and Yourno 1974) and *S.* Typhimurium TA1537 (Ames *et al.*, 1973) or base-pair substitution in the DNA of *S.* Typhimurium TA1535 (Barnes *et al.*, 1982) and potentially also in the DNA of other organisms.

On the other hand results obtained using *S.* Typhimurium TA100 and *S.* Typhimurium TA102 have shown that the numbers of spontaneous revertant colonies of these strains were within the ranges reported by Ames (1983), Mortelmans and Zeiger (2000) and Snowball (2002). Exposure of *S.* Typhimurium TA100 and *S.* Typhimurium TA102 to CAP for 5 and 10 seconds has significantly ($P < 0.05$) increased the number of revertant colonies of *S.* Typhimurium TA100 and *S.* Typhimurium TA102. This finding indicates that CAP species have the capability to induce base-pair substitution in the DNA of *S.* Typhimurium TA100 (Barnes *et al.*, 1982) and transition/transversion mutations in the DNA of *S.* Typhimurium TA102 (Levin *et al.*, 1982a) and potentially in the DNA of other bacteria.

In view of the fact that the Ames strains were specifically designed to detect agents that cause mutagenic effects and with high specificity, *S.* Typhimurium TA102 was designed to detect a number of oxidative mutagenic agents and have A.T base pairs at the critical site for reversion (Levin *et al.*, 1982). Furthermore, the test is used widely all over the world as an initial screening test for mutagenicity (OECD, 1997; ICH, 2012; ISO, 2012; EMA, 2013). The result obtained here with the *S.* Typhimurium TA100 and *S.* Typhimurium TA102 indicates that the CAP generated using helium and oxygen gas is capable of causing damage to the DNA of the bacteria. More specifically, CAP can induce base-pair substitution in the DNA of bacteria. This damage is similar to that found in the *dps* mutants after it had been treated with hydrogen peroxide which causes an increase in the level of G.C to T.A mutations in these (Martinez and Kolter, 1997). The results suggest that inactivation of bacteria using CAP is mainly a

result of oxidative stress damage and that the UV and thermal effects can be neglected as discussed earlier. This finding is in agreement with the results obtained in the previous chapter using OxyR regulon mutants and suggests that the inactivation of bacteria outside the direct effect of plasma etching and damage to cells is mainly a result of oxidative stress by reactive oxygen species.

Sequencing the DNA from the revertant colonies of Ames strains resulting from CAP treatment is an important research point which needs to be investigated in order to find out more about the kind of DNA damage occurring as result of CAP treatment; however due to time limits available for this study this was not possible.

CHAPTER 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

This study has investigated the effects of Cold Atmospheric Plasma (CAP) on different strains of Gram positive and Gram negative bacteria, the role of lipopolysaccharides (LPS) on resistance of bacteria to CAP treatment, the role of oxidative stress in bacterial inactivation and possible DNA damage or mutagenic effects of CAP.

To ensure the accuracy and the reliability of the results, it was necessary to carry out numerous confirmatory tests on each strain/mutant. The results of these tests have shown that the identity and the characteristics of each strain/mutant were confirmed as correct.

The results obtained in this study have shown that Cold Atmospheric Plasma (CAP) is capable of inactivating a wide range of Gram positive and Gram negative food-borne pathogenic bacteria. This finding is in agreement with those found by others (Montenegro *et al.*, 2002; Lee *et al.*, 2006; Morris *et al.*, 2007; Perni *et al.*, 2007; Niemira and Sites, 2008; Perni *et al.*, 2008b; Ma *et al.*, 2008; Song *et al.*, 2009; Yun *et al.*, 2010; Wang *et al.*; 2012). Nevertheless, it was difficult to compare the inactivation rates obtained in this study with those which were documented by others. This was due to different plasma systems, different gas mixtures and different treatment approaches (directly onto fruit, vegetable and meat slices or membrane filters, static exposure or movement of the samples under the plume) being used.

In this study, *E. coli* K-12 MG1655 was found to be more susceptible to CAP treatments than *E. coli* O157: H7 and *E. coli* H10407. When *E. coli* K-12 was compared with different strains of *L. monocytogenes,* no significant differences

between their susceptibility to CAP treatment were found. However, when *L. monocytogenes* WSLC 1042 was compared with *S.* Typhimurium LT2, the susceptibility of *L. monocytogenes* WSLC 1042 to CAP treatment was more than that of *S.* Typhimurium. Since *E. coli* K-12 MG1655 has no O-antigen, in its Lipopolysaccharide (LPS) structure (Klena, *et al.*, 1992; Liu and Reeves, 1994; Hobman *et al.*, 2007; Browning *et al.*, 2013) and the LPS structure is a unique feature of Gram negative bacteria (Caroff and Karibian, 2003; Murray *et al.*, 2009), the sensitivity of *E. coli* K-12 MG1655, and *L. monocytogenes* strains to CAP treatment was hypothesized to be due to their lacking the O-antigen and absence of LPS structures in the cell wall respectively. The LPS has been found to be a barrier to antibiotics such as novobiocin, spiramycin, and actinomycin D (Tamaki *et al.*, 1971) and hydrophobic dyes, detergents, fatty acids, phenols, and polycyclic hydrocarbons (Raetz and Whitfield, 2002). The LPS and enterobacterial common antigen (ECA) in STEC O157:H7 and *Salmonella* were also found to be essential to the resistance of these strains to acetic acid and other short chain fatty acids (Barua *et al.*, 2002). This finding also suggested that LPS might have a role in tolerance/increased resistance to CAP treatments and more investigations were conducted. In these investigations, *S. enterica* serovar Typhimurium LT2 was found to be more tolerant to CAP treatment than *S. enterica* serovar Typhimurium TA98. Since the latter is a deep rough mutant (McCann *et al.*, 1975), i.e. has an incomplete LPS structure, its increased susceptibility to CAP treatment could be attributed to defective LPS structure. However, this strain also has more genomic deletions that may contribute to this increased susceptibility. These deletions included the galactose operon, biotin operon, nucleotide-excision-repair *uvrB* gene

and chlorate-resistance genes (Ames *et al.*, 1973; Maron and Ames 1983). In more recent studies, this strain was also found to be missing other important genes, which include *mfdA* (encoding a multi-drug translocase), *mdaA* (a major nitroreductase), *dps* (encoding a DNA-binding protein), genes involved in molybdenum cofactor biosynthesis and a number of ORFs of unknown functions (Porwollika *et al.*, 2001). The *dps* gene in *E. coli* was found to be involved in oxidative stress resistance and is part of the OxyR regulon (Zheng *et al.*, 2001a). Hence, the susceptibly to CAP of *S. enterica* serovar Typhimurium TA98 might be the result of the strain lacking LPS, being more susceptible to oxidative stress or may be due to the synergetic effect of both incomplete LPS and of loss of oxidative stress defence.

No differences in susceptibility to CAP treatment were found between the related *E. coli* K-12 strains, *E. coli* W3110 and *E. coli* BW25113 (Hayashi, *et al.*, 2006; Baba *et al.*, 2006) and their isogenic mutants, *E. coli* W3110 (Δ*rfaC*), *E. coli* BW25113 (Δ*rfaE*) and *E. coli* BW25113 (Δ*lpcA*). Each of these mutants has a deletion of or in one of the genes involved in the biosynthesis of the inner core or the outer core part of the core polysaccharide region which means they have an incomplete core polysaccharides region (Heurlier, K., personal communication; Baba *et al.*, 2006). This result suggests that losing the capability to synthesise the core polysaccharide region, partially or completely, has not increased the strain's susceptibility to CAP treatment. In other words, the core polysaccharide region on its own does not give protection to the bacterial cells against CAP treatment. Similarly, no differences in the sensitivity to CAP between *E. coli* MG1655 (Str^R) and its isogenic mutant *E. coli* MG1655 (Str^R Km^R) (Δ*waaQ*) were found at low

exposure times to CAP treatment. However, at higher exposure times the mutant was more tolerant. Since the mutant was defective in the *waaQ* gene involved in the biosynthesis of the core polysaccharides region (Yethon, *et al.*, 1998; Møller *et al.*, 2003), this tolerance is possibly a result of clump formation as this mutant has the tendency to form clumps of cells (Møller *et al.*, 2003). The clumps might have acted as a shield preventing the CAP species from reaching the internal cells of the clumps. It has also been reported by Yethon *et al.* (2000) that a *waaO* mutant of *E. coli* F470, had wild-type levels of resistance to sodium dodecyl sulfate (SDS).

Moreover, no significant differences in the susceptibility to CAP treatment between *E. coli* K-12 MG1655 and its derivatives (DFB1655 L5 and DFB1655 L9) were found. *E. coli* K-12 MG1655 lacks the O-antigen (Klena, *et al.*, 1992; Liu and Reeves, 1994; Hobman *et al.*, 2007; Browning *et al.*, 2013) whereas *E. coli* K-12 DFB1655 L5 and *E. coli* K-12 DFB1655 L9 have a complete LPS structure, with L9 producing more O-antigen than L5 (Browning *et al.*, 2013). This demonstrates that even with restoration of the O-antigen to *E. coli* K-12 MG1655, its susceptibility to CAP treatments remain unchanged.

Additionally growing *E. coli* O157: H7 under conditions suitable for colanic acid production has not increased the strain's resistance to CAP treatments.

To summarize these findings, *E. coli* O157: H7 and *E. coli* H10407 were found to be more tolerant to CAP treatment than *E. coli* K-12 MG1655, meanwhile all of the *E. coli* K-12 derivatives (with and without O-antigen; with and without core polysaccharide) including *E. coli* K-12 MG1655 showed similar susceptibility to CAP treatments except *E. coli* MG1655 (Str^R Km^R) (ΔwaaQ) which has shown some tolerance. Moreover, *E. coli* K-12 MG1655 and *L. monocytogenes* strains

also showed similar susceptibility to CAP treatments. In contrast, *S. enterica* serovar Typhimurium LT2 was found to be more tolerant to CAP treatment than *L. monocytogenes* WSLC 1042 and *S. enterica* serovar Typhimurium TA98. Prevention of CAP gaseous species diffusion into the atmosphere has increased inactivation zones on the plates.

It can be concluded that LPS has no role in the tolerance to CAP treatment shown by *E. coli* O157: H7, *E. coli* H10407 and *S. enterica* serovar Typhimurium LT2, since derivatives of *E. coli* K-12 MG1655 (DFB1655 L5 and DFB1655 L9) have not shown such tolerance, this suggests that these strains might have different defence mechanisms against CAP treatment, especially since the complete genome sequences of the *E. coli* O157: H7 has shown many differences between this strain and *E. coli* K-12 strains (Hayashi *et al.*, 2001; Hobman *et al.*, 2007; Browning *et al.*, 2013). Similarly, the complete genome sequence of *E. coli* H10407 has shown that this strain, although it is similar to *E. coli* K-12, has a larger genome and four extra plasmids (Crossman *et al.*, 2010). *S. enterica* serovar Typhimurium TA98 was found to have multiple genomic deletions which make it incomparable to the parent strain *S. enterica* serovar Typhimurium LT2. The results obtained at this stage of the study have suggested the possible involvement of oxidative stress and more investigations were conducted to test this hypothesis using different mutants as sensors for oxidative stress.

The OxyR mutant was more sensitive to cold atmospheric plasma (CAP) treatment than the parental strain in both the K-12 and O157:H7 strains. These results are similar to those were found by Dr Neil Doherty using mutants of *E. coli* O157: H7, (Δ*soxS*, and Δ*oxyR*) (Doherty N., Personel comunication) and by Perni *et al.*

(2007) using an isogenic mutant of *E. coli* MG1655 (Δ*soxS*,). Since the *oxyR* gene is known to be involved in the resistance to oxidative stress (Yoon, *et al.*, 2002; Patil, *et al.*, 2011), this has led to more investigations into the role of oxidative stress using mutants of *E. coli* BW25113 (Baba *et al.*, 2006). Three mutants were found to be significantly ($P < 0.05$) more sensitive to CAP treatment than other mutants. These were *E. coli* BW25113 (Δ*dps*), *E. coli* BW25113 (Δ*grxA*) and *E. coli* BW25113 (Δ*sufC*). The genes deleted in these mutants are hydrogen peroxide-inducible and are part of the OxyR regulon (Zheng *et al.*, 2001a). The *dps* gene encodes a nonspecific DNA-binding protein (Dps) which was first isolated from starved cells. Dps plays an important role in gene expression and in protection of DNA during stationary phase from oxidative stress (Almiron *et al.*, 1992) and UV, Gamma irradiation, iron and copper toxicity, thermal stress, and acid and base shock (Nair and Finkel, 2004). It reduces the number of DNA single-strand breaks and transversion mutations (G.C to T.A) which are features of oxidative damage (Martinez and Kolter, 1997). Chodavarapu and co-workers have suggested that during oxidative stress, Dps reduces the initiations of DNA replication which gives opportunity to the DNA repair mechanisms to repair oxidative DNA damage and increases the probability of genetic mutations which might lead to survival under conditions of oxidative stress (Chodavarapu *et al.*, 2008). Dps was found to protect cells against oxidative stress by binding to Fe^{2+}, which can act as a Fenton reagent leading to generation of free radicals (Yamamoto *et al.*, 2011).

The *sufC* gene is one of the genes which encodes components of the ATP binding cassette (ABC) transporter (Zheng *et al.*, 2001a). The ATPase activity of *sufC*

gene is essential for iron acquisition through [Fe-S] cluster assembly under oxidative stress and iron limitation (Nachin *et al.*, 2003; Saini *et al.*, 2010).

The *grx*A gene encodes glutaredoxin 1 (Tao, 1997; Zheng *et al.*, 2001a; Zheng *et al.*, 2001b). Glutaredoxin 1 is part of the Glutaredoxin and thioredoxin systems which are involved in reducing disulfide bonds in cytoplasmic proteins (Prinz *et al.*,1997).

Interestingly, all mutants that showed higher sensitivity to CAP treatment have a deletion in genes that have very important roles in defending cells aginst oxidative stress damage. Moreover, these genes are not among those that were found to be also induced by heat (Morgan *et al.*, 1986). This has led to more investigation in order to confirm these findings and the Ames strains were used to detect possible DNA damage.

Results of the Ames mutagenicity test have shown that the number of revertant colonies of *S.* Typhimurium TA100 and *S.* Typhimurium TA102 has significantly ($P < 0.05$) increased after exposure of these strains to CAP treatment for 5 and 10 seconds. The numbers of spontaneous revertant colonies were within the ranges reported by Ames (1983), Mortelmans and Zeiger (2000) and Snowball (2002). This finding demonstrates that CAP species caused base-pair substitution mutations in the DNA of *S.* Typhimurium TA100 (Barnes *et al.*, 1982) and transition/transversion mutations in the DNA of *S.* Typhimurium TA102 (Levin *et al.*, 1982a) and potentially mutations in the DNA of other bacteria.

The Ames test is widely recognized (OECD, 1997; ICH, 2012; ISO, 2012; EMA, 2013) and the strains were specifically designed to detect agents that cause mutagenic effects and with high specificity. *S.* Typhimurium TA102 was designed

to detect a number of oxidative mutagenic agents and have A.T base pairs at the critical site for reversion (Levin *et al.*, 1982). The increase in the number of the revertant colonies of the Ames strains (*S.* Typhimurium TA100 and *S.* Typhimurium TA102) and the intolerance showed by the mutants of *E. coli* BW25113 (Δ *dps*, Δ*grxA* and Δ*sufC*) to CAP treatment clearly indicates that the CAP generated using helium and oxygen gas can cause damage to the DNA of the bacteria. More specifically, CAP can induce base-pair substitution in bacterial DNA. Interestingly the CAP-induced DNA damage detected in the Ames strains was similar to that which was found in a *dps* mutant after it had been treated with hydrogen peroxide, which was an increase in the level of G.C to T.A mutations in these mutants (Martinez and Kolter, 1997). Similar results were found by Wang and co- workers when atmospheric pressure glow discharge generated using pure helium was employed to induce mutations in *Streptomyces avermitilis* (Wang *et al.*, 2010).

The CAP used in this study was generated using helium and oxygen gas, and optical emission spectroscopy (OES) which was used to find out the plasma species in the range of 200-1000 nm showed that the plasma species found were O_2 species, He species, N_2 species OH radicals and UV. These species are in an excited state and in the presence of a humid environment thus, secondary species will be formed which include Superoxide ($O_2^{\cdot}$), Hydrogen peroxide (H_2O_2), Singlet oxygen ($^1O_2$), Ozone (O_3) Nitric oxide (NO) electrons and Positive ions (Brisset *et al.*, 2008; Sensenig *et al.*, 2010).

Moreover, according to the literature, the UV has no inactivation effect under such conditions (Laroussi and Leipold, 2004; Deng *et al.*, 2006; Ross *et al.*, 2006; Perni

et al., 2007) and the CAP temperature was always ~ 40 °C. Therefore, it can be concluded that the inactivation of bacteria outside the direct effect of plasma etching and damage to cells is mainly a result of oxidative stress caused by reactive oxygen species. The results suggest that inactivation of bacteria using CAP is mainly a result of oxidative stress damage. CAP species cause base-pair substitution mutations and transition/transversion mutations in the DNA of *S.* Typhimurium TA100 and *S.* Typhimurium TA102 respectively. This finding highlights some concerns about using CAP treatments in medical treatments such as dental and wound treatments. Mutants of *E. coli* K-12 BW25113 (Δ *dps*, Δ*grxA* and Δ*sufC*) are very efficient as sensors for agents that causes oxidative stress, and were more sensitive to CAP 'peripheral killing' than BW25113. It can also be concluded that the Ames test was successfully modified to be an appropriate test for detection of mutations caused by CAP treatement. The modifed test has the advantage that the amounts of limited histidine and biotin solution requirements are added to the MG agar directly therefore, no top agar layer is needed. This solves the problems encountered with using of top agar layer such as slipping of the layer out of place, uneven distribution of growth, inactivation of strains due to hot agar and solidfying of agar before plating (Mortelmans and Zeiger, 2000) in addition, it saves time needed for sample preparation.

The results have shown that low exposure times to CAP treatment have caused an increase in the percentage survival of bacterial strains. This is possibly due to the hormetic effect of CAP as it has been reported that low doses of many toxic substances stimulate the growth of biological cells including bacterial cells

(Calabrese and Blain, 2005 and Calabrese, 2008). Brugmann and Firmani (2005) found that low concentrations of acidified sodium nitrite stimulate the survival of clinical *Mycobacterium tuberculosis* isolates suggesting that reactive nitrogen intermediates have a hormetic effects on these bacteria. Similar hormetic effects of low concentration of Cu (II), Zn (II), Cd (II) and Cr (VI) on luminescence of marine and fresh water bacteria, *Photobacterium phosphorem* and *Vibrio qinghaiensis* were reported by Shen *et al.* (2009).

In many studies on the effect of CAP on spread plated samples very large error bars were seen. A number of factors were found to influence this variation. The sensitivities of bacterial strains to inactivation by CAP treatments were found to be affected by the dryness of agar plates. Bacteria plated on less dried plates were more sensitive to inactivation by CAP treatments than those plated on very dry plates. One possible reason for this is due to the formation of more hydrogen peroxide by the CAP in the presence of more humidity. To standardize the drying process, plates were kept in the laminar flow cabinet overnight and then on the bench for 1-2 days before use. For storing, plates were placed in sealed plastic bags and kept at 4 ^{o}C for not more than two weeks. It was also found that an uneven bacterial lawn led to variation between the replicates of the same treatment (large SD), therefore standardizing the plating procedure (type of spreaders, calibrated pipettes, time used, and direction of spreading movement) is crucial in minimizing the variations between the replicates.

Although the Ames test was efficient in detecting the type of mutation occurring due to CAP treatement however, it does not allow to find out what kind of other damage has ocurred. Therefore sequencing the DNA from the revertant colonies of Ames strains resulting from CAP treatment is an important research point which needs to be investigated in order to find out more about the kind of damage that CAP causes. Using reduction–oxidation-sensitive GFP (roGFP) also might allow more understanding to how the damage has occurred. Results obtained using OxyR regulon mutants has shown that some mutants were not significantly different from the parental strain *E. coli* BW25113. This could be due to the hypersensitivity to CAP of the K-12 parental strain making it difficult to detect differences. Therefore, construction of similar mutants in *E. coli* O157: H7 which has shown some tolerance to CAP might allow more differentiation in the sensitivity to CAP between the mutant and the parent strain. In addition, the *soxRS* regulon of *E. coli* is involved in the function of at least twelve genes in response to superoxide or nitric oxide stress (Demple, 1996). Knocking out a gene of these from *E. coli* could be a good sensor for reactive nitrogen species. Redox sensitive dyes could also be used. More investigation is needed to find out whether CAP treatments might induce mutations that make cells resistant to CAP or other antimicrobial treatments. This could be carried out by testing the sensitivity of the colonies which have survived CAP treatments to CAP and other antimicrobial treatments.

CAP has the capability to inactivate a wide range of microorganisms, including vegetative cells and spores, and biofilm organisms as well as proteins. It also can overcome some of the limitations that the conventional methods of food sterilization have, and therefore have the potential to satisfy the requirements of the food industry. CAP has the potential to be an effective control measure to prevent cross contamination with food-borne pathogens, which could be applied at critical control points in food processing plants. More specifically CAP could be used to control those pathogens which have often been found implicated in outbreaks associated with ready-to-eat foods such as *E. coli* O157: H7, *Listeria monocytogenes*, and *Salmonella*. These critical control points could be automated cutting knifes or defeathering machines used in the meat and poultry industry, or machines used in cutting, slicing, dicing and shredding of fruits and vegetables. Consequently, CAP can potentially ensure food safety and quality and ultimately could reduce the number of cases of food-borne diseases and losses in the food produced for human consumption. However, many factors have effects on the efficiency of CAP in microbial inactivation such as gas composition, gas flow rate, and configuration of the plasma rigs; substrate composition and the way the substrate is treated. Therefore, standardization of the inactivation conditions needs to be addressed.

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APPENDIX 1: PREPARATION OF CHEMICAL SOLUTIONS

1.1 Antibiotic solutions

Stock solutions of each antibiotic were prepared as described below. The appropriate amount was added to the broth media (to give final concentrations recommended for each strain before inoculation or to the molten (< 55 °C) agar media just before pouring 25 ml of medium into each Petri dish.

1.1.1. Ampicillin solution (0.8%, W/V)

Ampicillin solution was prepared by dissolving 200 mg of ampicillin powder in 25 ml of warm Milli-Q water (ultra pure water) at about 65 °C. Once the ampicillin was dissolved the solution was sterilized using a 0.45 μm membrane filter and then, aliquots of 1 ml were made and stored at -20 °C.

1.1.2. Tetracycline solution (0.8%, W/V)

Tetracycline solution was prepared by dissolving 200 mg of tetracycline powder in 25 ml of hydrochloric acid (0.02 N). Once the teteracycline was dissolved the solution was sterilized using a 0.45 μm membrane filter and then, aliquots of 1 ml were made and stored at -20 °C.

1.1.3. Kanamycin solution (50 mg/ml)

Kanamycin solution was prepared by dissolving 1.25 g of kanamycin sulphate in 25 ml of Milli-Q water. Once the kanamycin was dissolved the solution was

sterilized using a 0.45 μm membrane filter and then, aliquots of 1 ml were made and stored at -20 °C.

1.1.4. Streptomycin solution (100 mg/ml)

Streptomycin solution was prepared by dissolving 2.5 g of streptomycin in 25 ml of Milli-Q water. Once the streptomycin was dissolved the solution was sterilized using a 0.45 μm membrane filter and then, aliquots of 1 ml were made and stored at -20 °C.

1.2. Amino acid and vitamin solutions

1.2.1. Limited histidine and biotin solution (0.5mM)

D-biotin (F.W.247.3)	31.0 mg
L-Histidine. HCL (F.W.209.63)	26.3 mg

The above ingredients were added in the order indicated to 200 ml of warm Milli-Q water (about boiling) in a 250-ml volumetric flask. Each salt was thoroughly dissolved, by stirring on a magnetic stirrer until the mixture was very clear, before the next salt was added. The volume was adjusted to 250 ml using Milli-Q water. The solution was filter-sterilized using a 0.45 μm membrane filter. Aliquots of 20 ml were made and stored at 4 °C for several weeks.

1.2.2. Biotin solution (0.01%, W/V)

Biotin solution was prepared by adding 10 mg of D-biotin to 80 ml of warm Milli-Q water (about boiling) in a100-ml volumetric flask. The biotin was thoroughly dissolved by stirring on a magnetic stirrer. The volume was adjusted to 100 ml

using Milli-Q water, and then the solution was filter sterilized using a 0.45 μm membrane filter. Aliquots of 20 ml were made and stored at 4 °C for several weeks.

1.2.3. Histidine solution (0.5%, W/V)

Histidine solution was prepared by adding 547 mg of L-Histidine to 80 ml of Milli-Q water in a 100-ml volumetric flask. The histidine was thoroughly dissolved by stirring on a magnetic stirrer. The volume was adjusted to 100 ml using Milli-Q water. Aliquots of 20 ml were made in Universal glass tubes and autoclave sterilized at 121 °C for 20 minutes. Once the solution became cold, the tube caps were tightened and stored at 4 °C for several weeks.

1.3. Mutagenic solutions (positive controls for Ames strains)

1.3.1. Sodium azide

A stock solution of sodium azide (15 mg/ml) was made by dissolving 150 mg of sodium azide powder in 10 ml of sterile Milli-Q water. From the stock solutions, 100 μl was transferred into a 30-ml Universal tube (Sterilin, Ltd) containing 10 ml of sterile Milli-Q water in order to prepare a working solution of 0.15 mg/ml concentration. Both solutions were stored in the dark at 4 °C for several weeks.

1.3.2. Mitomycin C

A stock solution of mitomycin C (2 mg/ml) was made by adding 1 ml of sterile Milli-Q water into a vial containing 2 mg of mitomycin powder. A working solution of 0.05 μg/μl concentration was prepared by transferring 100 μl of

mitomycin stock solution into a 30-ml Universal tube (Sterilin, Ltd) containing 4 ml of sterile Milli-Q water. Both solutions were stored in the dark at 4 °C for no longer than one month.

1.4. Other chemical solutions

1.4.1. Vogel-Bonner (VB salts) medium E (50x)

Magnesium sulphate ($MgSO_4.7H_2O$)	10 g
Citric acid anhydrous ($C_6H_8O_7$)	91.4 g
Potassium phosphate, dibasic, anhydrous (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaHNH_4PO_4.4H_2O$)	175 g

The above salts were added in the order indicated to 650 ml of warm Milli-Q water (about 50 °C) in a 2-L Erlenmeyer flask. Each salt was thoroughly dissolved, by stirring on a magnetic stirrer, before the next salt was added. The volume was adjusted to 1 litre, then aliquots of 200 ml in a 250-ml media bottles (Duran) were made and autoclave sterilized at 121 °C for 20 minutes. Once the medium became cold, the bottles caps were tightened and stored in the dark at room temperature for several weeks.

1.4.2. Glucose solution 40%

Glucose solution (40%) was prepared by adding 400 g of D-glucose to 700 ml of Milli-Q water in a 2-L Erlenmeyer flask. The glucose powder was thoroughly dissolved by stirring. The volume was adjusted to 1 litre using Milli-Q water, then aliquots of 200 ml were made into a 250-ml media bottles (Duran) and autoclave

sterilized at 121 °C for 20 minutes. Once the solution became cold, the bottles caps were tightened and stored at 4 °C for several weeks.

1.4.3. Crystal violet solution (0.1%, W/V)

Crystal violet solution was prepared by adding 0.1 g of crystal violet to 100 ml of reverse osmosis (RO) water. The solution was thoroughly mixed and stored in the dark.

1.4.4. Magnesium sulphate solution (1.0 M)

Magnesium sulphate solution was prepared by dissolving 30.1 g of Magnesium sulphate ($MgSO_4$) in 200 ml of Milli-Q water. The solution was transferred into a 250-ml volumetric flask and the volume was adjusted to 250 ml using Milli-Q water.

1.4.5. Tris-acetate-EDTA (TAE) buffer (50X stock solution)

TAE buffer was prepared by dissolving 242 g of Tris base in RO water then 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) solution were added. The volume was adjusted to 1000 ml. From the 50X stock solution a 1X working solution was prepared by diluting the stock solution 50:1 with RO water.

APPENDIX 2: PREPARATION OF MEDIA

2.1. Luria- Bertani (LB) broth

Tryptone	10 g
Sodium chloride	5 g
Yeast extract	5 g

LB broth was prepared by dissolving the above ingredients in 1 litre of reverse osmosis (RO) water. To prepare LB agar, 15 g of agar No. 1 (Oxoid) was added. The medium was autoclave sterilized at 121 °C for 20 min. Agar plates were prepared by transferring 25 ml of agar medium, using a 25-ml pipette, to each Petri dish.

2.2. Maximum Recovery Diluent (MRD)

MRD was prepared by dissolving 9.5 g of MRD powder (Oxoid, Ltd), which was composed of 1 g of peptone and 8.5 g of sodium chloride, in 1 litre of RO water. The MRD solution was autoclave sterilized at 121°C for 20 minutes.

2.3. Brain Heart Infusion (BHI) broth/agar

BHI broth was prepared by dissolving 37 g of BHI powder (Oxoid, Ltd) (which was composed of 12.5 g calf brain infusion solids, 5g beef heart infusion solids,10g proteose peptone, 2.0 Glucose, 5.0 Sodium and 2.5 chloride disodium phosphate) in 1 litre of RO water. To prepare BHI agar, 15 g of agar No. 1 were added. The medium was autoclave sterilized at 121°C for 20 minutes. Agar plates were

prepared by transferring 25 ml of molten (> 55°C) agar media, using a 25-ml pipette, to each Petri dish (Difco Laboratories, 1998).

2.4. Nutrient broth

Nutrient broth was prepared by dissolving 25 g of Nutrient broth No. 2 powder (Oxoid, Ltd) which was composed of 10g 'Lab-Lemco' powder, 10g peptone and 5g sodium chloride in 1litre of Milli-Q water. Aliquots of 200 ml in a 250-ml media bottles (Duran) were made and autoclave sterilized at 121°C for 20. When the media became cold, the bottles caps were tightened and stored in the dark at room temperature.

2.5. Nutrient agar plates

Agar	15 g
Nutrient broth No. 2	25 g

The above ingredients were dissolved in 1 litre of Milli-Q water. The medium was autoclave-sterilized at 121 °C for 20 minutes. Agar plates were prepared by transferring of 25 ml of molten (> 55 °C) agar media, using a 25-ml pipette, to each plate.

2.6. Top agar supplemented with limited histidine and biotin

Agar	3 g
Sodium chloride	3 g

The above ingredients were added to 450 ml of Milli-Q water in a 500-ml media bottle. The mixture was heated until the agar was completely melted and then 50

ml of limited histidine and biotin solution (0.5 mM) were added. Aliquots of 3 ml were made and the medium autoclave-sterilized at 121 °C for 20 minutes (Mortelmans and Zeiger, 2000).

2.7. Minimum glucose (MG) agar plates

MG agar media were prepared by adding of 7.5 g of Agar to 450 ml of Milli-Q water in 500-ml media bottle. The mixture was autoclaved for 20 min at 121 °C. Once the agar had been cooled to about 65 °C, 10 ml of VB salt (50x), and 25 ml of 40% glucose solution were added aseptically in the order indicated above. Moreover, amounts of chemicals and antibiotics required for each strain (as indicated in Table 2.1) were also added. Thorough shaking was applied after the addition of each of these ingredients to the sterilized agar. Agar plates were prepared by transferring 25 ml of agar medium, using a 25-ml pipette, to each Petri dish (Mortelmans and Zeiger, 2000).

2.8. MG supplemented with limited histidine and biotin (MGLHB) agar plates

MGLHB agar plates were prepared in the same way the MG agar plates were prepared. However, 240 mg of sodium chloride were added to the agar-water mixture before autoclaving and, 4 ml of limited histidine/biotin solution (0.5mM) were added just before distribution of the media into the Petri dishes. These amounts of sodium chloride and limited histidine and biotin solution (0.5mM) were calculated to give a final concentration per plate equivalent to that which should be given by adding 2 ml of the top agar which had a concentration of 6 mg

/ml and 0.1 ml/ml of sodium chloride and limited histidine and biotin solution (0.5mM) respectively, i.e. the final concentration per plate that contains 25 ml of medium, was 12 mg/plate and 0.2 ml/plate of sodium chloride and limited histidine and biotin solution (0.5mM) respectively. It has been reported that these trace amounts of histidine are essential to let all the bacteria on the plate go through several cell divisions and this growth is necessary in several cases for mutagenesis to occur (Ames *et al*, 1973; Mortelmans and Zeiger, 2000).

2.9. Enriched MG agar plates

Enriched MG agar plates were prepared in the same way as the MG agar plates were prepared. However amounts of vitamins and amino acids, which are needed to prepare different types of enriched MG plates as indicated in the Table 2.1, were added before distribution of the media into the plates (Mortelmans and Zeiger, 2000).

Table 2.1: Chemicals (vitamins, amino acids) and antibiotics added in order to prepare different types of enriched MG agar plates (Mortelmans and Zeiger, 2000).

Type of plates	**Chemical and Antibiotic added to prpare 500 ml of MG medium**
Biotin supplemented MG Plates	4 ml of 0.01% biotin to give final concentration 0.8 μg/ml of medium
Histidine supplemented MG Plates	4 ml of 0.5% histidine to give final concentration 40 μg/ml of medium
Histidine and biotin supplemented MG Plates	4 ml of 0.01% biotin and 4 ml of 0.5% histidine to give final concentration 0.8 μg/ml and 40 μg/ml of medium
Histidine, biotin and ampicillin supplemented MG Plates	4 ml of 0.01% biotin, 4 ml of 0.5% histidine and 1.5 ml of ampicillin (8 mg/ml) to give final concentration 24 μg/ml of medium
Histidine, biotin and tetracycline supplemented MG Plates	4 ml 0.01% biotin, 4 ml of 0.5% histidine and 125 μl of tetracycline (8mg/ml) to give final concentration 2 μg/ml of medium
Histidine, biotin, ampicillin and tetracycline supplemented MG Plates	4 ml 0.01% biotin, 4 ml of 0.5% histidine , 1.5 ml of ampicillin (8 mg/ml) and 125 μl of tetracycline (8 mg/ml) to give final concentrations as above

2.10. M9 Minimal Salts (5X)

Disodium phosphate heptahyrate ($Na_2HPO_4.7H_2O$)	64.0 g
Potassium phosphate, monobasic (KH_2PO_4)	15 g
Sodium chloride (NaCl)	2.5 g
Ammonium chloride (NH_4Cl)	5 g

The above ingredients were added into a 2-L Erlenmeyer flask containing 1 litre of Milli-Q water and were thoroughly dissolved, by stirring on a magnetic stirrer. The volume was adjusted to 1 litre, and then aliquots of 200 ml were dispensed into 250-ml medium bottles which were autoclave sterilized at 121 °C for 20 minutes. Once the medium became cold, the bottlescaps were tightened and the media stored at room temperature (Davis *et al.*, 1994; Difco Laboratories, 1998).

2.11. M9 Minimal Salts Medium (MSM)

MSM was prepared by adding 200 ml of M9 (5X) to 750 ml of sterile Milli-Q water cooled to 45-50 °C. Twenty ml of filter sterilized 20% glucose solution and 2 ml of sterile 1.0 M $MgSO_4$ solution were added aseptically and thoroughly mixed, then the volume was adjusted to 1000 ml (Davis, *et al.*, 1994; Difco Laboratories, 1998).