Evaluation of the Sensitivity of Bacterial and Yeast Cells to Cold Atmospheric Plasma Jet Treatments

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Abstract

The focus of this research was firstly to determine the influence of the atmospheric plasma drive frequency on the generation of atomic oxygen species and its correlation with the reduction of bacterial load after treatment *in vitro*. The treatments were carried out using a helium-plasma jet source called PlasmaStream[TM]. The susceptibility of multiple microbial cell lines was investigated in order to compare the response of gram-positive, gram-negative as well as yeast cell lines to the atmospheric plasma treatment. It was observed for the source evaluated that at a frequency of 160 kHz, increased levels of oxygen-laden active species (i.e. OH, NO), were generated. At this frequency the maximum level of bacterial inactivation *In vitro* was also achieved. *Ex vivo* studies (using freshly excised porcine skin as a human analogue), were also carried out to verify the anti-bacterial effect of the plasma jet treatment at this optimal operational frequency and to investigate the effect of treatment duration on the reduction of bacterial load. The plasma jet treatment was found to yield a four log reduction in bacterial load after 6 minutes of treatment, with no observable adverse effects on the treatment surface. The gram-negative bacterial cell lines were found to be far more susceptible to the atmospheric plasma treatments, than the gram-positive bacteria. Flow cytometric analysis of plasma treated bacterial cells (*E. coli*) was conducted in order to attain

a fundamental understanding of the mode of action of the treatment on bacteria at a cellular level. This study showed that after treatment with the plasma jet that the stages of cell death that the *E. coli* cells progressed through the following stages of cell death - the inactivation of transport systems, followed by depolarisation of the cytoplasmic membrane and finally permeabilisation of the cell wall.

Introduction

While atmospheric plasmas have been widely applied for surface cleaning or activation prior to adhesive bonding [1], their use in biomedical applications is relatively recent [2] and is often termed Plasma Medicine. There has been growing interest in the use of atmospheric plasma sources for biological treatments as they have been shown to kill bacteria [3], enhance the rate of wound healing [4], and have also exhibited potential as a cancer treatment [5]. Cold Atmospheric Plasmas (CAP) have been shown to be especially effective as a low-temperature treatment for medical device decontamination [6]. A particular advancement of CAP-based medical sterilization is its ability to inactivate prions under specific conditions, a misfolded protein that is resistant to conventional heat and/or chemical germicides decontamination procedures [7, 8]. In addition, by using atmospheric plasmas, it is possible to treat substances that are not suitable for treatment under vacuum, such as living organisms. These plasma treatments are reported to achieve promising results without significant heating or painful sensations [9, 10].

Although the bactericidal effect of plasmas is widely acknowledged, the mechanisms of action are still not fully understood [11]. To date, there have been relatively few reports which systematically investigate the interaction of plasma and microorganisms, living tissues, and, in particular, potential applications in medicine [9, 11-15]. One of the main theories for the breakdown of the bacterial cells, is one first suggested by Mendis *et al.* and Laroussi *et al.* [16, 17]. which involves an electro physical mechanism. They established the idea that charged

particles could be majorly responsible for the rupture of the cell membrane following plasma treatment. Electrostatic tension builds up on the cell surface culminating in electrostatic disruption of the outer cell membrane. Reactive species such as –OH, NO_2 and O have also been reported to be responsible for bacterial load reduction using cold atmospheric plasma treatments [18, 19]. These reports have shown that oxygen-based and nitrogen-based species can cause strong oxidative stresses on the outer membranes of cells. The outer lipid bilayer of a cell envelope is susceptible to the effects of the hydroxyl radical and it is believed that this radical negatively impacts the function of the membrane, leading to cell lysis [20]. The inactivation of Gram positive bacteria by atmospheric plasma treatments is thought to be caused by the diffusion of reactive species produced by the plasma jet through the cell membrane, where they affect the intracellular compounds [16]. In contrast, the inactivation of Gram-negative bacteria is reported to be associated with charge accumulation on the outer surface of the cell membrane, which overcomes the tensile strength of the membrane leading to its rupture [21].

Recent studies to analyse the effect of atmospheric pressure plasma jets (APPJs) on mammalian cells have included the use of flow cytometry [22]. These relatively limited studies have demonstrated that the plasma jet treatment affects the cells on a sub-cellular level, namely decreasing the expression of cell surface integrins. Other work investigating the effect of APPJs on tumoral cells using flow cytometry showed up to 70 % apoptosis following plasma treatment [23].

The primary objective of this research is to develop an understanding of the effects of atmospheric plasmas on bacterial cells. Due to the bacterial diversity on wound surfaces, the aim is to obtain data that will facilitate greater application of these plasmas to wound care. The approach taken was three-fold. The first was to evaluate, *in vitro,* the effects of the plasma on various bacterial strains, and to determine the optimum operational parameters by which maximum bacterial inactivation could be achieved. Secondly, *ex vivo* studies were

performed using freshly excised porcine skin to help evaluate its effect on tissue. The skin was firstly inoculated and then treated with the plasma, in order to determine inactivation levels. Maximum safety thresholds were established to ensure no damage would be inflicted to healthy tissue at the site of application. Finally, intracellular changes in bacterial physiology were evaluated using flow cytometry after plasma treatments in order to develop a fundamental understanding of the processes by which the plasma induces cell death.

Experimental Section

Plasma Source

The cells were treated using a helium atmospheric pressure plasma jet (APPJ) manufactured by Dow Corning Plasma Solutions, which uses dual-pin parallel tungsten electrodes of end-type design [24]. This source has two electrode pins which are spaced 12 mm apart and positioned at one end of a quartz reactor tube (length 60 mm, 19 mm outer diameter, 16 mm inner diameter with a nozzle exit area of 2 cm^2). The internal volume of the nozzle is 12 cm^3, with a specific heat capacity of 700-733 J kg^{-1} K^{-1} at 300 K [25]. The process gas (helium) flows between the two pin electrodes down through the quartz tube and exits at the open orifice of the nozzle. The gas flow rate was fixed at 10 slm in this study. This APPJ set-up is completed with a C2000 Redline high voltage variable frequency generator, allowing the frequency to be varied from 1-500 kHz [26].

Plasma Characterization

Optical Emission Spectroscopy (OES) was used in order to investigate the intensity change for plasma-generated reactive species, as certain reactive species are believed to be the main factor influencing wound healing during atmospheric plasma treatments [27]. In this study, OES data was captured using an Ocean Optics USB4000 UV/VIS spectrometer. This system operates across the range of 200-850 nm and has a resolution of 1.5 nm FWHM (Full With at

Half Maximum). The OES lens was positioned approximately 5 cm from the bottom of the plasma applicator jet, while all measurements were taken at a 90 $^{\circ}$ angle to the quartz tube and 1 mm downstream from the nozzle exit orifice.

Bacterial Cell Growth

The effect of the plasma treatments on both Gram positive and Gram negative bacterial cell lines was investigated. The three Gram negative species used in this study were *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13884), *Pseudomonas aeruginosa* (ATCC 27853) and the two Gram positive species used were *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 29213). In addition, one yeast cell line was included in the study as a way of investigating how plasma effects differ due to cellular morphology. The cell line studied was *Saccharomyces cerevisiae*. All bacterial cell lines were grown overnight in tryptone soya broth at 37 $^{\circ}$C. Overnight stocks of the bacteria were prepared as follows: after flaming a sterile conical flask of 100 ml medium, a sterile loop was used to transfer one single colony of the bacteria from a tryptone soya agar plate to the flask. The bacteria were then allowed to incubate for 16 hrs with vigorous shaking (250 r/min) at 37 $^{\circ}$C. The yeast cell line investigated, *S. cerevisiae*, was cultured in high glucose broth medium as this cell line needs higher levels of sugar to survive.

In vitro Bacterial Treatment

1 ml aliquots of the bacterial solutions were pipetted into six well plates. These wells were treated individually with exposure times varying between 30 and 360 s. Tenfold dilution of the treated samples were plated on tryptone soya agar and incubated for 24 hrs. Using this serial dilution technique, the colonies on each plate could be counted. This data was then used to calculate colony-forming units (CFUs) /ml of treated sample, which when compared to

CFUs/ml of untreated control sample could be used to determine the effectiveness of the treatment in bacterial inactivation.

Ex vivo Porcine Model

The porcine skin used to model the plasma effects *ex vivo,* was obtained from a local abattoir (Ballon Meats, Carlow). All pig skin was freshly excised, cleaned and sterilised upon arrival in the lab. The sterilisation procedure was as follows: the skin was divided into 2 cm^2 samples, washed in a sterile laminar cabinet using aseptic techniques with methanol and ethanol and rinsed with sterile deionised water. Samples were then placed in individual sterile petri dishes and allowed to dry before testing. The cleaning procedure involved washing with both phosphate-buffered saline and ethanol and then returning to sterile petri dishes prior to plasma treatment. The plasma treatments of the excised skin were carried out within 2 days of animal slaughter. The choice of treatment parameters to use was based on the results of the *in vitro* experiment for the frequency (160 kHz), and on observation of microscopic damage to the porcine skin, the voltage used was 100 V.

50 μl aliquots of bacterial solution were pipetted onto the skin surface and the samples were returned to the incubator for 30 minutes to allow the bacteria to soak into the skin surface. Skin samples were then treated at 160 kHz for a treatment time of between 2 and 6 minutes. Following the treatments, the samples were placed in 10 ml sample tubes of sterile PBS for 30 minutes. 1 ml from each of these bottles was removed in order to perform serial dilutions and plated on agar plates, using the spread-plate method. These plates were then incubated overnight at 37 °C and the number of colony forming units per ml was determined using the colony count method [28]. All measurements were made in triplicate.

Flow cytometry analysis

A Beckman Coulter CyAn™ ADP Analyser was employed, with excitation using a 488 nm laser. The neutral density filter was removed to provide increased sensitivity in the forward

scatter channel. Detection of fluorescence emission from propidium iodide (PI) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX) was achieved using the FL4 (680/30) and FL1 (530/40) band-pass filters, respectively. Control experiments were carried out to establish treatment conditions that would clearly separate dye-positive from dye-negative cell populations. The negative population consisted of untreated *E. coli* (a 10 mL overnight culture in TSB was harvested by centrifugation and re-suspended in 20 mL tris-buffered saline, pH 7.5 (TBS), and 0.1 mL of this diluted to 1 mL with TBS). To prepare permeabilized cells for PI staining, cells were treated with 70% isopropanol (0.1 mL cell suspension + 0.2 mL TBS + 0.7 mL isopropanol) for 30 min at room temperature, whereupon they were centrifuged, rinsed once with 1 mL TBS, and re-suspended in 1 mL TBS. PI was made up to 200 µg/mL in distilled water, and 16.5 µL was added to the cell suspensions (final PI concentration 3.3 µg/mL) shortly before analysing the samples. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is an ionophore that disrupts transmembrane ion gradients, thereby causing membrane depolarization. 20 µL of 5 mM CCCP dissolved in DMSO was added to a 1 mL cell suspension (giving a final concentration of 100 µM), while 20 µL DMSO was added to control cells. After 5 min, 10 µL BOX stock solution (10 mg/mL in DMSO) was added to both samples (final concentration 100 µg/mL), and flow cytometry analysis was carried out. After plasma treatment of the test samples, the bacterial suspensions were removed from the 6-well plates and the wells rinsed with TBS. Cells were pelleted by centrifugation for 5 minutes and washed twice with TBS. After the second wash the samples were re-suspended again in 1 mL TBS and stained with the dyes as per the control cells above.

Results and Discussion

Optical Emission Spectroscopy (OES)

Preliminary investigations evaluated the effect of the plasma frequency on the level of active species generated by the helium plasma jet. OES was used to collect the localised emission

lines observed along the axis of the plume and to study their intensities and spatial variation. The plasma species selected were OH, O, He and NO_2. These reactive oxygen and nitrogen species were selected, because they were found to have the most significant effect on bacterial inactivation [29-31]. He gas lines were selected in order to monitor the change in the process gas.

As illustrated in Figure 1, a variation in intensity of OH, O, NO_2 and He with the frequency of the plasma was obtained. Two main harmonics can be identified. The first observed at 160 kHz corresponds to the maximum intensity of all the active species investigated within the plasma, while the second harmonic (320 kHz) shows a peak in the OH molecular band but not in the other three active species investigated.
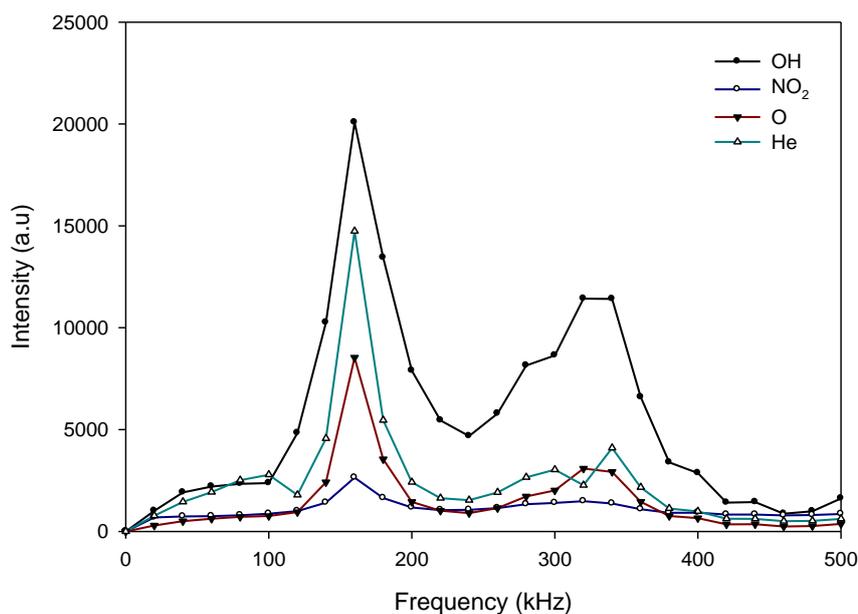
Figure 1: Optical emission spectroscopy intensity data for the four species shown, with changes in the frequency used to generate the He atmospheric plasma jet. Higher levels of active species production can be seen at both 160 kHz and at 320 kHz.

Atmospheric Plasma Treatment of Bacterial Cells *in vitro*

Initial bacterial treatment studies were performed using *E. coli*. This was the first strain investigated due to it being commonly found on wound surfaces, its ease of manipulation, culturability and short doubling time [32]. The study involved plasma treatment of 2 ml aliquots of bacterial solution in 6 well plates for 2 minutes at 160, 140 and 180 kHz. The latter frequencies were chosen for comparison as they are located 20 kHz, either side of this strong harmonic. With this lower intensity, the aim was to confirm the findings from the OES experiment, regarding the optimal treatment regime. Jet orifice-to-sample distance was kept constant at 4 mm, while the He flow rate was 10 slm. The number of surviving CFUs after treatment was determined using the serial dilution and pour plate method. Bacterial inactivation was observed for all three treatment conditions; however it is clear from Figure 2, that the optimal operational frequency for bacterial inactivation of those tested is 160 kHz. As expected this frequency corresponded to the highest intensity of active species using the OES measurements
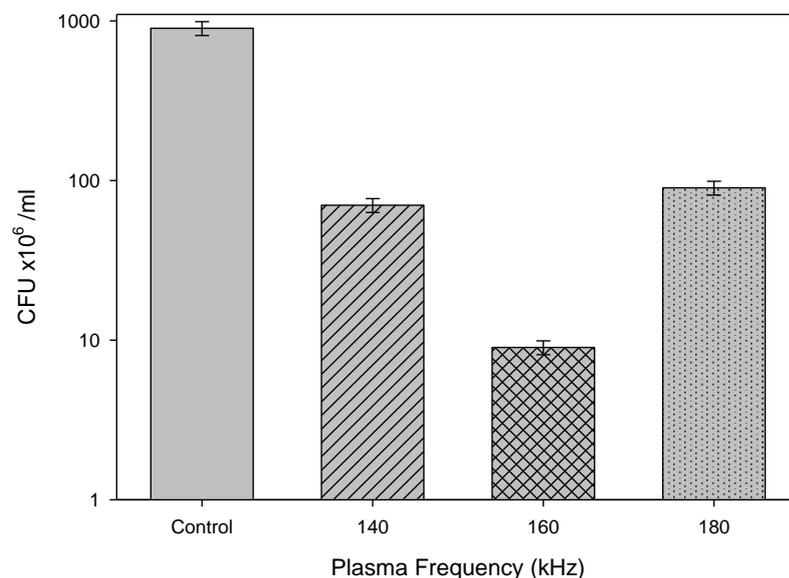
Figure 2**:** Bar graph showing the effect of varying plasma frequency on bacterial reduction of *E.coli*.

To further evaluate the effect of the reactive species on bacterial activation, a frequency of 100 kHz, determined from the OES (Figure 1), was chosen as a point with minimal production of reactive species in order to compare directly with its highly producing 160 KHz counterpart. These frequencies were subsequently tested over a number of time durations to evaluate the effect of administered plasma dose, Figure 3. A correlation between the plasma dose administered and the bacterial reduction was observed, whereby increasing treatment durations as expected led to a lower bacterial load. It was determined that although the reactive species created using the 160 KHz frequency clearly induced a faster reduction in bacterial load when compared to the 100 KHz, it was evident that these reactive species are not the only mechanism for the induction of cell death but are of significant importance.
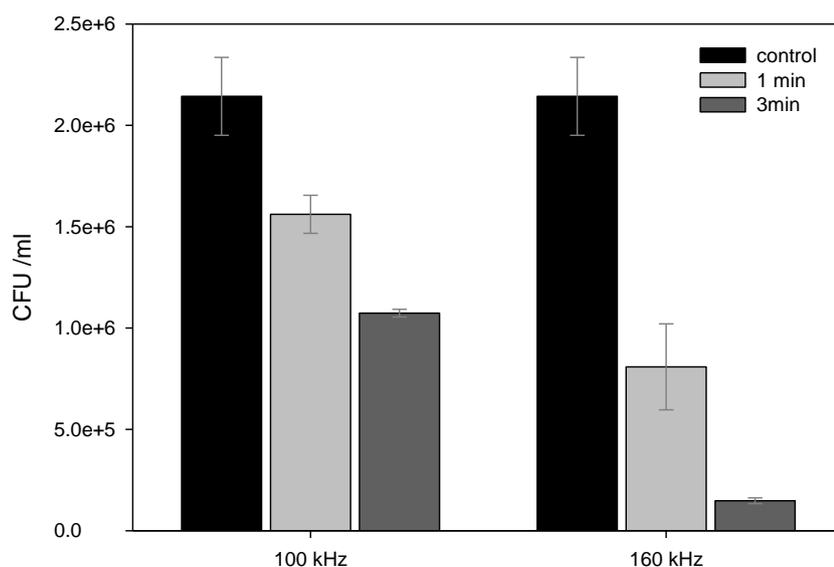
Figure 3: Colony forming units found to survive after plasma treatments of *E. coli* treated for 1 and 3 minutes at both 100 and 160 kHz.

Atmospheric Plasma Treatment of Bacterial Cells *ex vivo*

This experiment consisted of inoculating porcine skin samples and performing plasma treatments in order to determine the efficacy of plasma inactivation of bacteria in an environment closer to that observed in a human wound. The results from this experiment are

shown in Figure 4. It was observed that upon treating the pig skin for up to 6 minutes using the jet operating at 160 kHz, a 4 log reduction in bacterial load was achieved. Based on microscopic examination, no damage to the skin was observed under the conditions used. This experiment also confirms observations in earlier *in vitro* experiment regarding the correlation of the plasma dosage effect with bacterial reduction.
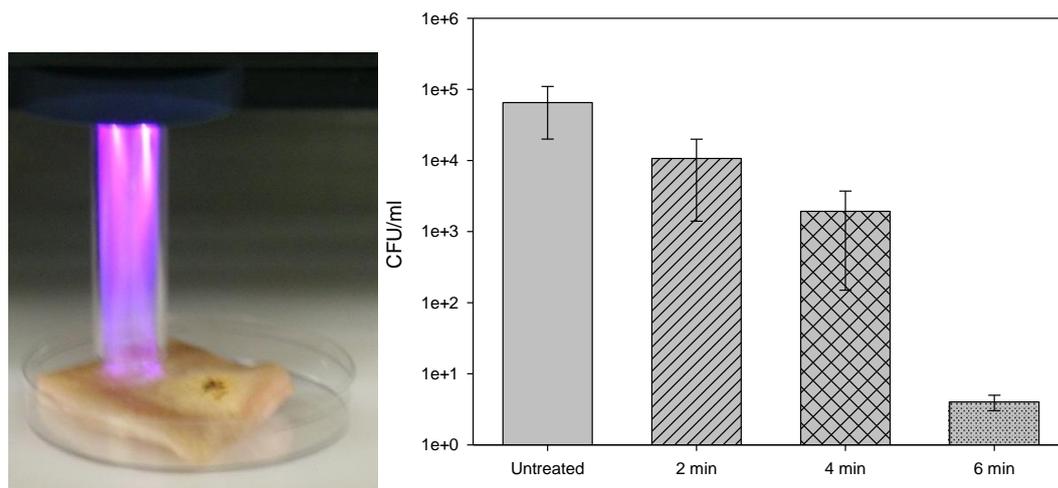
Figure 4: Photograph showing the treatment of pig skin using the He plasma jet (left). Graph of CFU/ml results obtained for plasma treating *E. coli* inoculated on fresh pig skin for the treatment times shown (right).

Cell Line Sensitivity

Within infected wounds, many strains of bacteria can be found [33] and so the ability to inactivate various distinct cell lines is desirable if a plasma source is to be effective as a wound treatment system. Cell line sensitivity was evaluated using both Gram positive *Bacillus subtilis* and Gram negative *Escherichia coli* were treated using the plasma jet. The investigation involved pipetting 2 ml aliquots of bacterial samples into 6-well plates followed by plasma treatments under the following treatment parameters: 160 kHz, 100 V, jet orifice-to-surface distance of 4 mm, Helium flow rate at 10 slm. Multiple treatment times were performed ranging from 10 to 120 seconds. Colony forming unit counts were determined

after less than one hour, as well as after 4 hours following plasma treatment, to determine the immediacy of the plasma inactivation. The results of this treatment are shown in Figure 5 (*E. coli* and *B. subtilis*). Comparing the results of these two bacteria it is clear that the *E. coli* is more sensitive to the plasma treatment than the *B. subtilis*. One explanation for the relative lack of structural damage to Gram-positive bacteria in comparison to Gram-negative in the current study is that while the latter bacteria have a double membrane, the Gram-positive bacteria have a thicker murein layer, which makes them more rigid thereby increasing their tensile strength [16]. Gram negative bacteria also have a far more irregular surface morphology than gram positive [17, 34, 35]. These irregularities could lead to higher localised electrostatic forces culminating in membrane rupture at lower doses than those needed for a similar result in gram positive cells.

Under the plasma processing conditions used, significant levels of bacterial cell death were not observed within the first hour after treatment (Figure 5). When samples were returned to the incubator however, and then tested again at 4 hours after treatment, cell viability was found to significantly decrease. This could indicate that the inactivation ability of the plasma does not take place instantly upon treatment and the survivability of the cell is not a clear situation of either dead or alive, i.e. the health of the cell may only be attenuated at first and gradually it succumbs to cell lysis. This observation concurs with previous literature [17, 36], in which it was established that survival curves of bacterial strains after plasma treatment in fact followed a biphasic inactivation kinetics curve, with initial inactivation levels being significantly lower than the second and far less resistant phase, i.e. the cells are initially inactivated slowly, but as time passes, after the plasma treatments, the cells exhibit a higher level of inactivation.
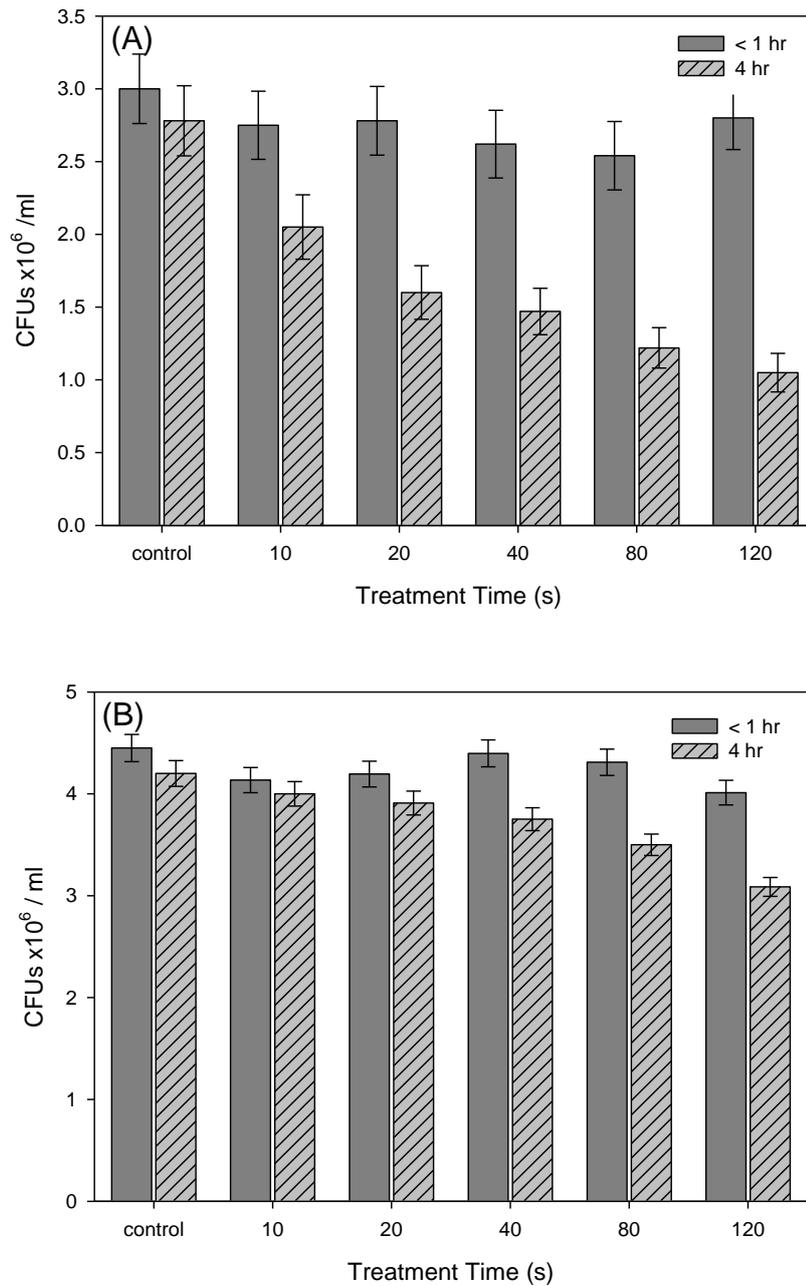
Figure 5: Colony forming units (CFU) /ml that survived plasma treatment of gram negative cell line *E. coli* (A) and gram positive cell line *B. subtilis* (B) after treatment times of up to 2 minutes. The untreated control is included for reference.

In order to further investigate the Gram-specific effect observed in the initial study, three additional cell lines were treated under the same conditions: two Gram-negative strains (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) and one Gram positive strain (*Staphylococcus aureus*). Also examined was the yeast cell line *S. cerevisiae*, this type of

cell, although similar to bacteria in many ways, differ significantly in morphology and their biological and chemical pathways [37]. The results of this experiment for the *S. cerevisiae* cell are given in Figure 6.
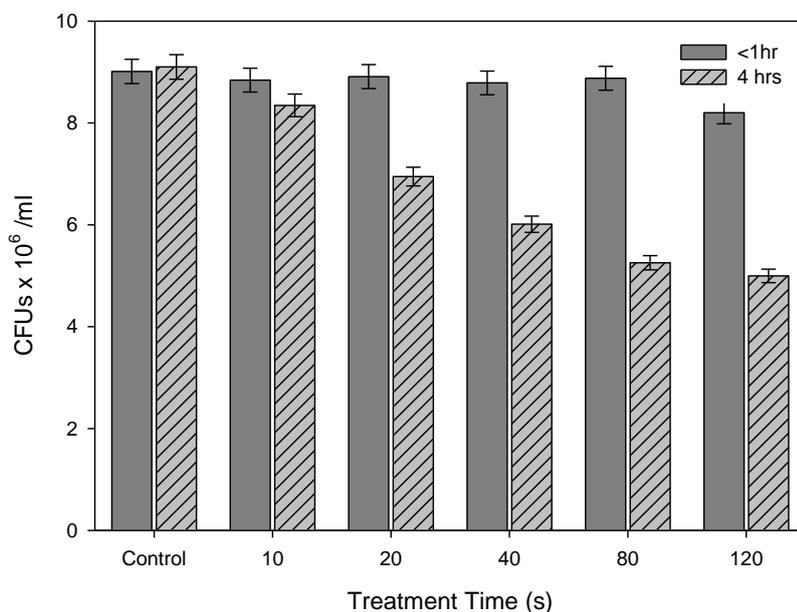
Figure 6: Colony forming unit that survived plasma treatment of yeast cell line *S. cerevisiae* after treatments of up to 2 minutes, the untreated control is included for reference

The trend for *S. cerevisiae* cell show in Figure 6 is similar to that observed for *E. coli* and *B. subtilis* cell lines described earlier. Similar decreases in colony forming units with treatment time were observed for *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Based on the level of reduction in colony forming units it is possible to rank the sensitivity of the bacteria and yeast cells investigated to the plasma treatments, this is shown in Table 1. From this study it is concluded that the Gram-negative strains succumb to cell death after plasma treatment more readily than the Gram-positive strains. It was also found that the yeast cell (*S. cerevisiae*) showed inactivation rates that fell between the gram negative bacteria *P. Aeurginosa* and *K. pneumoniae*.

Table 1: Sensitivity of Gram positive (G+) and Gram negative (G-) bacteria to plasma treatments

Decreasing Sensitivity to Plasma Treatment					
E. coli (G-)	*P. Aeurginosa* (G-)	*S. cerevisiae* (Yeast)	*K. pneumoniae* (G-)	*S. aureaus* (G+)	*B subtilis* (G+)

Flow Cytometry

The use of propidium iodide (PI) for flow cytometry analysis is a rapid and well-established method for monitoring cell death and is based on the principle that the intact membrane of viable cells excludes the propidium ion [38]. Loss of this permeability barrier represents irreparable damage leading to cell death [39]. BOX is a fluorescent stain that enters the cell only if the membrane is depolarised [40]. BOX staining allows for a measure of the cellular metabolic "stress" level. The presence of an intact polarised cytoplasmic membrane signifies a fully healthy cell. It is believed that if a cell is stressed energetically as opposed to physically, the active transport system will cease, the cytoplasmic membrane will become depolarised and eventually permeabilisation will occur, indicating cell death [41].

Before analysing the effect that plasma treatment has on *E. coli*, the staining procedure and instrument setup were optimised using control populations of cells – alcohol-permeabilised cells for PI staining, and CCCP-treated (depolarised) cells for BOX staining. Untreated cells served as negative controls in each case. Alcohol-permeabilised cells showed a marked increase in PI fluorescence, with over 99 % of cells occurring in the high fluorescence region, as compared to 0.2 % for untreated cells. The shift in fluorescence was less marked in the case of CCCP-depolarised cells stained with BOX. Nonetheless, almost 95 % of treated cells appeared in the high fluorescence region, compared to approximately 1 % for untreated cells, demonstrating that conditions were found whereby permeabilised and depolarised cells could clearly be differentiated from normal healthy cells.

With both positive and negative controls established, the staining and flow cytometry conditions could be applied to atmospheric pressure plasma jet-treated samples. The results given in Figures 7 and 8 show the effect that 5 minutes of plasma treatment had on the cells. These are scatter plots of log of PI fluorescence versus log of BOX fluorescence (membrane disruption versus membrane depolarisation). In Figure 7, untreated and plasma-treated cells are stained with each dye separately. In the upper panels, the occupancy of Region 4 increases from 1% (control cells, left) to 8.5 % (plasma-treated cells, right) when stained with PI, indicating membrane damage caused by plasma exposure. The lower panels present control cells (left) and plasma-treated cells (right) that have been stained by BOX to detect membrane depolarisation. These results show that plasma treatment brings about a significant level of membrane depolarisation (the occupancy of Region 1 increases from 0.2 to 20.4 %). When double staining of cells is carried out (Figure 8), it becomes clear that the permeabilised cell population is a subset of the 20 % that are depolarised (i.e. they stain for both dyes). It may well be that depolarization of the cell membrane takes place first and is followed by (or leads to) the full permeabilisation of the cell, resulting in cell death. According to the literature, only 1 % of cells whose membranes become permeabilised will form a colony when grown on agar plates [41]. 40-60 % of cells with depolarized membranes will still manage to form colonies.

To investigate if this reduction in the growth of colonies was achieved as a result of the atmospheric plasma treatment, serial dilutions were performed on samples treated for 5 minutes with the plasma. Treated cells and control samples were plated onto agar and left to incubate overnight. Colony forming units/mL were evaluated and it was found that ~60 % of cells formed colonies, which was considerably less than the ~86 % expected. This difference with the previous report in the literature [41] may be attributed to the fact that the plasma effect leading to cell death does not transpire immediately. Cells are not simply "zapped" by the plasma and permeabilised instantly. What is more likely is that it is a gradual effect that takes

place, where some cells might even receive a mild enough treatment they are capable of repairing themselves if plated on growth medium soon enough, while others experience significant levels of treatment and begin the process of cell lysis. This correlates well with the results from Figure 4, where *E. coli* cell counts were seen to exhibit very little change when determined immediately after plasma treatment, but when they were returned to incubation and measured four hours after treatment, significant levels of inactivation were observed.

It was hypothesized by Nebe-von-Caron *et al*. that if a cell is stressed energetically, the pathway towards cell death will begin with the active transport systems ceasing, followed by the cytoplasmic membrane becoming depolarised and culminating in the membrane becoming fully permeabilised [41]. The stresses they imposed on cells included heat treatment or energy starvation. The fluorescent staining methods utilised during this current research have determined aspects of the physiological state of individual bacterial cells following treatment with atmospheric plasma. This multicolour staining procedure has led to improved understanding of the intracellular processes taking place that result in bacterial inactivation after these treatments. The results obtained by this research validate the hypothesis that bacteria after treatment with atmospheric plasma follow a similar path towards cell death as that put forward by Nebe-von-Caron *et al* [41].
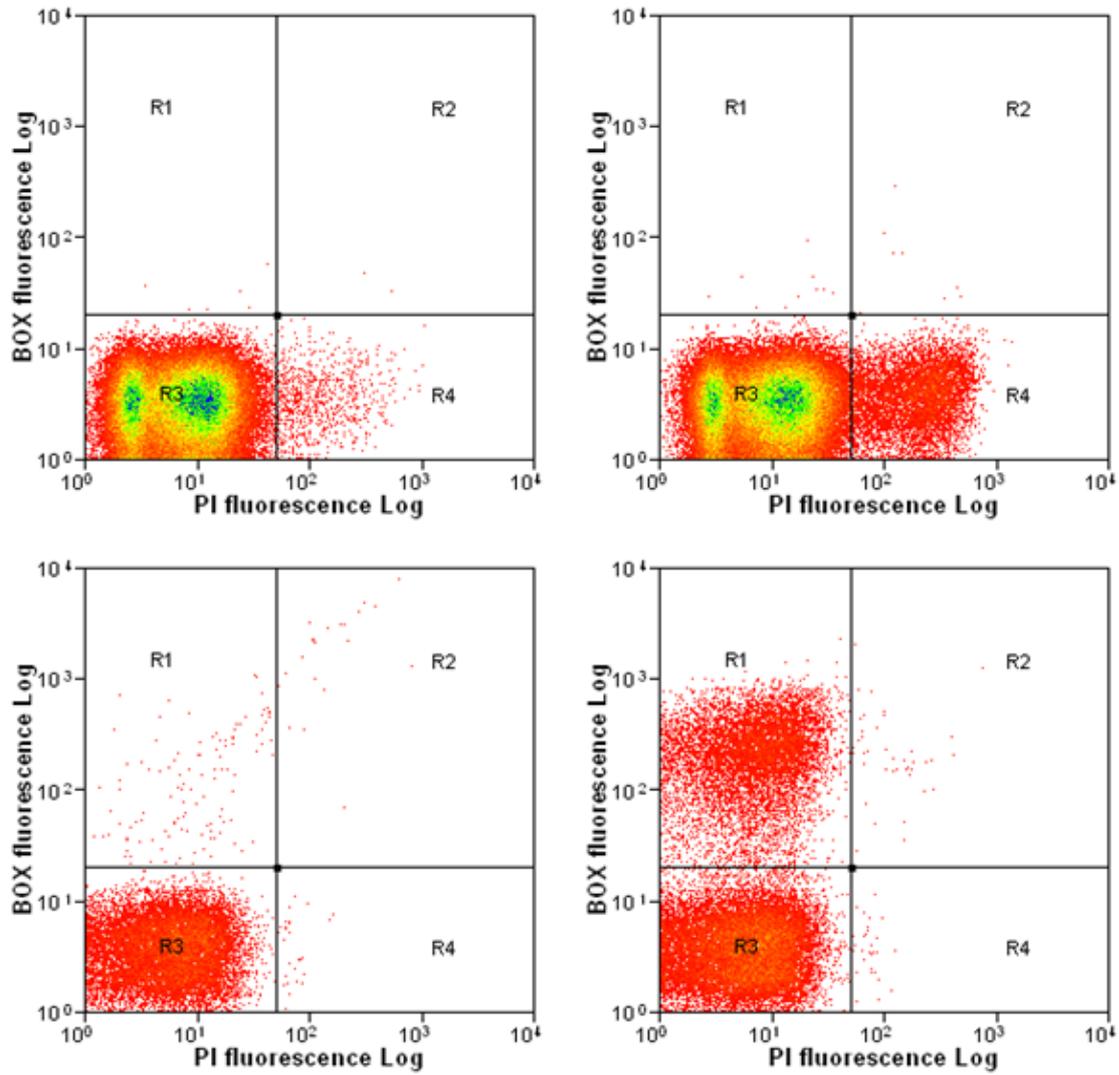
Figure 7: Flow cytometry analysis of single dye staining of control *E. coli* cells, and cells exposed to 160 kHz plasma for 5 min. Bi-variate plots are shown of PI versus BOX fluorescence for cells stained with one dye only. Dots correspond to individual cells. Where more than one cell co-locates, the colour of the plot changes progressively from red to blue.
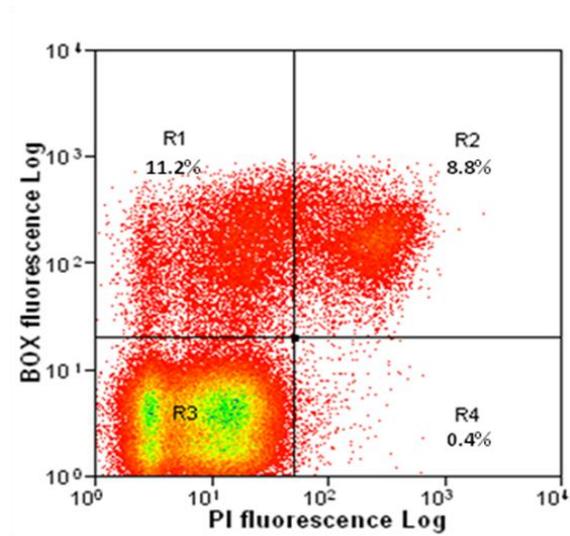
Figure 8: Flow cytometry of plasma-treated E. coli, double-stained for PI and BOX. The occupancy of the quadrants shows that plasma treatment results in 20 % of the cell population being BOX-positive (R1 + R2), therefore having depolarised membranes, and that almost all of the PI-positive (membrane disrupted) cells are also BOX-positive (R2 at 8.8 %, with R4 at 0.4 %).

Conclusions

This study investigated three aspects of the treatment of bacterial and yeast cells using atmospheric plasma. Firstly, the determination of the effect of plasma frequency on the generation of oxygen active species, and its effect on bacterial reduction. Once the optimised frequency for this system was determined, treatments were carried out both *ex vivo* (porcine skin) and *in vitro* (bacterial solution). The mode of operation of the plasma was then determined using flow cytometry.

Specific outcomes of this research are as follows:

- Real-time optical emission spectroscopy (OES) showed a variation in intensity of active species (OH, NO_2) believed to be responsible for bacterial inactivation with varying operational frequency of the plasma source. The largest harmonic, observed at

160 kHz, generates the maximum intensity of the active species and this frequency was therefore selected for the *in vitro* treatment studies.

- In order to determine the effect of frequency on the inactivation efficiency of the plasma, samples of *E. coli* were treated for 2 minutes at 140, 160 and 180 kHz and it was found that samples tested at 160 kHz exhibited the highest levels of bacterial inactivation.

- Cell line sensitivity to the helium plasma was found to be in the following order:

  E. coli ≥ *P. aeruginosa* ≥ *S. cerevisiae* ≥ *K. pneumoniae* >> *S. aureus* > *B. subtilis*

  Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *K. pneumoniae*) were found to be more susceptible to the plasma treatments than Gram-positive strains (*S. aureus*, *B. subtilis*). This sequence concurs with previous observations in literature that gram-negative cell lines were shown to be far more susceptible to the plasma treatments and it is believed that this is due to their thinner cell membranes.

- *Ex vivo* experiments carried out on *E. coli* inoculated on skin showed that the APPJ operating at 160 kHz achieved a four log reduction in bacterial load after 6 minutes of treatment (99.99 % reduction).

- Flow cytometric evaluation after treatment of *E. coli* showed that with increasing plasma intensity the progression of this bacterial cell line towards cell death was clearly evident. The mechanism observed was initially a decrease in cell membrane potential, culminating in full membrane permeabilisation.

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