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Atmospheric Cold Plasma Inactivation of Escherichia Coli, Salmonella Enterica Serovar Typhimurium and Listeria Monocytogenes Inoculated on Fresh Produce

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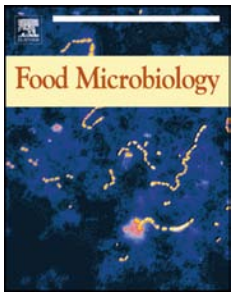

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Atmospheric Cold Plasma inactivation of *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* inoculated on fresh produce

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Abstract

Atmospheric cold plasma (ACP) represents a potential alternative to traditional methods for non-thermal decontamination of foods. In this study, the antimicrobial efficacy of a novel dielectric barrier discharge ACP device against *Escherichia coli*, *Salmonella enterica* Typhimurium and *Listeria monocytogenes* inoculated on cherry tomatoes and strawberries, was examined. Bacteria were spot inoculated on the produce surface, air dried and sealed inside a rigid polypropylene container. Samples were indirectly exposed (i.e. placed outside plasma discharge) to a high voltage ($70kV_{RMS}$) air ACP and subsequently stored at room temperature for 24 h. ACP treatment for 10, 60 and 120 s resulted in reduction of *Salmonella*, *E. coli* and *L. monocytogenes* populations on tomato to undetectable levels from initial populations of 3.1, 6.3, and 6.7 $\log_{10}$ CFU/sample, respectively. However, an extended ACP treatment time was necessary to reduce bacterial populations attached on the more complex

surface of strawberries. Treatment time for 300 s resulted in reduction of *E. coli*, *Salmonella* and *L. monocytogenes* populations by 3.5, 3.8 and 4.2 $\log_{10}$ CFU/sample, respectively, and also effectively reduced the background microflora of tomatoes.

Highlights:

A key advantage of this in-package non-thermal decontamination approach is the possibility to eliminate of post-processing contamination of the produce, thus increasing microbiological food safety and extension of produce shelf life. Inactivation was dependent on fresh produce surface features.

Key words: Atmospheric cold plasma, decontamination efficacy, pathogenic bacteria, fresh produce, ozone.

1. Introduction

The benefits associated with consumption of fresh produce maintain a high consumer demand for a wide range of pre-packed ready to use products. Nevertheless, fresh produce may contribute to the transmission of bacterial, parasitic and viral pathogens (Abadias *et al*. 2008). In recent years, foodborne human illnesses resulting from contaminated fresh produce have been widely reported globally. Most reporting countries identified *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. as the target pathogens capable of causing severe human infection and deaths (Rangel *et al*. 2005; Raybaudi-Massilia *et al*. 2009; Olaimat and Holley 2012; CDC, 2012). A wide range of fresh fruit and vegetable products have been implicated in foodborne infections, such as lettuce, sprouted seed, melon, tomatoes, radish, pepper, basil and other mixed salads (Fernandez *et al*. 2013; Olaimat and Holley 2012). Pathogens, such as *E. coli* O157:H7 and *Salmonella* may reside in protected sites on surface of the fresh produce and be able to survive for long periods of time beyond the expected shelf-life (Olaimat and Holley 2012). Flessa *et al*. (2005) reported that *L. monocytogenes* is capable of survival on the surface of fresh intact or cut strawberries

throughout the shelf life of the fruit and can survive on frozen strawberries for periods of 4 weeks. A health hazard to the consumers may also arise due to the possible presence of microbial toxins as a consequence of produce contamination with spoilage bacteria (Issa-Zacharia *et al*. 2010).

Raw fruits and vegetables can become contaminated while growing or during harvesting, postharvest processing, storage or distribution (Cevallos-Cevallos *et al*. 2012). How bacteria attach and the strength of attachment has not been well understood, but once attached to the surface of fresh produce it is difficult to remove the pathogens by washing (Berger *et al.* 2010; Warning and Datta 2013). Conventional postharvest washing and sanitising treatments are not highly effective for produce, often resulting in less than 2 log unit reductions of pathogens (Niemira 2012). Moreover, some low pH based preservation techniques may contribute to the bacterial adaption to acidic environment and subsequently increase their acid resistance (Roberts and Wiedmann 2005). Disinfection can become less effective when microorganisms are attached to produce surface include biofilm formation, concentration reduction of sanitizer near produce surface and accessibility of sanitizer to cells attached to rough surfaces (Wang *et al*. 2012). Pathogens can also attach to surface through interaction with epiphytic microflora and may be further protected by internalising which itself dependant on many produces phyllosphere characteristics (Erickson 2012).

Non-thermal antimicrobial treatments of fruits, vegetables and other food produce have been the subject of much research. Atmospheric cold plasma (ACP) technology is a relatively new approach aiming to improve microbiological safety in conjunction with maintenance of sensory attributes of the treated foods. A key process advantage is the minimal water usage. However, apart from issues associated with water mediated decontamination, it is likely that many of the features associated with minimal processing and phyllosphere of produce that impact on traditional washing decontamination, may also interact with the optimum

application of ACP. The antimicrobial efficacy and design of ACP systems including producer gas composition, electrode configuration as well as the type of bacteria and substrate varies widely among research studies (Fernandez *et al*. 2013, Niemira 2012; Noriega *et al*. 2011; Niemira and Sites 2008). The use of indirect plasma in conjunction with utilisation of closed chambers for decontamination of meat produce have been highlighted in recent studies conducted by Rod *et al.* (2012) and Frohling *et al*. (2012b). Our previous study also demonstrated the antimicrobial efficiency of indirect ACP exposure, where *E. coli* in a sealed package was readily inactivated within seconds (Ziuzina *et al.* 2013). However, there are limited numbers of reports based on in-package plasma decontamination of fresh fruits and vegetables (Fan *et al*. 2012; Klockow and Keener 2009). Therefore, the objective of this study was to evaluate the efficacy of indirect ACP generated inside a sealed package against *E. coli*, *Salmonella* and *L. monocytogenes* inoculated on cherry tomatoes and strawberries and to evaluate its potential to reduce background microflora present on cherry tomatoes and strawberries in order to increase the produce shelf life.

2. Materials and methods

2.1. Bacterial strains and inocula preparation

Three bacterial strains were used in this study. *Escherichia coli* NCTC 12900 was obtained from National Collection of type cultures of the Health Protection Agency (HPA, UK), *Salmonella enterica* Typhimurium ATCC 14028 and *Listeria monocytogenes* NCTC 11994 were obtained from the microbiology stock culture of the School of Food Science and Environmental Health of the Dublin Institute of Technology. Stock cultures were maintained at -70°C in the form of protective beads (Technical Services Consultants Ltd, UK). One protective bead of each culture was streaked onto separate tryptic soy agar (TSA, ScharlauChemie, Spain), incubated at 37°C for 24 h and further maintained at 4°C. A single isolated colony of each culture was inoculated in tryptic soy broth without glucose (TSB-G,

ScharlauChemie, Spain) and incubated at 37°C for 18 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min, washed twice in sterile phosphate buffered solution (PBS, Oxoid LTD, UK) and finally resuspended in PBS, resulting in concentration of 8-9 Log_{10} CFU/ml, which were further used as the working inoculum. The concentration of inoculum was confirmed by plating appropriate dilutions on TSA, followed by incubation at 37°C for 24 h for *E. coli* and *Salmonella* and 48 h for *L. monocytogenes*.

2.2. Preparation of produce

Whole fresh cherry tomatoes and strawberries (Class 1, Origin: Spain) were purchased from the local supermarket and stored at 4°C until use. The tomatoes were 2±0.5 cm in diameter and 5-15 g in weight. Strawberries weight was approximately 10-20 g. The same produce cultivar was used for each experiment. Cherry tomatoes were sterilized with 70% of ethanol (Klerwipe 70/30, Shield Medicare LTD, Farnham, UK) in order to reduce the background microbial load before surface inoculation of respective bacterial strain. Sterilized samples were then washed with sterile deionized water to remove any remaining ethanol residue and allowed to dry in the laminar flow safety cabinet at 23°C for 1 h prior to inoculation (Mattson *et al*. 2011). In order to assess ACP treatment efficacy for reduction of the background microflora, unsterilized tomatoes were also used.

2.3. Fresh produce inoculation procedure

For inoculation, tomatoes and strawberries were placed with the blossom end down on sterile petri dishes. The samples were spot-inoculated with bacteria applying either 50 µl or 100 µl of a culture on the tomato or strawberry surface, respectively (Das *et al*. 2006; Mahmoud *et al*. 2007). The droplets were deposited in several different locations, ensuring that the inoculum did not flow to the side of the samples. Inoculated samples were dried for 1 h in laminar flow safety cabinet to allow the attachment of bacteria on the surface of produce prior to the ACP treatment.

2.4. Experimental design

The ACP system utilised was a dielectric barrier discharge system previously described in Ziuzina *et al*. (2013), with a maximum high voltage output of 120 kV at 50 Hz. The distance between the two 15 cm diameter aluminium disk electrodes was 40 mm which was equal to the height of the polypropylene container (310 x 230 x 40 mm) utilised as both a sample holder and as a dielectric barrier.

Inoculated samples (four of either tomatoes or strawberries) were aseptically transferred on one of the corner of the container so as to expose the samples to indirect ACP discharge (Fig.1). The distance between the samples and centre of the electrodes was within the range from 140 mm to 160 mm. In order to evaluate ACP treatment efficacy against background microflora, uninoculated samples were used. After product loading, each container was sealed within a high barrier polypropylene film (Cryovac, B2630, USA) and placed between the aluminum electrodes of the transformer. The inoculated and uninoculated samples were treated with 70 kV_{RMS} for 30 s - 300 s in air and at atmospheric pressure. All samples were subjected to a post-treatment storage time of 24 h at room temperature. In order to evaluate any possible effect of storage on the bacterial growth, inoculated control samples were stored for 24 h under similar conditions. All experiments were performed in duplicate and replicated three times to ensure reproducibility of the experimental data and are reported as log_{10} CFU/sample.

2.5. Microbiological analysis

For microbiological analysis, inoculated untreated control samples (to estimate initial attached bacterial population), inoculated untreated samples stored for 24 h (to assess the effect of storage on microbial growth), uninoculated untreated control samples (to determine initial background microflora), and either inoculated or uninoculated ACP treated samples were analyzed. The samples were aseptically transferred into separate sterile stomacher bags

(BA6041, Seward LTD, UK) with 10 ml of sterile MRD and hand rubbed for 2-3 min. The resulting suspension was serially diluted in MRD. The surviving *E. coli*, *Salmonella* and *L. monocytogenes* populations were determined by agar overlay method (Mahmoud 2010). Briefly, aliquots of an appropriate dilution were surface plated on TSA, incubated for 2-4 h, and overlayed with the appropriate selective media: Sorbitol MacConkey agar (SMAC, ScharlauChemie, Spain) supplemented with Cefixime-Tellurite (CT, Oxoid LTD, England) for *E. coli*, Xylose Lysine Deoxycholate agar (XLD, ScharlauChemie, Spain) for *Salmonella*, and polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM, ScharlauChemie, Spain) supplemented with PALCAM Listeria Selective Supplement (Oxoid LTD, England) for *L. monocytogenes*. Plates were then incubated for 24-48 h at 37°C.

Surviving background microflora of the uninoculated samples was evaluated using non-selective media TSA for estimation of aerobic mesophilic bacteria and Potato Dextrose agar (PDA, ScharlauChemie, Spain) for estimation of yeasts and moulds, with further incubation of agar plates at 37°C and 25°C, for 48 h and 5 days, respectively. The limit of detection for bacterial recovery on food samples was 1.0 Log_{10} CFU/sample.

2.6. Ozone measurements

Ozone concentration inside the sealed package was measured using Gastec ozone detector tubes (Product #18M, Gastec Corporation, Japan). Measurements were taken immediately after plasma treatment and after 24 h of post treatment storage.

2.7. Scanning Electron Microscopy (SEM)

Attachment of different bacteria, namely *E. coli* and *L. monocytogenes*, attached on tomato and strawberry samples was observed using SEM. Inoculated strawberry samples were prepared as described by Gratao *et al.* (2008) with minor modifications. Briefly, the samples

were spot inoculated with either bacterium and dried under laminar flow at 23°C. The tissue from the inoculated sites of the fruit was excised forming 1 cm in diameter and 1 mm of thickness pieces. The cells were fixed in ice-cold 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH7.4) (SCB) for 2 h. The cells were washed with the same buffer three times and fixed in 1% osmium tetroxide for 2 h at 4°C. After 2 h of fixation, bacterial cells were washed with SCB followed by three washes with distilled water. The samples were dehydrated using increasing concentrations of ethanol (30%, 50%, 70%, 80%, 95%, and 99.5%) and freeze dried (Labconco, FreeZone 6; Mason Technology, Dublin, Ireland). In order to prevent surface charging by the electron beam, the samples were sputter-coated with gold particles using Emitech K575X Sputter Coating Unit resulting in a coating of 10 nm after 30 s. The samples were examined visually using a FEI Quanta 3D FEG Dual Beam SEM (FEI Ltd, Hillsboro, USA) at 5 kV.

2.8. Statistical Analysis

Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, USA). The surviving population of *E. coli*, *Salmonella* and *L. monocytogenes* and ozone concentration following ACP treatment were subjected to analysis of variance (ANOVA). Means were compared according to the method of Fisher's Least Significant Difference-LSD at the 0.05 level.

3. Results

Generally, indirect ACP treatment with subsequent 24 h of storage effectively reduced the numbers of microorganisms on either produce surface studied. On cherry tomatoes, treatments for 10 s, 60 s, and 120 s reduced populations of *Salmonella*, *E. coli* and *L. monocytogenes* to undetectable levels, respectively. However, an extended treatment time of 300 s was necessary to reduce bacterial populations attached on the more complex surface of strawberries.

3.1. Inactivation of bacteria on cherry tomatoes

The influence of ACP treatments on viability of *E. coli*, *Salmonella* and *L. monocytogenes* is represented in Figure 2. Tomato samples were inoculated with an average of 3.1 ±0.6 $\log_{10}$ CFU/sample for *E. coli*, 6.3 ±0.6 $\log_{10}$ CFU/sample for *Salmonella* and 6.7 ±0.6 $\log_{10}$ CFU/sample for *L. monocytogenes*. After treatment for 10 s and above *Salmonella* populations on tomato were undetectable. Treatment for 45 s reduced populations of *E. coli* and *L. monocytogenes* by 2 ±1.2 and 4.5 ±0.2 $\log_{10}$ CFU/sample, respectively. Further increasing treatment time from 45 s to 60 s reduced populations of *L. monocytogenes* by 5.1 ±0.5 $\log_{10}$ CFU/sample and reduced populations of *E. coli* to undetectable levels. Populations of *L. monocytogenes* were reduced to levels below detection limits after extended treatment for 120 s.

3.2. Inactivation of bacteria on strawberries

Reductions of *E. coli*, *Salmonella* and *L. monocytogenes* inoculated on strawberries are represented on Figure 3. The average initial attached population of *E. coli*, *Salmonella* and *L. monocytogenes* was 4.4 ±1.7, 6.6 ±1.2 and 7.3 ±0.3 $\log_{10}$ CFU/sample, respectively. After 60 s and 120 s of ACP treatment, populations of *E. coli* were reduced by 1.2 ±1.6 and 1.6 ±0.1 $\log_{10}$ CFU/sample, respectively, with significantly different reductions of 3.5 ±0.7 $\log_{10}$ CFU/sample achieved after treatment for 300 s ($P \leq 0.05$). Similarly, populations of *Salmonella* were reduced by 1.7 ±0.1 and 3.8 ±0.4 $\log_{10}$ CFU/sample after ACP exposure for 120 s and 300 s, respectively. No significant difference in antimicrobial efficacy of ACP treatments for either 120 s or 300 s against *L. monocytogenes* was observed where average reductions of approximately 4.2 ±0.5 $\log_{10}$ CFU/sample were recorded. No changes were noticed in the levels of bacterial populations attached on the untreated control tomato or strawberries samples after storage for 24 h.

3.3. Inactivation of background microflora on produce

The reductions of background microflora on cherry tomatoes and strawberries due to indirect ACP treatments are represented on Figure 3.

An average of initial background microflora on cherry tomatoes was 5 ±0.1 $\log_{10}$ CFU/sample (Fig. 4a). After 60 s of ACP treatment the aerobic mesophilic counts were reduced by 3 ±0.7 $\log_{10}$ CFU/sample while yeasts and moulds were reduced by 2.5 ±0.6 $\log_{10}$ CFU/sample. Further increase in treatment time to 120 s resulted in reductions of yeasts and moulds to undetectable levels while population of mesophilic bacteria was reduced of by 4.2 ±0.8 $\log_{10}$ CFU/sample. Mesophilic bacteria were not detected when the treatment time was increased to 300 s. Untreated and stored for 24 h samples showed no changes in the growth levels of background microflora on tomato samples.

Lower reduction levels of spoilage microorganisms by ACP treatment were observed in the case of strawberry samples (Fig. 4b). Significant decrease in mesophilic counts was observed after 60 s of ACP treatment, resulting in reductions by 1.6 ±0.9 $\log_{10}$ CFU/sample ($P \leq 0.05$) from the control 3.6 ±0.3 $\log_{10}$ CFU/sample. Populations of mesophilic bacteria did not decrease further when treatment time was extended from 60 s to either 120 s or 300 s. Populations of yeasts and moulds initially present on strawberries were 5.5 ±0.1 $\log_{10}$ CFU/sample. These levels decreased by 1.0 ±0.8 $\log_{10}$ CFU/sample after 120 s of ACP treatment. Extending the treatment time from 120 s to 300 s resulted in an additional 0.4 ±0.4 log reduction in the population of yeasts and moulds. It should be noted that the levels of mesophilic bacteria of untreated control strawberry samples increased by 1.8 ±1.0 $\log_{10}$ CFU/sample during 24 h storage, whereas populations of yeasts and moulds remained the same.

3.4. Ozone generation

Generation of ozone inside the sealed package containing either cherry tomatoes or strawberry samples as a function of ACP treatment time is represented in Figure 5. The ozone

concentration inside the package containing cherry tomatoes increased gradually with increasing the treatment time. All ACP treatment times studied resulted in significant increase of ozone concentration ($P \leq 0.05$) with maximum concentration of 5600 ppm achieved after 300 s of treatment. However, no significant difference in ozone concentration generated during the treatment of strawberry samples was observed. ACP treatment for 60 s resulted in an average of 2800 ppm, and further increasing treatment time from 60 s to 120 and 300 s resulted in an average of 3200 and 3500 ppm of ozone, respectively.

3.5. Scanning Electron Microscopy (SEM)

In order to examine if the complex substrate surface features had any effect on the bacterial adherence, and thus effect antimicrobial efficacy of ACP treatment, SEM analysis of untreated *E. coli* and *L. monocytogenes* inoculated on produce surface was conducted. Figure 6(a,b) represents the surface of strawberry and tomato, respectively, inoculated with *L. monocytogenes* where strong bacterial attachment in the form of clusters was noticed. On the contrary, only a small amount of individually attached bacterial cells of *E. coli* on the rough surface of strawberry was found (Fig. 6c).

4. Discussion

The indirect ACP treatment showed better inactivation efficacy against inoculated challenge bacteria and background microflora present on the surface of the two different products tested. Cherry tomatoes were selected as they have been associated with recent foodborne illness outbreaks and represent common raw food ingredients of commercial salads. Strawberries are also popular fruits and consumed raw. Moreover, these produce types present different surface decontamination challenges to the ACP system, i.e. tomato surface which is smooth, and the more complex surface of strawberry - uneven with numerous seeds.

In general, higher inactivation rates due to ACP treatment were achieved for bacteria inoculated on smooth surface of tomatoes. *Salmonella* and *E. coli* were more rapidly

inactivated on tomato than *L. monocytogenes.* Among the three bacteria studied, *Salmonella* was the most sensitive to ACP, where 10 s of treatment time reduced bacterial population to undetectable levels. For tomato, increasing treatment time enhanced the inactivation efficacy of ACP in the case of *E. coli* and *L. monocytogenes*. Increasing treatment time from 45 s to 60 s inactivated *E. coli* populations present on tomatoes, whereas inactivation to undetectable levels of *L. monocytogenes* was obtained only after an extended treatment time of 120 s. It is reported that Gram positive bacteria are more resistant to ACP treatments than Gram negative (Montie *et al.* 2000; Lee *et al.* 2006; Ermolaeva *et al*. 2011; Frohling *et al*. 2012a), which was also clearly demonstrated in the current study. *Salmonella* and *E. coli* are Gram negative bacteria with a thinner outer membrane compared to the Gram positive *L. monocytogenes*. The thicker membrane of the Gram positive bacteria may present a barrier to the diffusion of plasma reactive species through the bacterial cell wall, thus impacting antimicrobial efficacy. On the contrary, Fan *et al.* (2012) revealed greater sensitivity of Gram positive *L. innocua* than Gram negative *Salmonella* and *E. coli* inoculated on tomato surface. Interestingly, other comparative studies reported similar susceptibility between Gram positive and Gram negative bacteria to ACP with respect to inactivation (Kostov *et al.* 2010; Olmez and Temur 2010; Klampfl *et al*. 2012). Clearly, the target cell characteristics are important factors for inactivation efficacy, but no clear trend is apparent and complex interactions with the system, process, surface or medium may also impact on efficacy in combination with cell type.

In this study we observed that the difference in the initial levels of the attached bacterial populations complicates the comparison of the bacterial sensitivity to the ACP treatments based on bacterial cell membrane characteristics. It is widely accepted that high initial bacterial concentration may affect inactivation efficacy of plasma treatment. The study conducted by Fernandez *et al.* (2012) clearly demonstrated that increasing the concentration of *S. Typhimurium* from 5 to 8 $\log_{10}$ CFU/filter reduced the inactivation efficiency of ACP,

suggesting that the initial concentration of microorganisms present on foods plays an important role in the efficacy of plasma treatment. In the present work, the lower initial populations of *E. coli* attached on tomatoes surface did not necessarily contribute to the increased ACP bactericidal characteristics. Within 45 s of treatment populations of *E. coli* were reduced by 2 log from the initial 3.1 $\log_{10}$ CFU/sample, whereas this treatment time resulted in the reductions of *L. monocytogenes* populations by 4.5 log from the initial 6.7 $\log_{10}$ CFU/sample, and only 10 s was required to reduce *Salmonella* by 6.3 $\log_{10}$ CFU/sample. This indicates the importance of the mechanisms and strengths of bacterial attachment with respect to a decontamination procedure. It has also been demonstrated that the resistance to ACP may also vary between bacteria species. Despite the higher inoculation levels on tomato surface, *Salmonella* appeared to be more sensitive than *E. coli.* Similar results were achieved in the research conducted by Niemira and Sites (2008) where *Salmonella* Stanley was more sensitive to ACP than *E. coli* inoculated on both agar and apple surfaces.

The influence of the produce type on the overall antimicrobial efficacy of ACP was observed when results are compared with the strawberry decontamination study. Treatment for 120 s significantly reduced *L. monocytogenes* inoculated on strawberries. Increasing treatment time to 300 s did not yield any further reductions of bacteria. However, after 300 s of treatment, a proportional reduction of *E. coli* and *Salmonella* was achieved. Strawberry surface is more porous than the surface of tomato. Irregularities of the fruit surface may provide many niche areas for bacteria, providing physiological barrier or protection against ACP treatments. This factor probably contributed to the reduced ACP bactericidal effect on Gram negative bacteria on strawberries by comparison with tomatoes.

The influence of the complexity of the produce surface structure on inactivation efficacy of ACP was observed when treatments were evaluated for the reduction of background microflora naturally present on the produce. The causative agents of microbial spoilage in

fruits and vegetables can be bacteria (*Erwinia* spp., *Enterobacter* spp., *Propionibacterium chlohexanicum*, *Pseudomonas* spp., and lactic acid bacteria) as well as moulds and yeasts (*Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., and *Saccharomyces* spp., *Cryptococcus* spp., *Rhodotorula* spp.) (Raybaudi-Massilia *et al*. 2009). In recent study conducted by Jensen *et al.* (2013), 34 different species from 23 different genera for bacteria and 22 different species from 9 different genera for yeasts were identified in strawberry samples. Despite this potential diversity of indigenous microflora, an ACP treatment time of 120 s significantly reduced the numbers on smooth surface of tomatoes in our study. However, again ACP was not very effective for the reduction of background microflora on more complex surface of strawberries, although tomato and strawberries tend to share similar bacterial communities (Leff and Fierer 2013).

Current information available for characterisation of ACP suggests that plasma is a source of heat, UV radiation, charged particles and reactive oxygen and nitrogen based species (ROS and RNS, respectively) with a main role given to the ROS as prime plasma disinfectants (Laroussi and Leipold 2004; Laroussi 2009). In this study, it was demonstrated that increasing the treatment time resulted in increased antimicrobial efficacy of ACP against bacteria inoculated on produce. Moreover, the inoculated samples were indirectly exposed to plasma, i.e., at some distance to the plasma discharge (~160 mm from the centre of the plasma discharge). In case of indirect treatment the charged particles and the short-lived species would not be expected to play a role due to their potential to recombine before reaching the sample (Laroussi 2009). Therefore, ozone was expected to be one of the key factors contributing to antimicrobial efficacy of ACP treatments. It has been demonstrated earlier that considerable reductions of bacteria by indirect ACP occurred within seconds when extended post treatment storage was applied, suggesting diffusion of the reactive species into liquids during post-treatment storage, thereby affecting microbial cells (Ziuzina

et al. 2013). Extended 24 h post treatment storage time was also employed in the current study. It is likely that 24 h post treatment storage time facilitated ACP action on the bacterial cells by retaining generated reactive species within closed container, thus, promoting diffusion of the species inside the product tissue.

In the current work, as the treatment time increased, a significant increase in the ozone generated by plasma inside the package containing produce was noted ($P \leq 0.05$). However, it was also observed that the produce type influenced the concentration of ozone, where lower ozone levels were recorded for strawberry samples. Strawberries surface exhibit numerous pores, likely making the surface contact area larger than the area of tomato surface. This surface area differential may contribute to the increased dissolution rate of ozone generated inside the strawberry package, with subsequent reduced antimicrobial efficacy of ACP with regard to the all bacteria tested.

Considering the lower ozone concentrations and the consequent lower reductions of the challenge bacteria and background microflora on strawberries, it is likely that protection by more complex produce structures could be a critical parameter determining plasma treatment efficacy. Similarly, Fernandez *et al.* (2013) demonstrated that antimicrobial efficacy of plasma was influenced by produce surface features with higher bacterial reduction levels achieved on microbial filters than on more complex biotic surfaces.

As mentioned earlier, in this study, variations between initial populations of bacteria were apparent, with *Salmonella* and *L. monocytogenes* more readily attaching on the surface of either produce than *E. coli*. Regardless of the different surface features of the produce studied, SEM images confirmed the larger populations of *L. monocytogenes* adherent cells in addition to clusters of cells present. Despite the irregular nature of strawberry surface, which would probably facilitate bacterial attachment, *E. coli* populations visualised by SEM on the fruit surface were still less dense by comparison with *L. monocytogenes* images. A possible

374 explanation for the lower levels of attached *E. coli* is the presence and interaction with
375 naturally existing indigenous epiphytic bacteria. Depending on the types of epiphyte present
376 the survival of pathogens can be either enhanced or inhibited (Erickson 2012). For example,
377 Cooley *et al*. (2006) demonstrated that one epiphyte *Enterobacter asburiae* isolated from
378 lettuce inhibited colonisation of *E. coli*, whereas another epiphyte *Wausteria paucula* had the
379 opposite effect; enhancing *E. coli* survival.

380 Other factors that may affect microbial attachment to fresh produce are the different
381 morphology and chemistry of the produce as different fruits and vegetables offer different
382 microniches for the attachment, penetration and proliferation of bacteria (Keeratipibul *et al*.
383 2011). Motility of microorganisms facilitates pathogen entry into wounds, stomata and other
384 existing fruit surface openings (Deering *et al*. 2012). We observed in SEM images that
385 bacterial cells were likely adhered inside the natural crevices of produce surface or close to
386 these regions. Naturally existing crack and pits on the surface of produce provide bacteria
387 opportunity to internalise. Internalisation through the naturally existing opening is widely
388 described in literature and considered as one of the major route of pathogens entry to plant
389 tissue (Deering *et al*. 2012). Incidences of internalisation dependent upon concentration of
390 bacteria, their location on the plant, age, integrity and stages of plant development, as well as
391 indigenous agonistic/antagonistic bacteria present on plant have been reported (Erickson
392 2012; Shi *et al*. 2009). This study indicated that the decontaminating effect of ACP is a
393 function of produce type and the contaminating pathogen. The produce surface has an
394 influence on pathogen attachment, with the potential for internalisation particularly
395 associated with minimally processed fresh produce. Therefore the depth to which the plasma
396 generated chemical species are able to diffuse through a tissue in order to affect internalised
397 cells or those within a biofilm requires further investigation to elucidate how that diffusion
398 capability of ACP can be effectively harnessed. Overall, the results of this study indicated

399 that bacterial attachment and increased survivability on more complex surfaces following
400 ACP treatments should be considered as very important factors influencing treatment design.

401 **Conclusion**

402 In summary, the high voltage indirect ACP treatment was highly efficient for
403 decontamination of fresh produce inside a sealed package. Short treatment times of 10, 60 s
404 and 120 s resulted in reductions to undetectable levels of *Salmonella*, *E. coli* and *L.*
405 *monocytogenes*, respectively on cherry tomatoes. However, treatment times of up to 300 s
406 were required to attain substantial reductions on strawberry surfaces. Similarly,
407 yeasts/moulds and mesophiles on tomato surface were not detected after 120 to 300 s,
408 respectively. Thus, it can be concluded that ACP treatment with 24 h post treatment storage
409 can eliminate microorganisms on fresh produce surfaces inside a sealed package. In order to
410 achieve optimum decontamination efficiency by ACP, factors including type of produce,
411 their inherent surface characteristics, bacterial type, the strength and the nature of their
412 attachment as well as the diffusion capacity of the plasma species, to holistically address the
413 food safety issues associated with fresh produce, should be considered.

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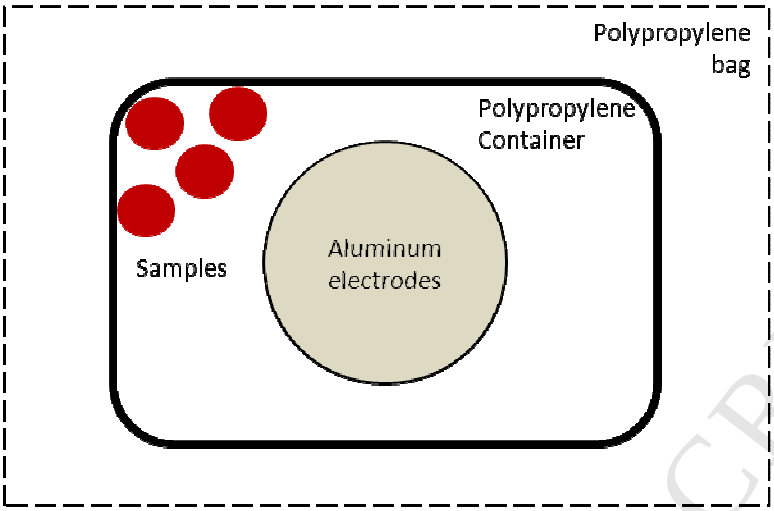
564 **Figures:**



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566 Fig.1: Schematic diagram of samples distributed within polypropylene container with respect
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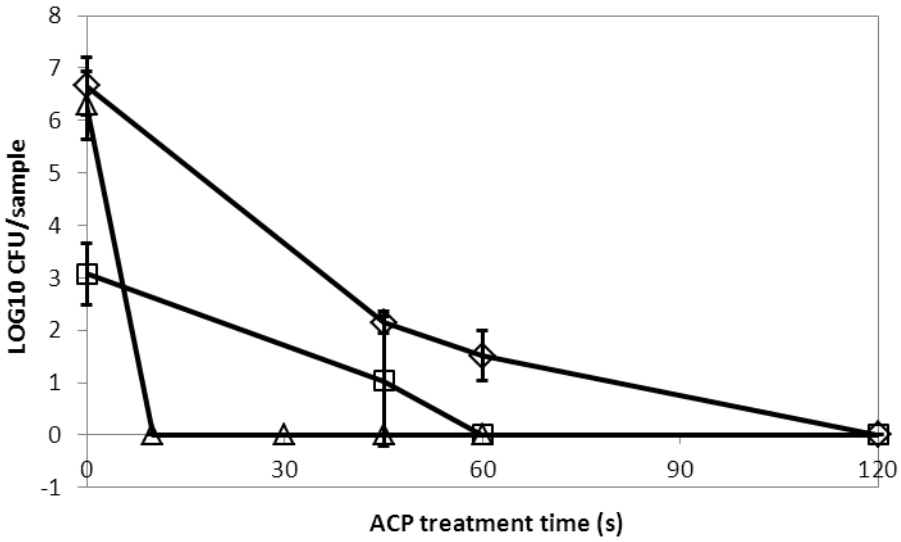

579

580 Fig.2: ACP inactivation efficacy against *E. coli* (□), *Salmonella* (△) and *L. monocytogenes* (◇)
581 inoculated on cherry tomatoes. Vertical bars represent standard deviation. Limit of detection
582 1.0 $\log_{10}$ CFU/sample.

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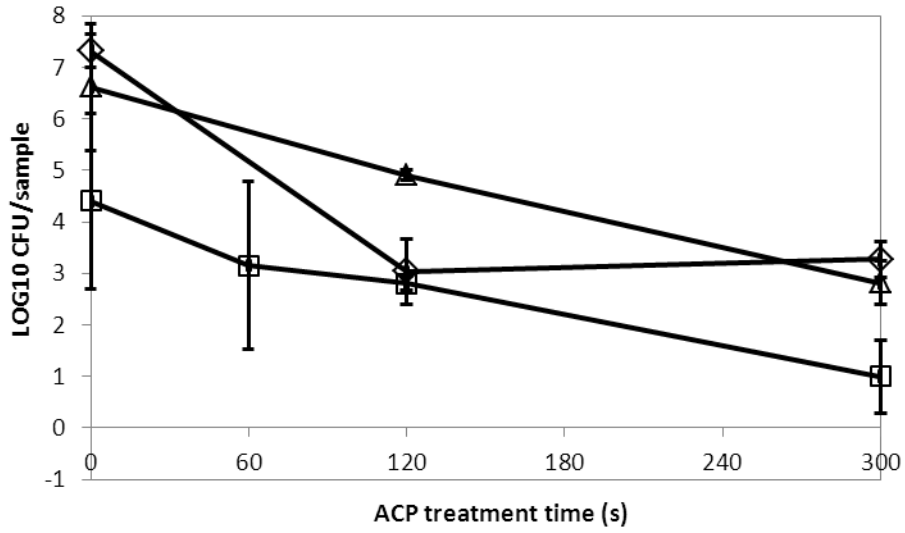
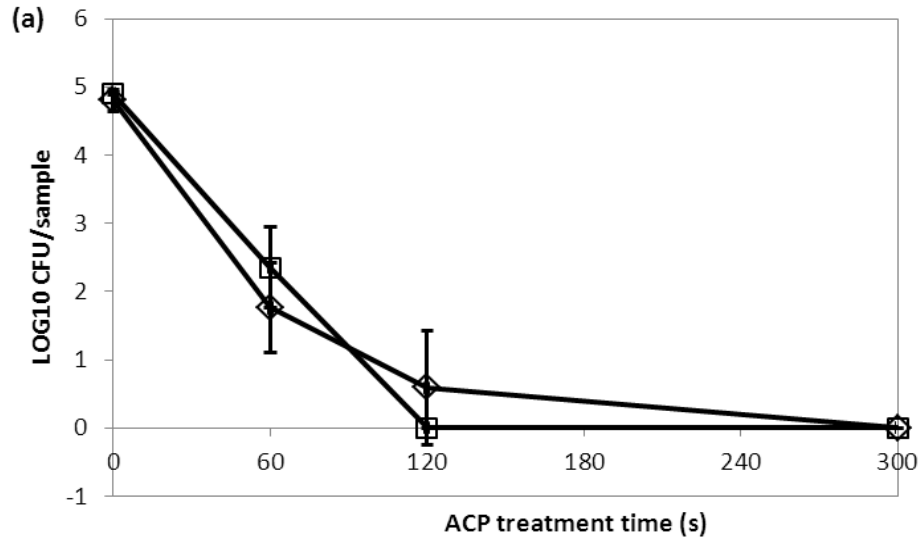


Fig.3: ACP inactivation efficacy against *E. coli* (□), *Salmonella* (△) and *L. monocytogenes* (◇) inoculated on strawberries. Vertical bars represent standard deviation. Limit of detection 1.0 $\log_{10}$ CFU/sample.



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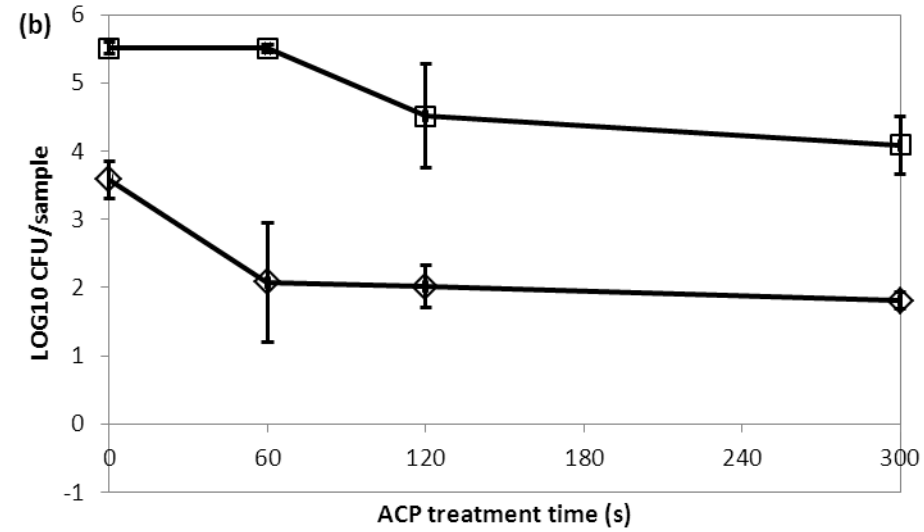

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617 Fig.4: ACP inactivation efficacy against aerobic mesophilic bacteria (◇) and yeasts and
618 moulds (□) on (a) cherry tomatoes and (b) strawberries. Vertical bars represent standard
619 deviation. Limit of detection 1.0 $\log_{10}$ CFU/sample.

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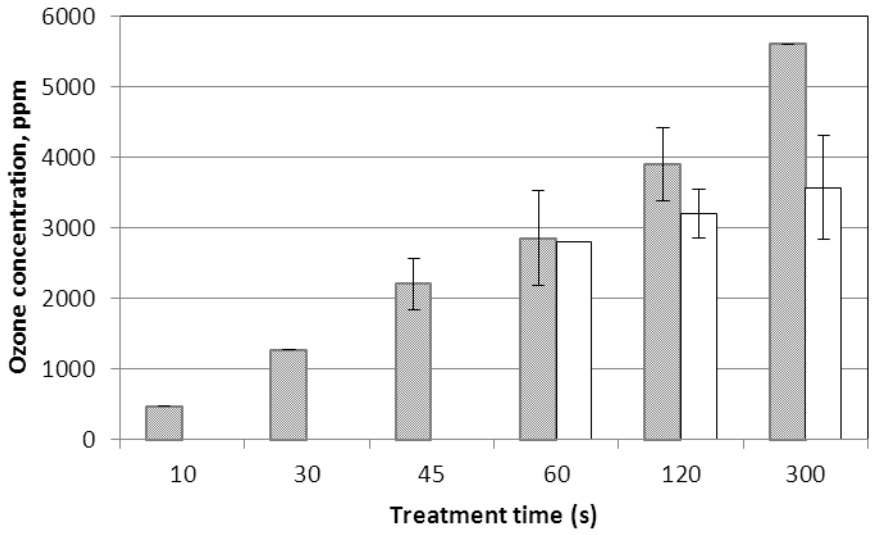

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626 Fig.5: Generation of ozone inside a sealed package during ACP treatment of either inoculated
627 or uninoculated samples of cherry tomatoes (▩) and strawberries (□). Vertical bars represent
628 standard deviation.

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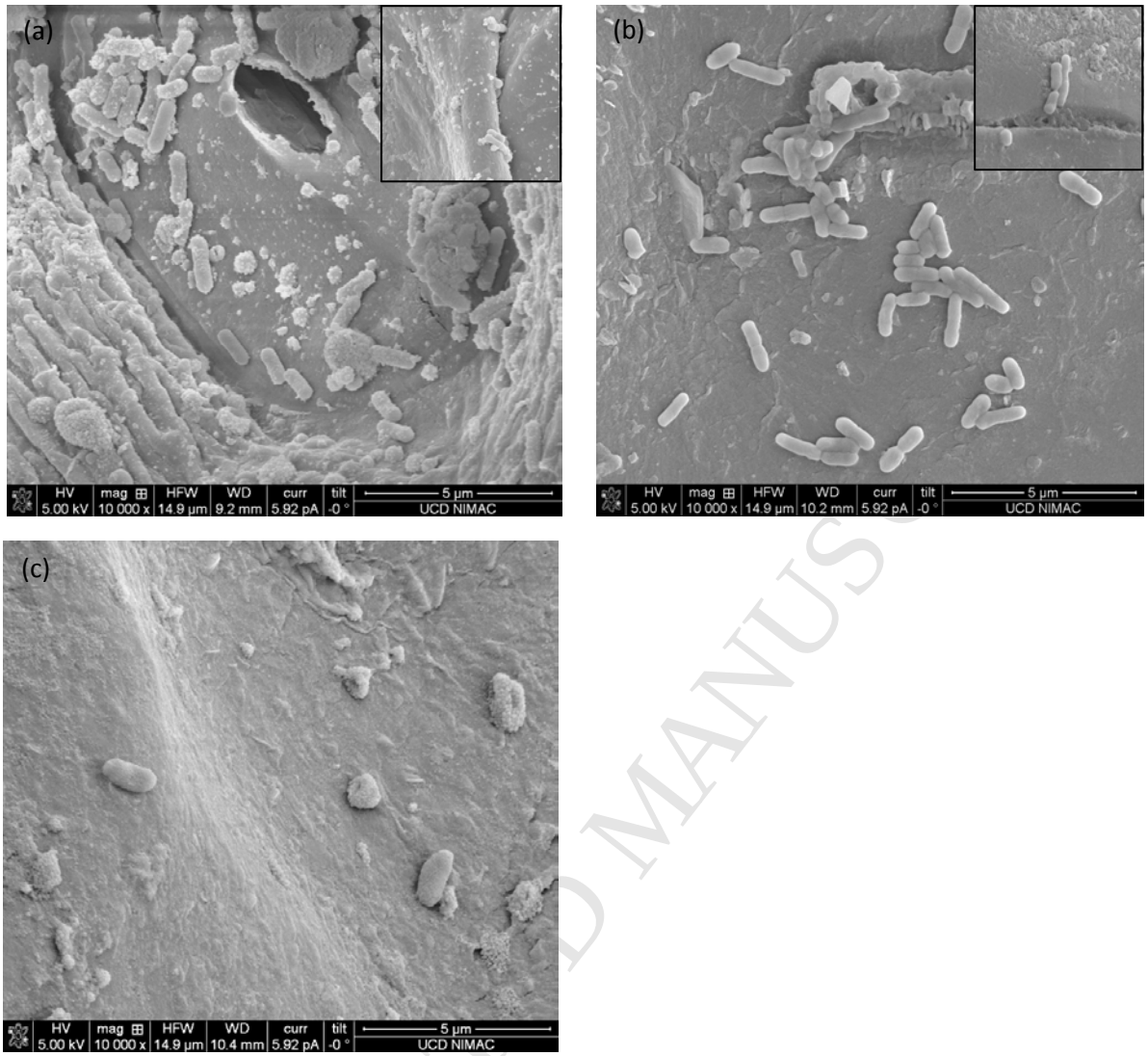

Fig.6: SEM images of untreated *L. monocytogenes* on (a) strawberries and (b) cherry tomatoes, and (c) *E. coli* inoculated on strawberry.

YFMIC 2105 Food Microbiology

Atmospheric Cold Plasma inactivation of *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* inoculated on fresh produce

Highlights

- In this study antimicrobial efficacy of ACP against *Escherichia coli*, *Salmonella enterica* Typhimurium and *Listeria monocytogenes* inoculated on cherry tomatoes and strawberries was evaluated.
- A key advantage of this high voltage level treatment for in-package non-thermal decontamination approach is the possibility to eliminate post-processing contamination of the produce.
- This approach has potential to provide both increased microbiological food safety and extension of produce shelf life.
- Inactivation was however, dependent on fresh produce surface features and pathogen type.