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High voltage atmospheric cold air plasma control of bacterial biofilms on fresh produce

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ABSTRACT

Atmospheric cold plasma (ACP) offers great potential for decontamination of food borne pathogens. This study examined the antimicrobial efficacy of ACP against a range of pathogens of concern to fresh produce comparing planktonic cultures, monoculture biofilms (*Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes*, *Pseudomonas fluorescens*) and mixed culture biofilms (*Listeria monocytogenes* and *Pseudomonas fluorescens*). Biotic and abiotic surfaces commonly occurring in the fresh food industry were investigated. Microorganisms showed varying susceptibility to ACP treatment depending on target and process factors. Bacterial biofilm populations treated with high voltage (80 kV) ACP were reduced significantly ($p < 0.05$) in both mono- and mixed species biofilms after 60 s of treatment and yielded non-detectable levels after extending treatment time to 120 s. However, an extended time was required to reduce the challenge mixed culture biofilm of *L. monocytogenes* and *P. fluorescens* inoculated on lettuce, which was dependent on biofilm formation conditions and substrate. Contained treatment for 120 s reduced *L. monocytogenes* and *P. fluorescens* inoculated as mixed cultures on lettuce ($p < 0.05$) by 2.2 and 4.2 Log_{10} CFU/ml respectively. When biofilms were grown at 4 °C on lettuce, there was an increased resistance to ACP treatment by comparison with biofilm grown at temperature abuse conditions of 15 °C. Similarly, *L. monocytogenes* and *P. fluorescens* exposed to cold stress (4 °C) for 1 h demonstrated increased tolerance to ACP treatment compared to non-stressed cells. These finding demonstrates that bacterial form, mono versus mixed challenges as well as environmental stress conditions play an important role in ACP inactivation efficacy.

1. Introduction

Recently, there has been increasing concern for the number of food associated illness worldwide (WHO, 2015). The ability of microorganisms to survive and grow under processing and storage conditions followed by continuous adaptation and increasing tolerance of microbial population to antibiotics and disinfectants has made it even more difficult to control their levels in food. Several types of bacterial population play major roles in the food industry such as *Escherichia coli*, *Salmonella*, *Shigella*, *Listeria* and spoilage bacteria such as *Erwinia carotovora*, *Pseudomonas* spp. which are commonly associated with food contamination and spoilage (Tournas, 2005). One of the important factors associated with fresh produce contamination and increased resistance to antimicrobials is its ability to form biofilms (Murray et al., 2017). Many foodborne pathogens have been identified as biofilm producers which may form biofilms on food matrixes, on food industry infrastructure as well as in water distribution system that could further contributes to food spoilage and cross contamination between the facilities and spread of foodborne pathogens (Zhao et al., 2017).

Bacterial biofilms are complex microbial multicellular communities embedded in an organised matrix mostly composed of extracellular polymeric substances (EPS) that traps several microorganism and other excreted cellular products including lysed cell debris and macro-molecules (Stoodley et al., 2002). Biofilm formation is one of the universal ways of microbial communities to develop coordinated structural and survival strategies. The ability of the biofilm to resist extreme environmental conditions such desiccation, low temperature and antimicrobials treatment (chemical and advanced treatments such as UV radiation) makes them of high importance to food safety (Borucki et al., 2003).

L. monocytogenes can thrive under a wide range of adverse environmental conditions: acids, high salt and refrigerated conditions. In addition, it can adhere to several biotic and abiotic surfaces and form biofilms (Renier et al., 2011). Pseudomonas spp. are versatile

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psychrotrophs commonly associated with spoilage of fresh foods because of their widespread existence in water, soil and vegetation under both optimal as well as refrigeration temperatures. They are documented as good producers of EPS including polysaccharides, nucleic acids and proteins, forming thick biofilms. Although much attention has focused on mono-culture biofilm, in nature most biofilms are comprised of multiple species (Tan et al., 2017). The interspecies interactions can develop structural and functional dynamics of these communities different from single species populations. Mixed culture biofilms have gained attention in the recent years due to observances of resistance by comparison with single species biofilm (Lohse et al., 2017; Pang et al., 2017; Parijs and Steenackers, 2018). This is possibly due to presence of higher extracellular polymer substance (EPS) production, differences in physiological status or due to interspecies cross protection (Jahid and Ha, 2014; Stewart, 2015). Sustained co-existence of multi-species in the same biofilm implies compatibility between species and the possibility for cooperative interaction between them. In this study, mixed biofilm comprising *L. monocytogenes* and *P. fluorescens* was studied. The interactions between these bacterial species has been previously been studied in planktonic and biofilms by Sasahara and Zottola (1993) and Buchanan and Bagi (1999). In addition to biofilm formation, bacteria species are influenced by the food components as well as the food storage conditions. Food preservation conditions such as low pH, high osmotic pressure or low temperature could be adverse for some microorganisms which could induce stress responses and enhance bacterial resistance to antimicrobial treatments (Chen et al., 2017; Vivijs et al., 2016). Short term exposures may alter cellular physiology and bacteria become resistant to subsequent challenges such as food disinfection or food preservation techniques (Calvo et al., 2017; Patil et al., 2010; Rodriguez-Romo and Yousef, 2005). The assessment of ACP as an industrial level food decontamination strategy requires information on microbial plasma resistance after food environmental stress conditions. Both *L. monocytogenes* and *Pseudomonas* species are able to grow at low temperatures, they may jointly form biofilm on variety of raw materials and food or food contact surfaces, which makes them ideal to investigate its growth and indeed control as multispecies biofilms.

A clearer understanding of physiological behaviour of multispecies biofilm communities formed by bacteria on abiotic or biotic surface in food processing environment could provide useful information necessary for controlling the contamination of food products. Atmospheric cold plasma is a form of ionised gas generated at atmospheric pressure and under non-thermal conditions (Pankaj and Keener, 2017). The gas discharge triggers a complex network of biological and cellular responses by generating various active agents such as high energy UV photons, charged particles (positive, negative and free electrons), and reactive species (namely, ROS, RNS, hydrogen peroxides) (Misra et al., 2018). Accumulation of these charged particles and reactive species could damage bacterial cell membrane and intracellular components via different modes of action that have lethal effects on microorganisms (Bourke et al., 2017). Cold plasma treatment is capable of surface sterilization by inactivating the microbial population through series of physio-chemical mechanisms; however the exact mechanism responsible for bactericidal activity is still elusive. ACP treatment has been demonstrated for microbial decontamination of produce (Misra et al., 2014; Ziuzina et al., 2015b). This study aimed at (i) determining the influence of plasma and treatment parameters on bacterial inactivation (ii) influence of short term exposure of acid and cold stress shock on the inactivation by ACP (iii) inactivation of key foodborne pathogens and spoilage microorganisms as mono or mixed species biofilm in model product media as well as on lettuce and (iv) to examine the effect of different temperature storage conditions on biofilm formation on fresh produce and its effects on ACP antimicrobial efficacy.

2. Methods and materials

2.1. Culture maintenance

The bacterial strains used in this study, *Listeria monocytogenes* NCTC 11994, *Escherichia coli* NCTC 12900, (non-toxigenic O157:H7), *Salmonella enterica Typhimurium* ATCC 14028 and *Pseudomonas fluorescens* LZB065 were obtained from the microbiology stock culture of the School of Food Science and Environmental Health of the Dublin Institute of Technology. *Erwinia carotovora* was obtained from Leibniz Institute DSMZ, Germany. All strains were maintained at −80 °C in the form of protective beads (Pro-Lab Microbank®, UK). Each bacterial strain was streaked onto tryptic soy agar (TSA, Biokar Diagnostics, France) and incubated at 26 °C (*E. carotovora*), 30 °C (*P. fluorescens*) and 37 °C (*S. enterica*, *E. coli*, *L. monocytogenes*) for 24 h. The bacterial culture plates were then maintained at 4 °C.

2.2. Lettuce sample preparation

Lettuce (Irish iceberg) was purchased on the day of the experiment from the local supermarket and stored in refrigerator at 4 °C until use. The outer leaves were removed, and inner leaves were used for the experiment. Lettuce pieces were cut (5 × 5 cm) aseptically and immediately rinsed with sterile distilled water to clean and to remove unattached bacteria. Washed lettuce samples were dried in biosafety cabinet for 20 min.

2.3. Preparation of lettuce broth (LB) media

Lettuce juice was prepared as described in Ziuzina et al. (2015b) with minor modifications. Briefly, lettuce juice was extracted from cored, whole iceberg lettuce that was processed using a stomacher. The extracted juice was centrifuged (10,000 rpm for 10 min at 4 °C) twice to remove coarse particles. The supernatant obtained was membrane filter sterilized (0.2 μm, pore size, Millipore, Ireland) and was diluted to 12% using sterile distilled water. All broths were freshly prepared before each experiment and stored at 4 °C until use.

2.4. Inoculation procedure

To prepare inoculum, single colony of each bacterial strain was transferred to tryptic soy broth (TSB, Biokar Diagnostics, France) and incubated at respective temperature depending on the bacteria for 18 h until stationary phase was reached. Cells were harvested by centrifugation at 10,000 rpm for 10 min and the obtained pellet was washed thrice with phosphate buffer solution (PBS, Sigma Aldrich). After washing, the final pellet was re-suspended in PBS. The concentration of bacterial inoculum was adjusted to 0.5 McFarland standard (BioMérieux, Marcy-l'Etoile, France) corresponding to 7.0 Log_{10} CFU/ml which was confirmed by plating on TSA.

For stress exposure experiments, washed bacterial cells of *L. monocytogenes* and *P. fluorescens* were suspended in either lettuce broth acidified to pH 4 using 33% acetic acid (Sigma-Aldrich Co., Ireland) then incubated for 1 h at 37 °C or in lettuce broth and stored in 4 °C for 1 h. The bacterial cells inoculated into plain lettuce broth incubated 37 °C for 1 h served as negative non-stress control.

The lettuce inoculation procedure was carried out by Ziuzina et al. (2015b) method with minor modification. The cut lettuce pieces were submerged in a beaker containing 300 ml of bacterial suspension of *L. monocytogenes* and *P. fluorescens* (mixed in equal ratio 1:1) for 2 h in laminar biosafety cabinet. Following incubation, the samples were washed with sterile distilled water in separate beaker in order to remove any unattached bacteria. The lettuce pieces were drained of excess water and were allowed to dry on sterile aluminium foil for 15 min each side in laminar air flow. Following air drying, the samples were transferred to sterile petri-dish and stored for 48 h at either 4 °C or

15 °C.

2.5. Biofilm formation

A 96 well microtitre plate method was used for biofilm formation. The prepared bacterial cultured suspension (200 µl) was dispensed into the 96 well microtitre plates and incubated for 24 h and 48 h at 30 °C respectively. The supernatant (with non-adherent cells) from each well was replaced with fresh broth after of 24 h incubation. Sterile LB without inoculum was used as a negative control.

For multispecies biofilm formation, *P. fluorescens* and *L. monocytogenes*, bacteria were selected to investigate individual contribution and biofilm forming capacity of each bacteria under mixed bacterial conditions. These bacteria are important strong biofilm formers in fresh and minimally processed produce. The individual inoculum was prepared as previously described and mixed in equal volume (1:1 ratio) while adjusting the bacterial concentration to 7.0 Log_{10} CFU/ml. Bacterial biofilms were produced similarly like mono-species biofilm by adding 200 µl of prepared mixed bacterial suspension into wells of microtitre plate and incubated at 30 °C for 24–48 h to form biofilms under static condition.

At the end of incubation, the supernatant was carefully aspirated and rinsed thrice with PBS to remove non-adherent cells. Prior to each experiment the biofilm grown on microtitre plate was air dried for 60 min.

2.6. Experimental system

The ACP device used in this study was Dielectric barrier discharge (DBD) system custom built in Dublin institute of technology generating maximum high voltage output of 120 kV at 50 Hz. The device is elaborately described in previous studies by Ziuzina et al. (2015a). The atmospheric air was used to generate ACP. The distance between the two electrodes was 26.6 mm. Samples were placed in a sealed container and treated by direct or indirect form of plasma treatment. For direct plasma treatment, the sample container was placed directly between the two electrodes i.e. within the range of plasma discharge. The distance between the top electrode and the sample for direct treatment was approximately 10 mm. While in case of indirect plasma treatment, the sample was placed at the corner of the container to achieve treatment away from plasma discharge range. The distance between the sample and the centre of the electrode was maintained approximately between 120 and 160 mm. For plasma treatment, each container containing the sample were sealed with a high barrier polypropylene bag (B2630; Cryovac Sealed Air Ltd., Dunkan, SC, USA) and placed between the aluminium electrodes.

For planktonic studies and stress studies, bacterial suspensions (100 µl) in PBS or lettuce broth were dispensed in 96 well micro-titre plates, placed at the centre of the polypropylene plastic container. Separate experimental sets were prepared: 1) Bacterial cells in PBS were treated at variable voltage range of 60–80 kV and treatment time of 15–300 s with post treatment storage time (PTST) of 24 h at room temperature; 2) bacterial cells in PBS treated at 80 kV subjected to 1 h and 24 h PTST; 3) acid and cold stressed cells in lettuce broth were treated at 80 kV for 60 s and 120 s; 4) mono-cultured biofilms were subjected to direct plasma treatment for 60 s, while the dual species biofilms were treated for 60 s, 120 s and 300 s; 5) the inoculated lettuce samples were exposed to indirect ACP treatment for 2 min and 5 min. Unless otherwise stated, all samples were treated at 80 kV with a PTST of 24 h at 4 °C. The effect of post storage period and storage temperature was evaluated; samples were stored for 1 h or 24 h post plasma treatment at 4 °C and 15 °C. Experiments were performed in duplicate and replicated twice. The bacterial suspension without any plasma treatment was kept as a negative control and stored under similar conditions.

2.7. Post treatment analysis

To assess the effect of ACP treatment on bacterial suspension in PBS or LB, corresponding samples were collected into sterile Eppendorf tubes and populations of surviving bacterial cells were estimated by plating appropriate dilutions on TSA.

Following the ACP treatment and 24 h post storage period, the bacterial biofilm in 96 well plate was re-suspended in 200 µl of sterile PBS. In order to disrupt the adhered biofilm, the plate was sonicated using water table sonicator (Bransonic 5510E-MT, USA, Mexico) for 10 min. The suspension was then pooled in sterile Eppendorf tube and serially diluted in sterile maximum recovery diluent (MRD, Scharlau Chemie, Spain). The antibacterial effect of applied ACP treatment on bacterial biofilm was quantification by plate count (PC) and XTT assay. The mono-culture biofilm was surface plated on TSA while to evaluate the individual contribution of bacterial species in dual culture biofilm, the bacterial biofilm suspensions were plated on selective media plates: Polymyxin acriflavine- LiCl-ceftazidime-aesculin-mannitol PALCAM (Scharlau Chemie, Spain) supplemented with PALCAM Listeria Selective Supplement (Oxoid Ltd., England) for *L. monocytogenes* and Pseudomonas Agar Base (PAB, Oxoid) supplemented with CFC selective agar supplement with Cetrimide Fucidin Cephalosporin (CFC, Oxoid Ltd., England) for *P. fluorescens*. Plates were incubated at appropriate incubation temperature as mentioned in Section 2.1 for 24–48 h. To examine the effect of ACP treatment on metabolic activity of bacterial cells in biofilms a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)[phenylamino)car-bonyl]-2H-tetrazolium hydroxide assay (XTT, 1 mg/ml, Sigma-Aldrich Co., Ireland) was utilised as described by Peeters et al. (2008). The percentage of surviving bacterial population was calculated as: $((A_{ACP} - A_C) / A_0) \times 100\%$. Comparing the absorbance of treated samples (A_{ACP}) with absorbance representing as negative control ($A_{C;}$ sterile broth media without inocula) and untreated control biofilms (A_0).

To study the effect of ACP treatment on bacteria attached on lettuce, control/treated lettuce samples were aseptically transferred into separate sterile stomacher bags (BA6041, Seward Ltd., UK) with 10 ml of MRD and homogenised for 3 min in the stomacher (Model: BA6020, England). The surviving *L. monocytogenes* and *P. fluorescens* populations were plated on appropriate selective agar plates. Experimental data were reported as Log10 CFU/ml for bacterial counts in lettuce broth/PBS, while log10 CFU/sample for bacterial counts on lettuce where each sample weighed approximately 1.4–1.7 g. Results are reported as Log_{10} CFU/ml for bacterial recovered in lettuce broth/PBS or Log_{10} CFU/sample for bacterial reductions on lettuce. The limit of detection for bacterial recovery was 1.0 Log_{10} CFU/sample (plated volume 0.1 ml and 1 ml of diluted or undiluted samples) or Log_{10} CFU/ml (plated volume 0.1 ml and 1 ml of diluted or undiluted samples).

2.8. Statistical analysis

Statistical analysis was performed using IBM SPSS statistical tool 23.0 software (SPSS Inc., Chicago, USA). Means were compared according to the method of Fisher's Least Significant Difference-LSD at the 0.05 level.

3. Results

3.1. Effect of critical control parameters on ACP inactivation efficiency

The study evaluated the effect of varying voltage levels (60, 70, 80 kV) on antimicrobial efficacy of ACP with PTST of 24 h against *S. enterica*, *L. monocytogenes* and *P. fluorescens* in PBS (Table 1). The highest voltage level of 80 kV showed significantly greater inactivation by comparison to 60 kV or 70 kV ($p < 0.05$). ACP treatment at 70 kV reduced *S. enterica* and *P. fluorescens* counts by 2.3 ± 0.10 and 3.0 ± 0.06 Log_{10} CFU/ml reduction after 120 s of treatment while

Table 1
Effect of voltage on plasma inactivation efficacy in PBS.

Applied voltages	Plasma treatment time (s)	*S. enterica*		*L. monocytogenes*		*P. fluorescens*	
		Cell density (Log_{10} CFU/ml)	SD	Cell density (Log_{10} CFU/ml)	SD	Cell density (Log_{10} CFU/ml)	SD
60 kV	C1	8.06^{a}	0.02	7.29^{a}	0.04	7.50^{a}	0.06
	C2	8.14^{b}	0.04	6.84^{b}	0.10	8.09^{b}	0.12
	30	7.38^{c}	0.05	6.32^{c}	0.09	6.30^{c}	0.09
	60	6.97^{f}	0.11	3.71^{e}	0.17	6.00^{c}	0.02
	120	5.69^{i}	0.18	ND^{g}	–	5.70^{e}	0.17
	300	ND^{k}	–	ND^{h}	–	ND^{h}	–
70 kV	C1	8.40^{a}	0.04	7.29^{a}	0.06	7.50^{a}	0.06
	C2	8.30^{b}	0.12	6.84^{b}	0.02	8.09^{b}	0.12
	30	6.95^{d}	0.05	5.30^{d}	0.06	6.0^{c}	0.10
	60	6.30^{g}	0.18	ND^{f}	–	5.8^{c}	0.01
	120	5.68^{i}	0.10	ND^{g}	–	4.50^{f}	0.06
	300	ND^{k}	–	ND^{h}	–	ND^{h}	–
80 kV	C1	8.19^{a}	0.02	7.29^{a}	0.05	7.50^{a}	0.06
	C2	8.04^{b}	0.05	6.84^{b}	0.14	8.09^{b}	0.12
	30	6.69^{e}	0.02	5.00^{d}	0.10	6.1^{c}	0.20
	60	5.79^{h}	0.03	ND^{f}	–	3.7^{d}	0.05
	120	ND^{j}	–	ND^{g}	–	ND^{g}	–
	300	ND^{k}	–	ND^{h}	–	ND^{h}	–

C1: Control without storage, C2: Control with storage, SD: Standard deviation, ND: Non-detectable (below detection limit of 1.0 Log_{10} CFU/ml), Different letters indicate significant difference between voltage levels and treatment time. Each experiment was conducted in duplicate and replicated twice.

complete inactivation was achieved using 80 kV voltage level. *L. monocytogenes* was reduced by 3.6 ± 0.17 Log_{10} CFU/ml after 60 s of ACP exposure at 60 kV, but again was more susceptible to the higher voltage levels.

The effect of contained post treatment storage time is a known important process factor for microbial inactivation and is presented in Fig. 1. ACP treatment for 120 s with 1 h PTST yielded ± 2.7 Log_{10} CFU/ml reduction of *P. fluorescens*, and ± 1.1 Log_{10} CFU/ml reduction of *S. enterica*. Extending the treatment time from 120 s to 300 s while retaining the PTST at 1 h, lead to further significant reductions in population density (Fig. 1a). However, greater ACP inactivation efficiency was obtained with 24 h PTST ($p < 0.05$) and 120 s treatment time, with cells below detection limit after 120 s of ACP exposure (Fig. 1b).

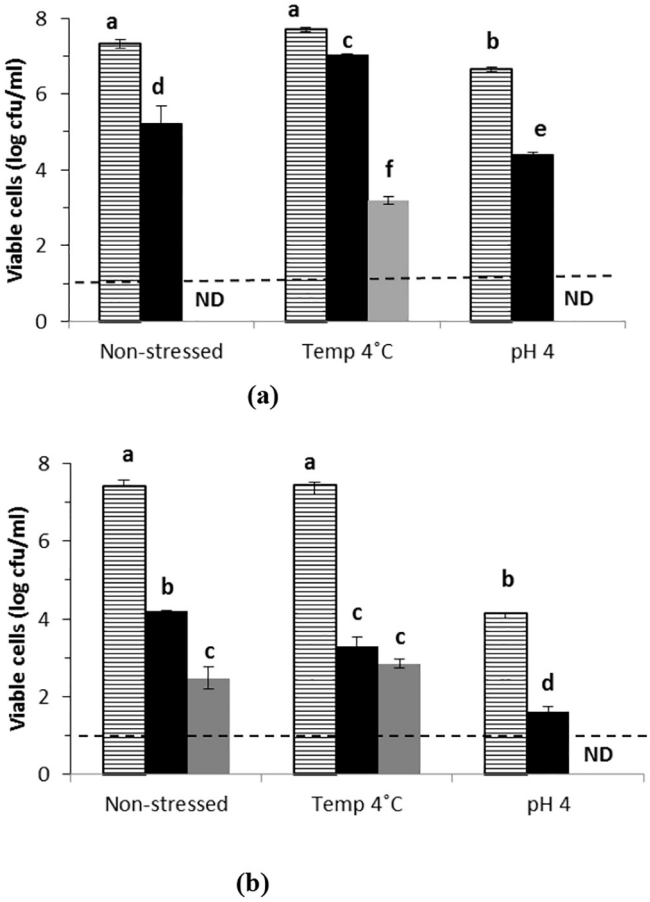

Fig. 2. Effect of acid stress and cold shock on the resistance of (a) *L. monocytogenes* and (b) *P. fluorescens* to ACP treatment (▤: Control untreated, ■: 60 s ACP, ■: 120 s ACP) when suspended in lettuce broth. Experiments were performed in duplicate and replicated three times (n = 6). Columns with different letters indicate a significant difference between controls and ACP treated samples ($p < 0.05$).

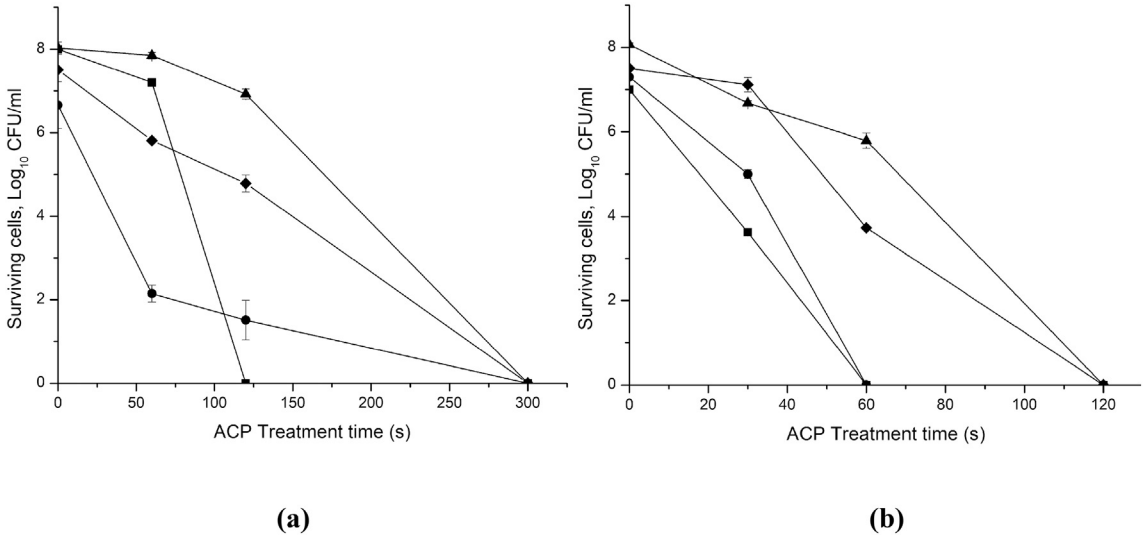

Fig. 1. ACP inactivation efficacy against planktonic (▲) *S. enterica*, (■) *E. coli*, (●) *L. monocytogenes*, (◆) *P. fluorescens*, with 30 s, 60 s, 120 s, 300 s of treatments at 80 kV with either (a) 1 h and (b) 24 h post treatment storage time. Experiments were performed in duplicates and replicated twice. Limit of detection was 1.0 Log_{10} CFU/ml.

3.2. Effect of food and storage conditions on ACP bacterial inactivation efficiency

To investigate the effect of food product and storage conditions on ACP bacterial inactivation efficiency, acid and temperature stresses were applied to *L. monocytogenes* and *P. fluorescens* inoculated in lettuce broth (model product media) and treated with ACP (Fig. 2). In general, the inactivation rates for *L. monocytogenes* and *P. fluorescens* obtained from short term exposure to acid (pH 4) stress were quicker than non-stressed control bacterial cells. *P. fluorescens* exposed to acid stress became highly sensitive and was reduced to 1.6 Log_{10} CFU/ml after 60 s of plasma exposure. Exposure to cold stress (4 °C) did not significantly influence the effectiveness of ACP treatments on *P. fluorescens*, both stressed and control cells had similar inactivation patterns. However, *L. monocytogenes* exposed to low temperature, showed significantly higher resistance to ACP than non-stressed population. Exposure to environmental stress such as acid or temperature could trigger stress responses in *L. monocytogenes* (Bergholz et al., 2012; Venkitanarayanan et al., 2017) thus demonstrating enhanced bacterial resistance to ACP treatment than cells not exposed to acid/temperature stress.

3.3. Effect of ACP treatment on mono-culture bacterial biofilms

Preliminary studies demonstrated ACP to effectively inactivate principle planktonic microorganisms relevant to fresh foods. Inactivation of bacterial biofilms is of special interest in food industry due to their resistance and increasing source of contamination in food products, leading to food spoilage, reducing shelf life of products, or transmission of diseases (Phillips, 2016). This study investigated the potential of ACP treatment for inactivation of bacterial biofilms formed by *E. coli*, *L. monocytogenes*, *S. enterica* and *P. fluorescens*. Since 48 h culture incubation yielded increased biomass with mature biofilms (Fig. S1), 48 h biofilm were utilised for challenge biofilm inactivation studies. The impact of ACP inactivation on biofilm cells were analysed by plate count and XTT assay. Surviving population of *E. coli*, *L. monocytogenes*, *S. enterica* and *P. fluorescens* 48 h old mono-culture biofilm are represented in the Fig. 3. Biofilm formation for 48 h in 12% lettuce broth resulted in average attached population of 5.4 ± 0.4 Log_{10} CFU/ml. According to both plate count and XTT assays, significant reduction of bacterial biofilms was observed after ACP treatment. ACP treatment reductions were 3.76, 4.14 and 2.6 Log_{10} CFU/ml for *L. monocytogenes*, *S. enterica* and *P. fluorescens* respectively. Highest inactivation levels were observed for *E. coli*, where ACP treatment reduced bacterial population to undetectable levels, analysed by plate count method. However, XTT assay demonstrated 34% metabolic activity when compared to control untreated (0 h). While, in case of *L. monocytogenes*, *S. enterica* and *P. fluorescens*, a better correlation was observed between the colony count and XTT assay, metabolic activity was reduced by 72%, 42% and 35% respectively.

3.4. Effect of ACP treatment on mixed culture bacterial biofilm

Bacterial contamination of food or food processing environment is more likely to exist as multi-species biofilms, therefore subsequent investigations focussed on a mixed species biofilm model. The significant increase in the biofilm biomass was observed in mixed culture biofilm (Fig. S1) as compared to monoculture biofilms. ACP was also employed to treat mixed species biofilms to investigate their antimicrobial effects in food processing environment. The Fig. 4 showed the size of the surviving population as a function of duration of ACP treatment for monoculture and mixed species biofilm of *L. monocytogenes* and *P. fluorescens*. In case of dual species biofilms, interspecies interaction was found to have small effect on antimicrobial efficacy of ACP treatment. Both *L. monocytogenes* and *P. fluorescens* in dual species showed around 4.2 ± 0.2 log reduction after ACP treatment of 60 s and 24 h PTST. Similar trend was observed by XTT assay, the percentage of metabolically active cells significantly decreased to an average of 52.5 ± 10.4%, 23.9 ± 2.3% and 3.5 ± 2.4% after 60, 120 and 300 s of treatment. Both species under mono culture or mixed conditions were found to be sensitive to ACP treatment.

3.5. Effect of ACP on bacterial populations inoculated on lettuce

The antimicrobial efficacy of ACP against dual bacterial biofilms of *L. monocytogenes* and *P. fluorescens* grown on lettuce at 4 °C or 15 °C is presented in Fig. 5. After 5 min of treatment, the concentration of cells in 48 h biofilm formed at 15 °C were significantly reduced to undetectable levels compared with untreated controls ($p \leq 0.05$). In the current study, different inactivation patterns were observed for 48 h biofilms grown at 4 °C and 15 °C. Increased resistance to ACP treatment was observed for 48 h biofilm formed at 4 °C with 4 Log_{10} CFU/ml reduction achieved for *L. monocytogenes* and 2.1 Log_{10} CFU/ml for *P. fluorescens*. Even though the average initial bacterial biofilms formed by *L. monocytogenes* (7.0 ± 0.3 Log_{10} CFU/ml) and *P. fluorescens* (5.5 ± 0.2 Log_{10} CFU/ml) was similar at both the temperatures, the biofilms grown at 4 °C were more resistant to inactivation by ACP. These findings imply that environmental stressors could influence antimicrobial efficacy of ACP treatment.

Storage temperature is an important factor in maintaining the quality and the shelf-life of the fresh products. A difference in overall lettuce appearance was observed at lettuce stored at different refrigeration temperatures prior plasma treatment; signs of slight browning observed only on lettuce stored at 15 °C. No difference in relation to colour or texture of lettuce was visually visible due to the plasma treatment and post 24 h storage. This research with lettuce stored at refrigeration and or abuse temperatures presents opportunity to further explore the effects of cold plasma on the physico-chemical and sensory properties of the fresh food products in future.

4. Discussion

In summary, results from this study show potential of ACP to treat both pathogenic and spoilage bacterial in mono or dual biofilms. The study demonstrates the impact of different parameters influencing ACP inactivation efficacy which includes: the bacterial type, physiological state of cells, substrate on which the bacteria are present and ACP process parameters such as applied voltage, treatment time and post treatment storage environment conditions.

Increasing applied voltage to 80 kV largely resulted in higher bacterial inactivation to undetectable levels within short period of treatment time. Similar results were obtained with Han et al. (2016), where the author also reported increased Reactive Oxygen Species (ROS) levels along with increased voltage levels have resulted faster inactivation. Atmospheric air plasma generates ROS and RNS species including ozone, hydrogen peroxide and nitrates; which are among the most commonly detected species using in-package Dielectric Barrier Discharge (DBD)-ACP system (Boehm et al., 2017; Han et al., 2015). The reactive species generated by plasma have inhibitory effect on bacterial population causing oxidative damage of macromolecules like DNA, proteins and lipids (Bourke et al., 2017). Additionally, PTST emerged as critical treatment parameter for bacterial inactivation with this system. Reports from Niquet et al. (2018) established that increasing the PTST helped retain the reactive species generated from plasma treatment inside the container, increasing the diffusion and interaction time with the microbial targets which further enhanced ACP's antimicrobial efficacy (Niquet et al., 2018). The study also reported high concentration of hydrogen peroxide using in-package DBD-ACP system which increased over time of post treatment storage time of 24 h (Niquet et al., 2018). Similarly, the influence of storage time on plasma efficiency at short treatment duration was clearly observed in our study. The PTST of 1 h or 24 h facilitates retention of short- or long-lived reactive species that enhances the antimicrobial efficacy for decontamination. The

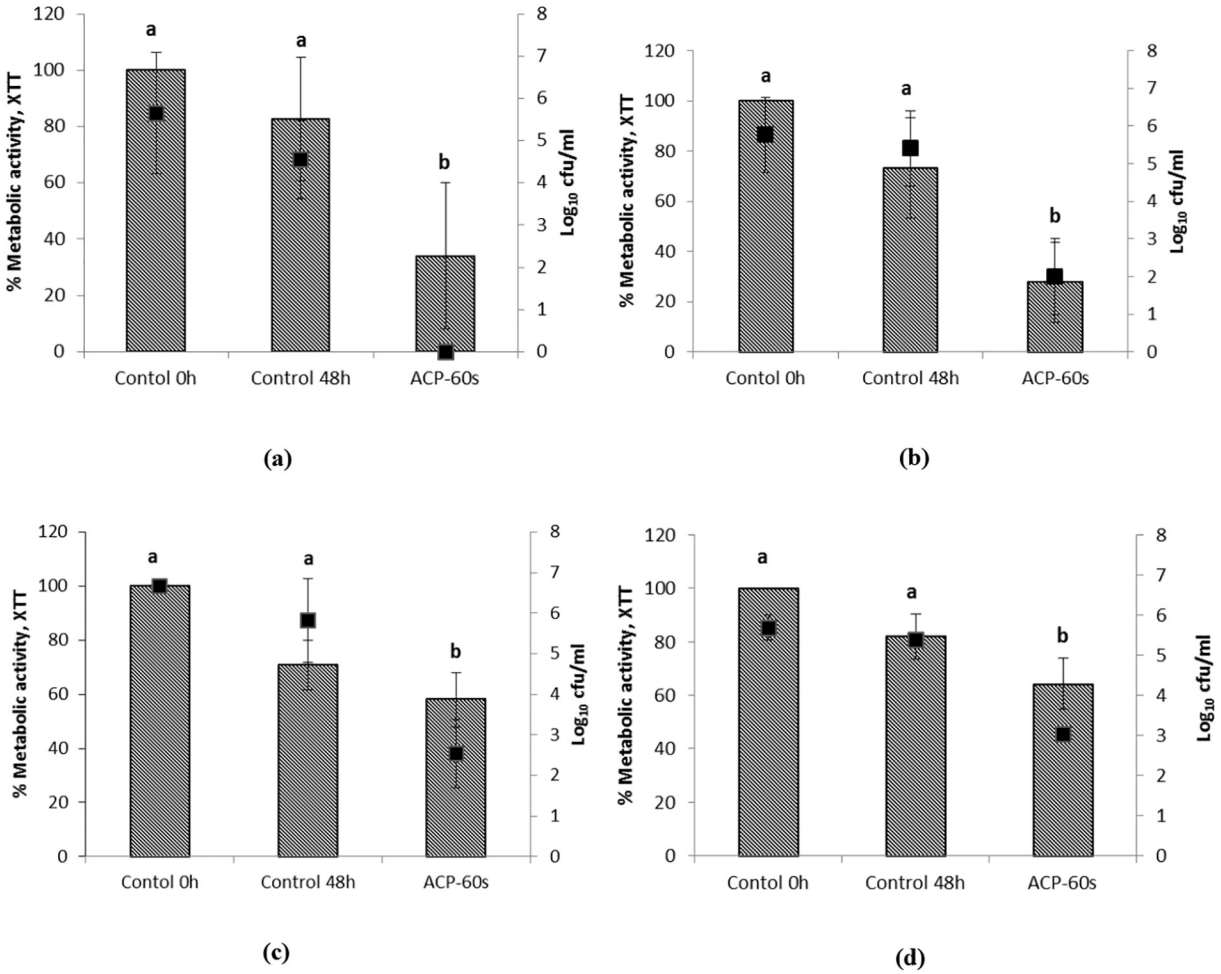

Fig. 3. Surviving populations of 48 h bacterial mono-culture bacterial of (a) *E. coli*, (b) *L. monocytogenes*, (c) *S. enterica* and (d) *P. fluorescens* assessed by (■) Plate count, (▨) XTT assay after 60 s of ACP treatment and 24 h PTST. Vertical bars represent standard deviation. Limit of detection for plate count was 1.0 Log_{10} CFU/ml. Values represented as different letters indicate significant difference between bacterial populations of the control and ACP treated samples analysed by plate count method. Each experiment was conducted twice in duplicate (n = 4), while XTT was performed twice in triplicate (n = 6).

interaction between the plasma species and bacteria has been reported (Han et al., 2015; Julák et al., 2012). In this study, high voltage of 80 kV along with 24 h PTST, was efficient to reduce most of the bacterial species with short treatment times thus these parameters were used for further analysis.

To simulate real fresh produce processing conditions, a lettuce model broth was used to analyse the influence of substrate components and storage conditions in relation to fresh produce on antimicrobial efficacy of treatment. It is known in food processing environment, most food substrate either enhance or reduce, bacterial attachment and subsequent biofilm formation (Papaioannou et al., 2018). Also, food-borne microorganisms are known to encounter variety of stress (acid, oxidative, cold or heat shock induced mutations) within the food chain, including production, harvest, postharvest handling, processing, disinfection and storage (Delaquis and Bach, 2012) which may increase tolerance or resistance in bacteria. Therefore, this study investigated the influence of short-term exposure of acid or cold stress on inactivation of common food pathogen and spoilage organism inoculated in lettuce broth by ACP. Both strains were sensitive to ACP post acid exposure showing significant reduction ($p < 0.05$) within 120 s of plasma exposure. *L. monocytogenes* exposed to cold stress (4 °C) for 1 h resulted in higher resistance than non-stressed control cells, potentially giving cross-protective effect against ACP treatment. While, *P. fluorescens* exposed to cold stress (4 °C) did not show significant difference to values obtained for control non-stressed cells. *L. monocytogenes* and *P. fluorescens* both are psychrotrophic bacteria known to survive and grown under refrigeration temperatures. Microbial adaptation to stress is also known to extend tolerance to multiple other lethal stresses, referred to as cross protection (Johnson, 2002). Microorganism can utilise cross-protection as a defence mechanism against many food disinfection or food preservation techniques (Rodriguez-Romo and Yousef, 2005). Therefore, the cold stress induced cross protection to cold plasma observed here should be taken into account when designing and implementing minimal processing regimes that rely on refrigeration temperature.

The physiological state of bacteria has been reported to play an important role in its resistance under adverse environment conditions. Several microbial pathogens or spoilage bacteria are able to form biofilm on wide variety of surfaces which may possess a potential risk for continuous reservoir of contamination in food processing environment. In general, biofilms are the dominant life style of bacteria which can grow and survive in all environments and have enhanced resistance to several antimicrobial agents (Sanchez-Vizuete et al., 2015). Fresh produce lettuce model broth was used to form biofilm on microplate surface to analyse the influence of substrate components in relation to fresh produces on antimicrobial efficacy of treatment. ACP treatment was effective against challenge mono species biofilms which was determined using direct plating technique combined with XTT assay to gain insight into both culturable and metabolic active cells of the bacterial biofilm. According to colony counts, treatment for 60 s

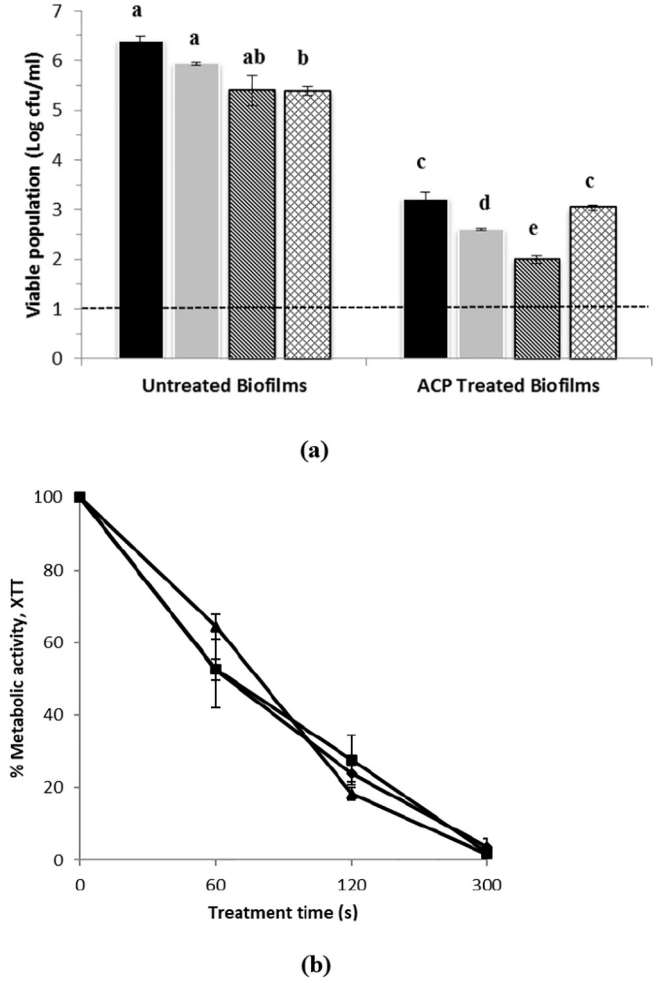

Fig. 4. Surviving populations of bacterial biofilms after ACP treatment at 80 kV and 24 h PTST assessed by (a) Colony count assay: (■) *L. monocytogenes* dual-biofilm, (▮) *P. fluorescens* dual-biofilm, (▨) *L. monocytogenes* mono-biofilm and (▩) *P. fluorescens* mono-biofilm. Detection limit of 1.0 Log_{10} CFU/ml; (b) XTT assay: (▮) *L. monocytogenes* single biofilm, (▲) *P. fluorescens* single biofilm, (◆) dual biofilm of *L. monocytogenes and P. fluorescens*. Each experiment was performed in duplicate and replicated twice (n = 4).

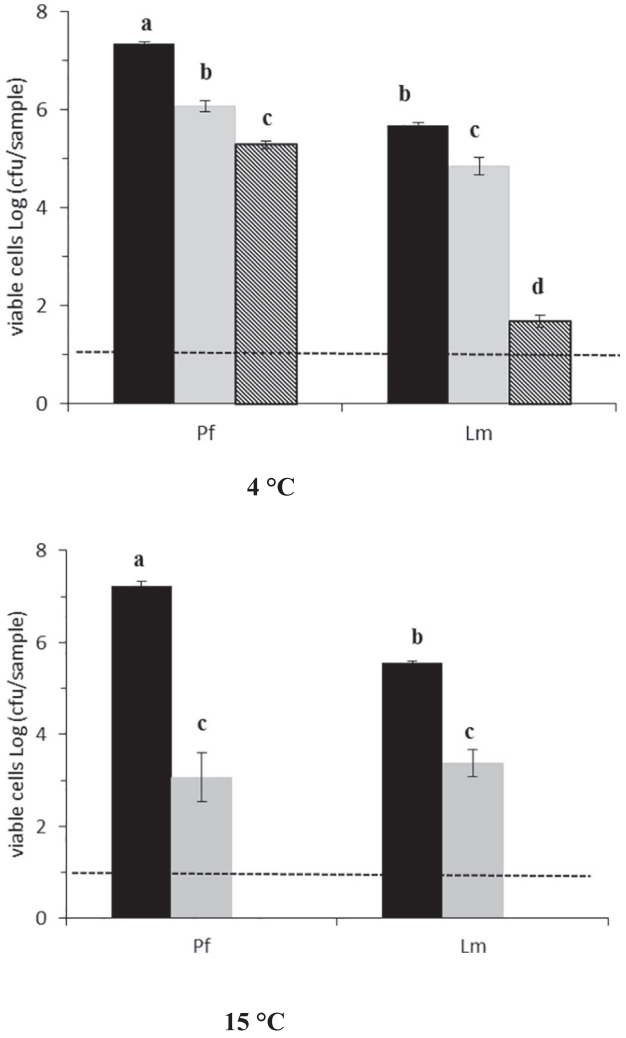

Fig. 5. Effect of ACP on 48 h dual bacterial biofilms of *L. monocytogenes* (Lm) and *P. fluorescens* (Pf) formed on lettuce at 4 °C and at 15 °C. (■) untreated control, (■) 2 min and (▨) 5 min ACP treated samples. ND: not detectable; limit of detection 1.0 Log_{10} CFU/ml. Experiments were performed in duplicate and replicated twice. Different letters indicate significant difference between bacterial populations of the control and ACP treated samples ($p < 0.05$). Vertical bars represent standard deviation.

reduced *E. coli* population to undetectable levels, whereas this treatment was less effective against *L. monocytogenes*, *S. aureus* and *P. fluorescens*. Percentage reduction values based on XTT assay showed good correlation with plate count method for *L. monocytogenes*, *S. aureus* and *P. fluorescens*. While in the case of *E. coli* biofilms, even though plate count results demonstrated bacterial cell count to undetectable levels, 34% metabolically active cells were detected after 60 s of ACP exposure. This could be due to oxidative stress encountered from reactive species generated by ACP treatment, bacteria may enter the viable but non-culturable (VBNC) state where bacteria cells are still alive but are not able to grow on bacteria media. VBNC is a strategic state adopted by most prokaryotes when subjected to adverse environmental conditions (Arana et al., 2010). There was a loss of bacteria culturability but the cells were still metabolically active under stress conditions and with the adoption of VBNC phenotype they may be able to retain their virulence factors that could contribute further to contamination (Barcina and Arana, 2009; Oliver, 2010).

Interestingly in multispecies biofilm, both *P. fluorescens* and *L. monocytogenes* displayed significantly higher biofilm cells compared to its pure culture biofilm. The main proposed factor for *P. fluorescens* and *L. monocytogenes* co-operation is the EPS production, large amount of Pseudomonas EPS that would fix, embed and protect *L. monocytogenes* (Puga et al., 2014). Several factors could be involved for the tolerance factor of multispecies biofilm which includes the higher extracellular polymer substance (EPS) production, differences in physiological status, interspecies cross protection among the species, internalization into the food (Stewart, 2015). Considering this, mixed biofilm species represent more challenging environment than single species biofilm. Previous studies by Norwood and Gilmour (2000) & Saá Ibusquiza et al. (2012) investigated resistance of single and mixed species biofilms of *Listeria* and *Pseudomonas* against different chemical disinfectants. Their research demonstrated that *Listeria* and *Pseudomonas* species grown in mixed species under most conditions were more resistant to disinfectants than single species. The interspecies interactions that take place in multi-species biofilm significantly modify the matrix complex if compared with mono-cultures. In contrast, in our current studies, interspecies interaction did not seem to have any effect on antimicrobial resistance of biofilm of each individual species. A significant reduction in mixed biofilms formed by *L. monocytogenes* and *P. fluorescens* after 60 s of treatment and showed complete inactivation after prolonging the treatment time to 120 s. The results obtained demonstrate that ACP treatment obstruct the association capacity between the mixed biofilms,

giving rise to mixed biofilm that is significantly ($p < 0.05$) less resistant to ACP. However, an extended treatment time of 300 s was necessary in order to achieve significant reductions of challenge mixed bacterial culture biofilms inoculated on lettuce for 48 h. Bacterial pathogens can rapidly attach to different plant parts (stomata, veins, lenticels, plant cuts) and persists for longer periods which are much more complex for antimicrobial treatments to reach (Warning and Datta, 2013). Also, the organic components in the fresh produces such as proteins, vitamins, could scavenge the reactive species generated by the plasma exposure thus defending the microbial cells from oxidation and cell death. Additionally, studies by Ziuzina et al. (2015b) demonstrated that the storage conditions, such as temperature, light and time had interactive effects on bacterial proliferation and susceptibility to the ACP treatment. The fluctuation in the temperature or light intensity may induce bacterial attachment, biofilm formation and internalization in plants (Ziuzina et al., 2015b). Consistent storage temperature is difficult to maintain throughout the distribution process of fresh produces. Therefore, in this work, effect of temperature (4 °C and 15 °C) on bacterial biofilm formation and its susceptibility to ACP treatment was evaluated. The temperature 4 °C was chosen as the general refrigeration temperature used for fresh produce storage and 15 °C was selected to be close to the thermal conditions encountered in many food production environments. The results demonstrated that high voltage ACP treatment for 300 s significantly reduced 48 h biofilm grown at 15 °C to undetectable levels, however there was significant difference between biofilm grown at 4 °C. Mixed biofilm formed at 4 °C showed increased resistance to treatment, *P. fluorescens* particularly was found to present higher resistance than *L. monocytogenes* to ACP treatment. Similarly, the resistance of the *L. monocytogenes* exposed to with chlorine (0.465%) or peroxyacetic acid (2%) increased with incubation time of stainless steel coupons at 4 °C (Belessi et al., 2011). Furthermore, previous studies have demonstrated bacteria present in mixed species biofilm have increased understanding of interactions and dynamics of surface attachment. The bacterial populations attached may not contribute at the same level towards biofilm formation or under environmental stress conditions, some of bacterial strain is able to dominate over the others, depending on the surrounding conditions and develop resistance (Giaouris et al., 2013; Kostaki et al., 2012). The more characteristic feature of the dual *L. monocytogenes* and *P. fluorescens* biofilms is the layering, *L. monocytogenes* is located at the bottom layer of the dual biofilm (Puga et al., 2014). The bacterial population located at bottom in the biofilm structure undergo several anaerobic and starvation stresses (Lungu et al., 2010). Depth and slow doubling time could contribute to highly increased resistance to antimicrobials as observed for *L. monocytogenes* in multispecies.

In conclusion, ACP was effective against planktonic population effectively eliminating viable cells present in mature biofilm formed in lettuce broth or on lettuce under different conditions (mono or mixed biofilm). ACP remains a promising technology for decontamination of pathogenic or spoilage bacteria in biofilms present on fresh fruits and vegetables on fresh produce and associated food processing environments. However, caution must be used in cognisance of the environmental stresses previously encountered by microbial risk and the potential for cross protection to ACP from low temperature exposure. All the results presented surely highlight the complexity of mono/mixed biofilm present, the influence of interspecies interaction between pathogenic and spoilage bacteria in biofilm, culture and biofilm setup conditions, temperature, substrate surface, composition of substrate. The fresh produce industry currently lacks efficient control methods to ensure elimination of food-borne pathogens from minimally processed food products. ACP could be part of an efficient control mechanism against bacterial contamination in fresh cut produce or minimally processed products which could help extend shelf-life while maintaining quality. Further investigation of the molecular mechanism behind the stress responses and the relationship to ACP treatment is needed to provide useful information for optimisation and implementation of effective HV-ACP decontamination regimes. Also, food quality studies are important for effective commercialization of the technology at industrial scale, current studies are in process to quantify the effect of the ACP technologies in combination with Modified atmospheric packaging (MAP) on the microbiology and quality of the selected fresh products under real storage conditions and validate the expected benefits of the technologies in terms of shelf-life.

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References

Arana, I., Muela, A., Orruño, M., Seco, C., Garaizabal, I., Barcina, I., 2010. Effect of temperature and starvation upon survival strategies of *Pseudomonas fluorescens* CHA0: comparison with Escherichia coli. FEMS Microbiol. Ecol. 74, 500–509. https://doi.org/10.1111/j.1574-6941.2010.00979.x.

Barcina, I., Arana, I., 2009. The viable but nonculturable phenotype: a crossroads in the life-cycle of non-differentiating bacteria? Rev. Environ. Sci. Biol. Technol. 8, 245–255. https://doi.org/10.1007/s11157-009-9159-x.

Belessi, C.E.A., Gounadaki, A.S., Psomas, A.N., Skandamis, P.N., 2011. Efficiency of different sanitation methods on *Listeria monocytogenes* biofilms formed under various environmental conditions. Int. J. Food Microbiol. https://doi.org/10.1016/j.ijfoodmicro.2010.10.020.

Bergholz, T.M., Bowen, B., Wiedmann, M., Boor, K.J., 2012. *Listeria monocytogenes* shows temperature dependent and independent responses to salt stress, including responses that induce cross-protection to other stresses. Appl. Environ. Microbiol. AEM-07658. https://doi.org/10.1128/AEM.07658-11.

Boehm, D., Curtin, J., Cullen, P.J., Bourke, P., 2017. Hydrogen peroxide and beyond-the potential of high-voltage plasma-activated liquids against cancerous cells. Anti-Cancer Agent Me. https://doi.org/10.2174/1871520617666170801110517.

Borucki, M.K., Peppin, J.D., White, D., Loge, F., Call, D.R., 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.69.12.7336-7342.2003.

Bourke, P., Ziuzina, D., Han, L., Cullen, P.J., Gilmore, B.F., 2017. Microbiological interactions with cold plasma. J. Appl. Microbiol. https://doi.org/10.1111/jam.13429.

Buchanan, R.L., Bagi, L.K., 1999. Microbial competition: effect of *Pseudomonas fluorescens* on the growth of *Listeria monocytogenes*. Food Microbiol. 16 (5), 523–529.

Calvo, T., Alvarez-Ordóñez, A., Prieto, M., Bernardo, A., López, M., 2017. Stress adaptation has a minor impact on the effectivity of Non-Thermal Atmospheric Plasma (NTAP) against *Salmonella* spp. Food Res. Int. https://doi.org/10.1016/j.foodres.2017.09.035.

Chen, J.-Q., Regan, P., Laksanalamai, P., Healey, S., Hu, Z., 2017. Prevalence and methodologies for detection, characterization and subtyping of *Listeria monocytogenes* and *L. ivanovii* in foods and environmental sources. Food Sci. Human Wellness 6, 97–120. https://doi.org/10.1016/j.fshw.2017.06.002.

Delaquis, P., Bach, S., 2012. Resistance and sublethal damage. In: Decontamination of Fresh and Minimally Processed Produce. Wiley-Blackwell, Oxford, UK, pp. 77–86. https://doi.org/10.1002/9781118229187.ch4.

Giaouris, E., Chorianopoulos, N., Doulgeraki, A., Nychas, G.J., 2013. Co-culture with *Listeria monocytogenes* within a dual-species biofilm community strongly increases resistance of *Pseudomonas putida* to benzalkonium chloride. PLoS One. https://doi.org/10.1371/journal.pone.0077276.

Han, L., Patil, S., Boehm, D., Milosavljević, V., Cullen, P.J., Bourke, P., 2015. Mechanisms of inactivation by high-voltage atmospheric cold plasma differ for *Escherichia coli* and *Staphylococcus aureus*. Appl. Environ. Microbiol. 82, 450–458. https://doi.org/10.1128/AEM.02660-15.

Han, L., Ziuzina, D., Heslin, C., Boehm, D., Patange, A., Sango, D.M., Valdramidis, V.P., Cullen, P.J., Bourke, P., 2016. Controlling microbial safety challenges of meat using high voltage atmospheric cold plasma. Front. Microbiol. https://doi.org/10.3389/fmicb.2016.00977.

Jahid, I.K., Ha, S.-D., 2014. The paradox of mixed-species biofilms in the context of food safety. Compr. Rev. Food Sci. Food Saf. https://doi.org/10.1111/1541-4337.12087.

Johnson, E., 2002. Microbial adaptation and survival in foods. In: Microbial Stress Adaptation and Food Safety. CRC Press. https://doi.org/10.1201/9781420012828.ch4.

Julák, J., Scholtz, V., Kotúčová, S., Janoušková, O., 2012. The persistent microbicidal effect in water exposed to the corona discharge. Phys. Med. https://doi.org/10.1016/j.ejmp.2011.08.001.

Kostaki, M., Chorianopoulos, N., Braxou, E., Nychas, G.J., Giaouris, E., 2012. Differential biofilm formation and chemical disinfection resistance of sessile cells of *Listeria monocytogenes* strains under monospecies and dual-species (with *Salmonella enterica*) conditions. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.07099-11.

Lohse, M.B., Gulati, M., Johnson, A.D., Nobile, C.J., 2017. Development and regulation of single- and multi-species *Candida albicans* biofilms. Nat. Rev. Microbiol. 16, 19–31.

https://doi.org/10.1038/nrmicro.2017.107.
Lungu, B., Saldivar, J.C., Story, R., Ricke, S.C., Johnson, M.G., 2010. The combination of energy-dependent internal adaptation mechanisms and external factors enables *Listeria monocytogenes* to express a strong starvation survival response during multiple-nutrient starvation. Foodborne Pathog. Dis. https://doi.org/10.1089/fpd.2009.0408.
Misra, N.N., Han, L., Tiwari, B.K., Bourke, P., Cullen, P.J., 2014. Nonthermal plasma technology for decontamination of foods. Nov. Food Preserv. Microb. Assess. Technol. 155.
Misra, N.N., Martynenko, A., Chemat, F., Paniwnyk, L., Barba, F.J., Jambrak, A.R., 2018. Thermodynamics, transport phenomena, and electrochemistry of external field-assisted nonthermal food technologies. Crit. Rev. Food Sci. Nutr. 58, 1832–1863. https://doi.org/10.1080/10408398.2017.1287660.
Murray, K., Wu, F., Shi, J., Jun Xue, S., Warriner, K., 2017. Challenges in the microbiological food safety of fresh produce: limitations of post-harvest washing and the need for alternative interventions. Food Qual. Saf. https://doi.org/10.1093/fqsafe/fyx027.
Niquet, R., Boehm, D., Schnabel, U., Cullen, P., Bourke, P., Ehlbeck, J., 2018. Characterising the impact of post-treatment storage on chemistry and antimicrobial properties of plasma treated water derived from microwave and DBD sources. Plasma Process. Polym. 15 (3), 1700127. https://doi.org/10.1002/ppap.201700127.
Norwood, D.E., Gilmour, A., 2000. The growth and resistance to sodium hypochlorite of Listeria monocytogenes in a steady-state multispecies biofilm. J. Appl. Microbiol. 88, 512–520. https://doi.org/10.1046/j.1365-2672.2000.00990.x.
Oliver, J.D., 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol. Rev. 34, 415–425. https://doi.org/10.1111/j.1574-6976.2009.00200.x.
Pang, X., Yang, Y., Yuk, H.G., 2017. Biofilm formation and disinfectant resistance of Salmonella spp. in mono- and dual-species with *Pseudomonas aeruginosa*. J. Appl. Microbiol. 123, 651–660. https://doi.org/10.1111/jam.13521.
Pankaj, S.K., Keener, K.M., 2017. Cold plasma: background, applications and current trends. Curr. Opin. Food Sci. 16, 49–52. https://doi.org/10.1016/j.cofs.2017.07.008.
Papaioannou, E., Giaouris, E.D., Berillis, P., Boziaris, I.S., 2018. Dynamics of biofilm formation by *Listeria monocytogenes* on stainless steel under mono-species and mixed-culture simulated fish processing conditions and chemical disinfection challenges. Int. J. Food Microbiol. 267, 9–19. https://doi.org/10.1016/j.ijfoodmicro.2017.12.020.
Parijs, I., Steenackers, H.P., 2018. Competitive inter-species interactions underlie the increased antimicrobial tolerance in multispecies brewery biofilms. ISME J. https://doi.org/10.1038/s41396-018-0146-5.
Patil, S., Valdramidis, V.P., Cullen, P.J., Frias, J.M., Bourke, P., 2010. Ozone inactivation of acid stressed *Listeria monocytogenes* and *Listeria innocua* in orange juice using a bubble column. Food Control. https://doi.org/10.1016/j.foodcont.2010.04.031.
Peeters, E., Nelis, H.J., Coenye, T., 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J. Microbiol. Methods 72, 157–165. https://doi.org/10.1016/j.mimet.2007.11.010.
Phillips, C.A., 2016. Bacterial biofilms in food processing environments: a review of recent developments in chemical and biological control. Int. J. Food Sci. Technol. 51, 1731–1743. https://doi.org/10.1111/ijfs.13159.
Puga, C.H., SanJose, C., Orgaz, B., 2014. Spatial distribution of *Listeria monocytogenes* and *Pseudomonas fluorescens* in mixed biofilms. In: *Listeria Monocytogenes*: Food Sources, Prevalence and Management Strategies.
Renier, S., Hébraud, M., Desvaux, M., 2011. Molecular biology of surface colonization by *Listeria monocytogenes*: an additional facet of an opportunistic Gram-positive foodborne pathogen. Environ. Microbiol. https://doi.org/10.1111/j.1462-2920.2010.02378.x.
Rodriguez-Romo, L.A., Yousef, A.E., 2005. Inactivation of *Salmonella enterica* serovar Enteritidis on shell eggs by ozone and UV radiation. J. Food Prot. https://doi.org/10.4315/0362-028X-68.4.711.
Saá Ibusquiza, P., Herrera, J.J.R., Vázquez-Sánchez, D., Cabo, M.L., 2012. Adherence kinetics, resistance to benzalkonium chloride and microscopic analysis of mixed biofilms formed by *Listeria monocytogenes* and *Pseudomonas putida*. Food Control 25, 202–210. https://doi.org/10.1016/j.foodcont.2011.10.002.
Sanchez-Vizuete, P., Orgaz, B., Aymerich, S., Le Coq, D., Briandet, R., 2015. Pathogens protection against the action of disinfectants in multispecies biofilms. Front. Microbiol. 6, 705. https://doi.org/10.3389/fmicb.2015.00705.
Sasahara, K., Zottola, E., 1993. Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems. J. Food Prot. 56, 1022–1028.
Stewart, P.S., 2015. Antimicrobial tolerance in biofilms. Microbiol. Spectr. 3. https://doi.org/10.1128/microbiolspec.MB-0010-2014.
Stoodley, P., Sauer, K., Davies, D.G., Costerton, J.W., 2002. Biofilms as complex differentiated communities. Annu. Rev. Microbiol. https://doi.org/10.1146/annurev.micro.56.012302.160705.
Tan, C.H., Lee, K.W.K., Burmølle, M., Kjelleberg, S., Rice, S.A., 2017. All together now: experimental multispecies biofilm model systems. Environ. Microbiol. 42–53. https://doi.org/10.1111/1462-2920.13594.
Tournas, V.H., 2005. Spoilage of vegetable crops by bacteria and fungi and related health hazards. Crit. Rev. Microbiol. 31, 33–44. https://doi.org/10.1080/10408410590886024.
Venkitanarayanan, K., Upadhyay, A., Nair, M.S., Upadhyaya, I., 2017. The effects of environmental conditions and external treatments on virulence of foodborne pathogens. In: Foodborne Pathogens. Springer, Cham, pp. 305–332.
Vivijs, B., Aertsen, A., Michiels, C.W., 2016. Identification of genes required for growth of Escherichia coli MG1655 at moderately low pH. Front. Microbiol. 7, 1672. https://doi.org/10.3389/fmicb.2016.01672.
Warning, A., Datta, A.K., 2013. Interdisciplinary engineering approaches to study how pathogenic bacteria interact with fresh produce. J. Food Eng. https://doi.org/10.1016/j.jfoodeng.2012.09.004.
World Health Organization. (WHO), 2015. WHO estimates of the global burden of foodborne diseases. Available at: http://www.who.int/foodsafety/publications/foodborne_disease/fergreport/en/ (Accessed 30 June. 2018).
Zhao, X., Zhao, F., Wang, J., Zhong, N., 2017. Biofilm formation and control strategies of foodborne pathogens: food safety perspectives. RSC Adv. https://doi.org/10.1039/C7RA02497E.
Ziuzina, D., Boehm, D., Patil, S., Cullen, P.J., Bourke, P., 2015a. Cold plasma inactivation of bacterial biofilms and reduction of quorum sensing regulated virulence factors. PLoS One 10, e0138209. https://doi.org/10.1371/journal.pone.0138209.
Ziuzina, D., Han, L., Cullen, P.J., Bourke, P., 2015b. Cold plasma inactivation of internalised bacteria and biofilms for *Salmonella enterica* serovar *Typhimurium*, *Listeria monocytogenes* and *Escherichia coli*. Int. J. Food Microbiol. 210, 53–61.