Dublin Institute of Technology
ARROW@DIT

Articles | School of Food Science and Environmental Health

2013

Atmospheric Cold Plasma Inactivation of Escherichia Coli in Liquid Media Inside a Sealed Package

Dana Ziuzina
Dublin Institute of Technology, dana.ziuzina@dit.ie

Sonal Patil
Dublin Institute of Technology, sonalpatil81@gmail.com

Paula Bourke
Dublin Institute of Technology, paula.bourke@dit.ie

Kevin Keener
Purdue University, kkeener@purdue.edu

Patrick Cullen
Dublin Institute of Technology, pjcullen@dit.ie

Follow this and additional works at: http://arrow.dit.ie/schfsehart

Part of the Food Science Commons

Recommended Citation

Ziuzina, D., Patil, S., Bourke, P., Keener, K. and Cullen, PJ. Atmospheric cold plasma inactivation of Escherichia coli in liquid media inside a sealed package. Journal of Applied Microbiology, 114(3):778-87, Mar. 2013. doi: 10.1111/jam.12087. Epub 2013 Jan 7.

This Article is brought to you for free and open access by the School of Food Science and Environmental Health at ARROW@DIT. It has been accepted for inclusion in Articles by an authorized administrator of ARROW@DIT. For more information, please contact yvonne.desmond@dit.ie, arrow.admin@dit.ie, brian.widdis@dit.ie.

This work is licensed under a Creative Commons Attribution-Noncommercial-Share Alike 3.0 License

Journal of
Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Atmospheric cold plasma inactivation of *Escherichia coli* in liquid media inside a sealed package

D. Ziuzina[1], S. Patil[1], P.J. Cullen[1], K.M. Keener[2] and P. Bourke[1]

1 School of Food Science and Environmental Health, Dublin Institute of Technology, Dublin, Ireland
2 Purdue University, Nelson Hall of Food Science, West Lafayette, IN, USA

Keywords
atmospheric cold plasma, dielectric barrier discharge, *Escherichia coli*, inactivation efficacy, ozone, scanning electron microscopy.

Correspondence
Paula Bourke, School of Food Science and Environmental Health, Dublin Institute of Technology, CathalBrugha Street, Dublin 1, Ireland. E-mail: paula.bourke@dit.ie

2013/1388: received 2 August 2012, revised 31 October 2012 and accepted 20 November 2012

doi:10.1111/jam.12087

Abstract

Aims: The main objective of this study was to determine the inactivation efficacy of dielectric barrier discharge atmospheric cold plasma (DBD-ACP) generated inside a sealed package for *Escherichia coli* ATCC 25922.

Methods and Results: A plasma discharge was generated between two circular aluminium electrodes at 40 kV. *E. coli* suspensions (10^7 CFU ml^{-1}) in either maximum recovery diluent (MRD) or phosphate buffered saline (PBS) were treated in a 96-well microtitre plate inside a sealed package. The effects of treatment time, post-treatment storage time, either direct or indirect samples exposure to the plasma discharge and suspension media were studied. Regardless of the media tested, 20 s of direct and 45 s of indirect plasma treatment resulted in complete bacterial inactivation (7 log CFU ml^{-1}). At the lower plasma treatment times (10–30 s) investigated, the effects of suspension media and mode of exposure on the inactivation efficacy were evident. The inactivation efficacy was also influenced by the post-treatment storage time.

Conclusions: It was demonstrated that the novel DBD-ACP can inactivate high concentrations of *E. coli* suspended in liquids within sealed packages in seconds.

Significance and impact of the Study: A key advantage of this in-package nonthermal novel disinfection approach is the elimination of post-processing contamination.

Introduction

In recent years, atmospheric cold plasma (ACP) achieved increased attention among advanced nonthermal technologies as an alternative approach for the elimination of spoilage microorganisms and pathogens from contaminated objects, including fresh and processed food surfaces, and medical devices. It has numerous advantages over more conventional methods such as low process operational costs, short treatment time at low temperatures, nontoxic nature, significant reduction of water consumption throughout disinfection processes, and its application for a wide variety of goods (Song *et al.* 2009; Chiang *et al.* 2010; Korachi *et al.* 2010). To date, atmospheric pressure plasma, through utilizing various systems for its generation, has been used for surface modification, water disinfection, and biomedical applications (Conrads and Schmidt 2000; Tendero *et al.* 2006; Moreau *et al.* 2008; Bárdos and Barankova 2010; Oehmigen *et al.* 2010). Dielectric barrier discharge (DBD) is one of the most convenient ways for plasma generation, and it provides an option for a broad range of applications and fundamental studies, through its flexibility of configuration with respect to electrode geometrical shape, the type of dielectric material employed, and the comparatively straightforward approach to scale-up to larger industrial installations (Kogelschatz 2000). The presence of oxygen in air leads to the formation of reactive oxygen species (ROS). ROS tend to react with each other as well as of oxygen molecules resulting in the formation of hydrogen peroxide, hydroxyl radical, superoxide, singlet oxygen, atomic oxygen and ozone (Schwabedissen *et al.* 2007; Klockow and Keener 2009; Oehmigen *et al.* 2010; Arjunan and Clyne 2011). The presence of nitrogen in air leads to generation of reactive nitrogen species (RNS) by plasma in gaseous/aqueous phase which are present in the form of peroxynitrite, nitric

 © 2012 The Society for Applied Microbiology

oxide and nitrite (Laroussi and Leipold 2004; Burlica *et al.* 2006; Tang *et al.* 2008; Arjunan and Clyne 2011; Du *et al.* 2012). These chemical reactive species, in turn, have the ability to inactivate microorganisms. The generation of these species depends on the critical control parameters such as gas pressure and composition, temperature, moisture and plasma excitation properties (Ehlbeck *et al.* 2011; Misra *et al.* 2011).

Mechanisms of bacterial inactivation by ACP are proposed in the literature (Gallagher *et al.* 2007; Cooper *et al.* 2010; Korachi *et al.* 2010; Fernandez and Thompson 2012; Frohling *et al.* 2012), but to date they are not entirely understood. However, the mechanism of microbial inactivation by molecular ozone and free radicals produced by its breakdown have been well studied, with the main modes of action at the bacterial cell wall and membrane disintegration causing surface lesions (Green *et al.* 2012). Plasma-generated free radicals can also be adsorbed on the surface of bacteria forming volatile compounds such as CO_2 which are then eliminated from the cells. Free radicals diffusing through the cell membrane may damage proteins and nucleic acids (Fernandez and Thompson 2012; Green *et al.* 2012).

The bactericidal effect of ACP has been investigated by many researchers. Several experimental parameters were demonstrated to be involved in the bactericidal efficiency of plasma such as the gap between the electrodes (Yun *et al.* 2010; Miao and Yun 2011), gas composition (Lee *et al.* 2011; Kim *et al.* 2011; Du *et al.* 2012), supply voltage (Yun *et al.* 2010), treatment time (Miao and Yun 2011), type of bacteria and initial bacterial population (Montie *et al.* 2000; Yu *et al.* 2006; Fernandez *et al.* 2012). However, to date, few studies have reported the efficacy of plasma treatment for microbiological control inside a sealed package (Schwabedissen *et al.* 2007; Eto *et al.* 2008; Klockow and Keener 2009; Chiper *et al.* 2011; Leipold *et al.* 2011; Rod *et al.* 2012). Moreover, by comparison with previous studies, the current study employs higher voltage and a large gap between electrodes in the system set-up. The aim of the present study was to investigate the in-package inactivation efficacy of direct and indirect dielectric barrier discharge atmospheric cold plasma (DBD-ACP) on *Escherichia coli* suspended in different media. Other parameters assessed in this study were post-treatment storage time and the rate of bacterial inactivation as a function of treatment time.

Materials and methods

DBD-ACP system set-up

The DBD-ACP device (Fig. 1) is a novel prototype atmospheric low temperature plasma generator. The system consists of a variable high voltage transformer with an input voltage of 230 V at 50 Hz and a maximum high voltage output of 60 kV at 50 Hz. The two 15-cm-diameter aluminium disc electrodes were separated by a polypropylene container which served both as a sample holder and as a dielectric barrier with wall thickness of 1·2 mm. The distance between the two electrodes was 22 mm, equal to the height of the container. Voltage was monitored using an InfiniVision 2000 X-Series Oscilloscope (Agilent Technologies Inc., Santa Clara, CA, USA). All experiments were performed at 40 kV peak to peak at ambient air and atmospheric pressure conditions.

Preparation of bacterial cell suspensions

Escherichia coli ATCC 25922 (generic strain) was obtained from the microbiology stock culture of the School of Food Science and Environmental Health of the Dublin Institute of Technology. Stock cultures of *E. coli* were maintained using protective beads (Technical Services Consultants Ltd, Lancashire, UK) at −70°C. One protective bead was used to inoculate tryptic soy agar (TSA; ScharlauChemie, Barcelona, Spain) plate and incubated at 37°C overnight. An isolated colony of *E. coli* ATCC 25922 was inoculated into 10-ml tubes containing tryptic

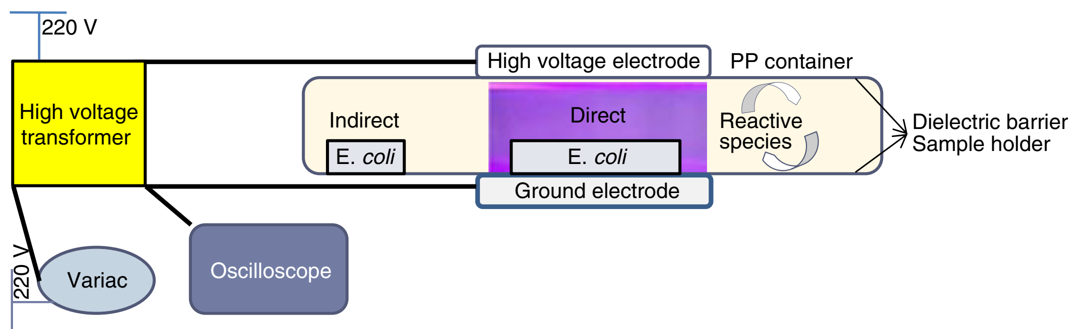

Figure 1 Dielectric barrier discharge atmospheric cold plasma generator set-up.

soy broth (TSB; ScharlauChemie) and incubated overnight at 37°C. The overnight culture (18 h) was harvested by centrifugation at 8720 ***g*** for 10 min. The cell pellet was washed thrice with sterile phosphate buffered saline (PBS; Oxoid Ltd, UK) and finally resuspended in either 10 ml of PBS or maximum recovery diluent (MRD). The bacterial density was determined by measuring absorbance at 550 nm using the McFarland standard (BioMérieux, Marcy-l'Etoile, France) to allow a working inoculum corresponding to $1{\cdot}0 \times 10^8$ CFU ml^{-1} to be prepared. For plasma treatment, cells were adjusted to a density of $1{\cdot}0 \times 10^7$ CFU ml^{-1} in PBS and MRD.

Experimental design

A working concentration (100 μl) of 1×10^7 CFU ml^{-1} *E. coli* ATCC 25922 in either MRD or PBS was dispensed into the wells of a microtitre plate. A row of either MRD or PBS without inoculum was used as controls, respectively. Microtitre plate with samples was placed in the centre of the rigid polypropylene plastic container directly between the electrodes within the plasma discharge for direct plasma treatment. For indirect plasma treatment, a separate container was used and microtitre plate was placed so as to achieve treatment outside the plasma discharge (Fig. 1). The distance between the sample and top electrode for direct treatment was 10 mm. For indirect treatment, the distance between the samples and centre of the electrodes varied from 120 to 160 mm owing to sample distribution in the microtitre plate. Each container was sealed with the high barrier polypropylene bag (B2630; Cryovac Sealed Air Ltd, Dunkan, SC, USA). Air was used as the working gas for the generation of plasma. Bacterial samples were then treated with plasma for 10, 20, 30, 45, 60 and 300 s at 40 kV peak-to-peak voltage. To assess the retention effect of plasma generated reactive species over time, samples were stored at room temperature for 0, 15, 30 and 60 min following 300 s of plasma treatment or 1 and 24 h after 60 s of treatment. Samples treated with shorter treatment intervals (10, 20, 30 and 45 s) were stored at room temperature for 24 h.

Microbiological analysis

The effect of the plasma treatment on the microbial load was determined in terms of reduction in viable counts. Plasma treated samples (of either PBS or MRD) were pooled together from the wells into sterile eppendorf tubes, serially diluted in MRD, and 0·1-ml aliquots of appropriate dilutions were surface plated on TSA. The plates were incubated at 37°C for 24 h. To detect a further possible increase in the formation of visible colonies, the plates were further incubated for 2–3 days. Results were presented as surviving bacterial population in $\log_{10}$ CFU ml^{-1} units. All experiments were carried out in duplicate and replicated at least twice.

Ozone, temperature and samples pH measurements

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

Thermal imaging was used to determine the temperature variations of the electrodes surface and the analysed samples. The temperature was measured before and after the 300 s of direct plasma treatment.

The pH of a series of uninoculated sample wells was monitored using a glass electrode pH-meter (420A; Orion Research Inc., Boston, MA, USA) prior to and after plasma treatment. Samples were pooled for analysis. To determine any effect of reduced pH on the microbial inactivation, experiments were conducted by inoculating *E. coli* (10^7 CFU ml^{-1}) in MRD with pH level (adjusted with 0·1 mol l^{-1} HCl) of 3·5, 4·5 and 5·5. Inoculated MRD samples were incubated at room temperature for 0, 6 and 24 h. Bacterial population incubated in MRD with pH ~ 7·0 was used as a control. Samples were serially diluted in MRD, and *E. coli* population densities were determined by surface-spreading on TSA.

Scanning electron microscopy

Bacterial samples treated in MRD with plasma for 30 s were assessed for cellular damage using scanning electron microscopy (SEM). Bacterial cells were prepared as described by Thanomsub *et al.* (2002) with minor modifications. Briefly, the samples were concentrated by centrifugation at 8720 ***g*** for 10 min. The cells were fixed in ice-cold 2·5% glutaraldehyde in 0·05 mol l^{-1} sodium cacodylate buffer (pH 7·4) (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 (50, 70, 80, 90, 95 and 99·5%) and freeze-dried (Labconco, FreeZone 6; Mason Technology, Dublin, Ireland). 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, OR, USA) at 5 kV.

Optical emission spectroscopy

Optical emission spectroscopy (OES) of the discharge within empty packages was acquired with a Stellarnet EPP 2000C-25 spectrometer with an optical fibre input. The fibre optic from the OE spectrometer was placed behind a quartz window incorporated into the centre of the side wall of the polypropylene container. The fibre had a numerical aperture of 0·22 and was optimized for use in the ultraviolet and visible portion of the spectrum with a wavelength range of 190–850 nm.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Data from microbiological evaluations, ozone concentration and pH analysis were subjected to analysis of variance (ANOVA). Means were compared using Fisher's least significant difference – LSD – at the 0·05 level.

Results

Effect of treatment time and post-treatment storage time on DBD-ACP inactivation efficiency

Survival curves of *E. coli* ATCC 25922 in MRD and PBS treated with plasma for 300 s followed by post-treatment storage for different time intervals are shown in Fig. 2. Direct plasma treatment of bacterial cells in MRD resulted in complete inactivation, irrespective of post-treatment storage time of 0–60 min (Fig. 2a). However, when the treatment was applied in PBS, direct exposure significantly reduced the population by 2 log cycles with no post-treatment storage time ($P \leq 0{\cdot}05$) and caused complete inactivation within post-treatment storage time of 15–60 min (Fig. 2a). Indirect plasma treatment of bacterial cells in MRD and PBS with no post-treatment storage (0 h) resulted in a reduction by 1 and 2 log cycles, respectively (Fig. 2b). Observations for indirect exposure after extending the post-treatment storage time to 15 min were similar to that of direct plasma exposure and post-treatment storage time of 0 min in MRD and PBS. Further increases in post-treatment storage time resulted in complete bacterial inactivation in both media. Thus, plasma inactivation efficiency was found to be dependent on the post-treatment storage time, which allowed for diffusion and action of the residual reactive species in liquids, regardless of the type of media used.

The effect of reducing treatment time to 60 s on DBD-ACP inactivation efficiency using two different post-treatment storage time intervals (1 and 24 h) is shown in Fig. 3. The influence of storage time on plasma efficiency

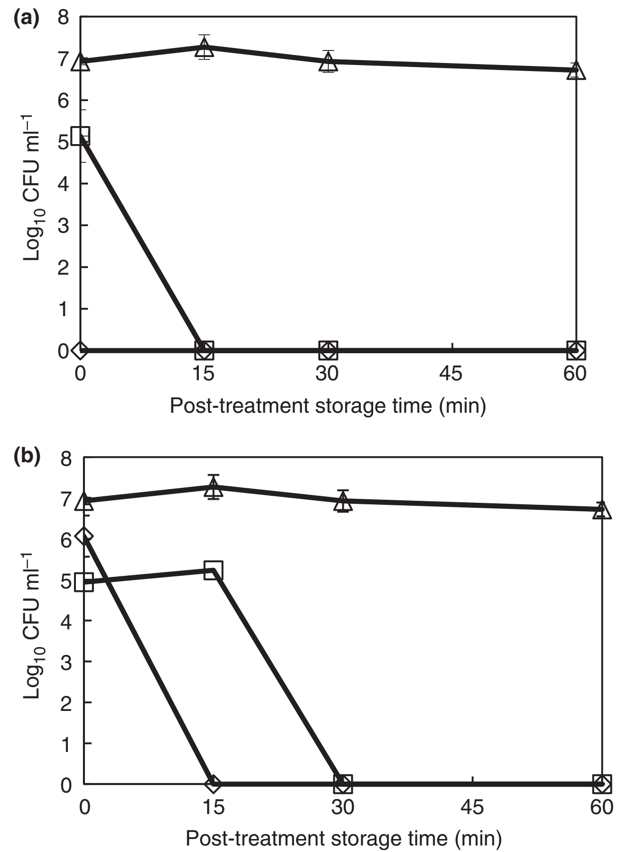

Figure 2 Effect of post-treatment (300 s) storage time on inactivation efficiency of *Escherichia coli* ATCC 25922. (a) Direct plasma exposure: (Δ) control; (◊) maximum recovery diluent (MRD); (□) phosphate buffered saline (PBS). (b) Indirect plasma exposure: (Δ) control; (◊) MRD; (□) PBS.

at shorter treatment duration of 60 s was clearly observed (Fig. 3a,b). *Escherichia coli* populations decreased by 5·2 and 6·5 log cycles in MRD and PBS, respectively, after 60 s of direct plasma treatment with 1 h of post-treatment storage. The media effect was evident in the case of indirect plasma treatment, where the bacterial population was reduced only by 1 log cycle in MRD and 6 log cycles in PBS with post-treatment storage for 1 h. Extending the post-treatment storage time to 24 h nullified any media effect, with complete inactivation recorded in both media, irrespective of the mode of plasma exposure (Fig. 3a,b). Considering these results, the effect of further reduced treatment time (<60 s) on plasma inactivation efficacy was assessed using only 24 h of post-treatment storage time.

Reduction in direct plasma treatment time to 20 s yielded complete inactivation in MRD. However, with indirect plasma treatment, a gradual reduction of *E. coli* population was observed in MRD with respect to the treatment time. A significant difference in bacterial

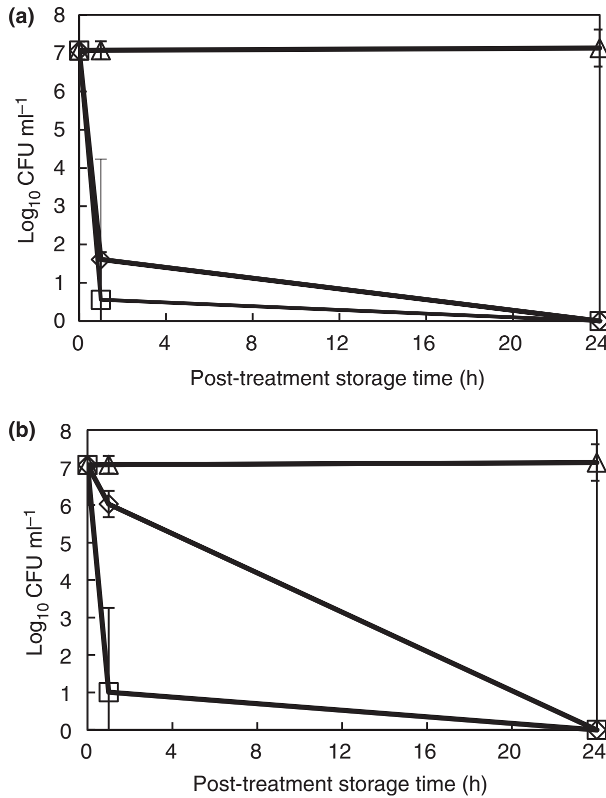

Figure 3 Effect of plasma treatment (60 s) and storage time (1 and 24 h) on inactivation efficiency of *Escherichia coli* ATCC 25922. (a) Direct plasma exposure: (Δ) control; (◊) maximum recovery diluent (MRD); (□) phosphate buffered saline (PBS). (b) Indirect plasma exposure: (Δ) control; (◊) MRD; (□) PBS.

reduction following indirect plasma treatment of 20 s to 45 s was noted ($P \leq 0{\cdot}05$). Complete inactivation in PBS was recorded, irrespective of the mode of exposure or treatment time (Fig. 4a,b).

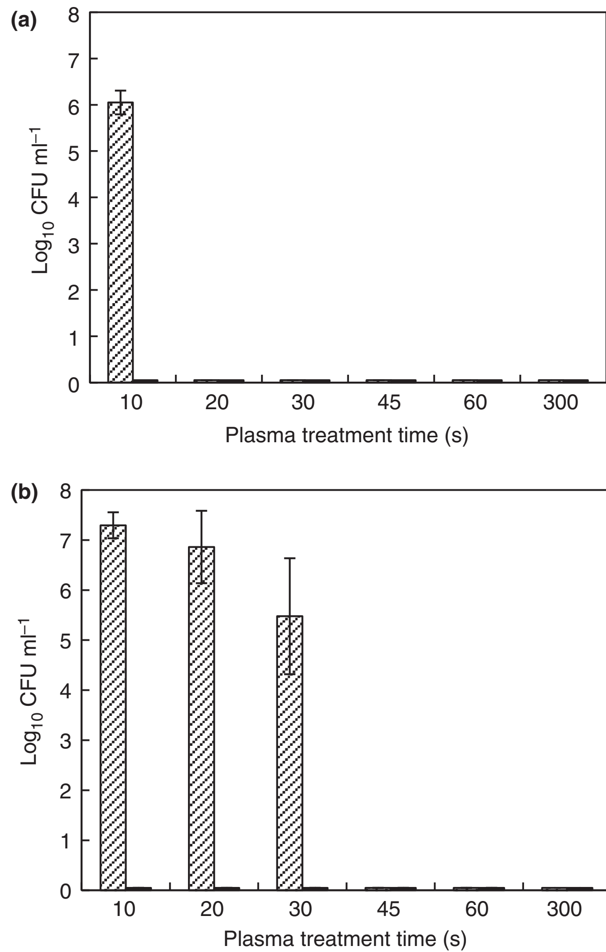

Figure 4 Effect of plasma treatment time (s) on inactivation efficiency of *Escherichia coli* ATCC 25922 (post-treatment storage time 24 h). (a) Direct plasma exposure: (■) phosphate buffered saline (PBS); (⁄) maximum recovery diluent (MRD). (b) Indirect plasma exposure: (■) PBS; (⁄) MRD.

Effect of ozone concentration, temperature and samples pH

Ozone

The concentration of ozone was related to the plasma treatment time studied (Table 1). Thus, immediately after 300 s of direct plasma treatment, an ozone concentration of approximately 4000 ppm was found, with 530 ppm detected when 10 s of plasma treatment was applied ($P \leq 0{\cdot}05$). However, in this study, no significant difference was observed between ozone concentrations generated after 300 or 60 s of either direct or indirect plasma treatment.

Temperature

The change in the temperature of the electrodes and the bacterial samples were measured using thermal imaging

Table 1 Effect of atmospheric cold plasma (ACP) treatment times on maximum recovery diluent (MRD) pH

	MRD pH	
ACP treatment time (s)	Direct ACP	Indirect ACP
10	$4{\cdot}2^{a}$	$4{\cdot}5^{a}$
20	$3{\cdot}8^{b}$	$3{\cdot}9^{b}$
30	$3{\cdot}7^{c}$	$3{\cdot}9^{b}$
45	$3{\cdot}4^{d}$	$3{\cdot}4^{c}$
60	$3{\cdot}3^{d}$	$3{\cdot}4^{c}$
300	$2{\cdot}4^{e}$	$2{\cdot}9^{d}$

Different letters in each column indicate a significant difference in pH.

camera (Hotfind LT, Dublin, Ireland). The maximum recorded temperature of the electrodes and the samples after extended plasma treatment to 15 min with 40 kV was 36 and 20°C, respectively.

Effect of pH

A considerable decrease in MRD pH was observed after plasma treatment with post-treatment storage of 24 h (Table 2). Direct and indirect plasma treatment of 60 s followed by 1 h of post-treatment storage reduced the MRD pH from 6·9 to 4·1 and 4·5, respectively. Direct plasma treatment time of 300 s followed by 1 h of post-treatment storage reduced the MRD pH to 3·5. In contrast, insignificant decreases in pH levels in PBS were observed after extended plasma treatment (data not shown). A separate set of experiments was conducted to investigate the effects of low pH, which indicated no significant effect of lower pH values on microbial inactivation.

Scanning electron microscopy

SEM images of DBD-ACP-treated and DBD-ACP-untreated *E. coli* cells are shown in Fig. 5. Smooth bacterial cells were observed in *E. coli* samples before plasma treatment (Fig. 5a). Following 30 s of either direct or indirect plasma treatment (Fig. 5b,c, respectively), the morphology of cells was changed compared to the untreated cells. Most of the *E. coli* cells exhibited surface roughness, indentations and small fractions of cells have either been deformed (arrows 1) or developed holes on their surface (arrows 2). Overall, DBD-ACP-treated cells appeared to be shrunken and dehydrated.

Optical emission spectroscopy

Emission spectrum of the filamentary discharge in air at 40 kV over the range of 180–900 nm is presented in Fig. 6. Most of the distinct peaks obtained in the near UV region corresponded to strong emissions from N_2 and N_2^+ excited species which was previously reported by Machala *et al.* (2007). However, the major peaks of active oxygen could not be identified on the light emission spectra, probably because active oxygen with a relatively

Table 2 Atmospheric cold plasma (ACP) treatment times and corresponding concentrations of ozone

	Ozone concentration (ppm)	
ACP treatment time (s)	Direct ACP	Indirect ACP
10	530[a]	1200[a]
20	1770[b]	2800[b]
30	2200[b]	2300[ab]
45	1870[b]	3200[bc]
60	4070[c]	4400[cd]
300	4100[c]	5200[d]

Different letters in each column indicate a significant difference in ozone concentrations.

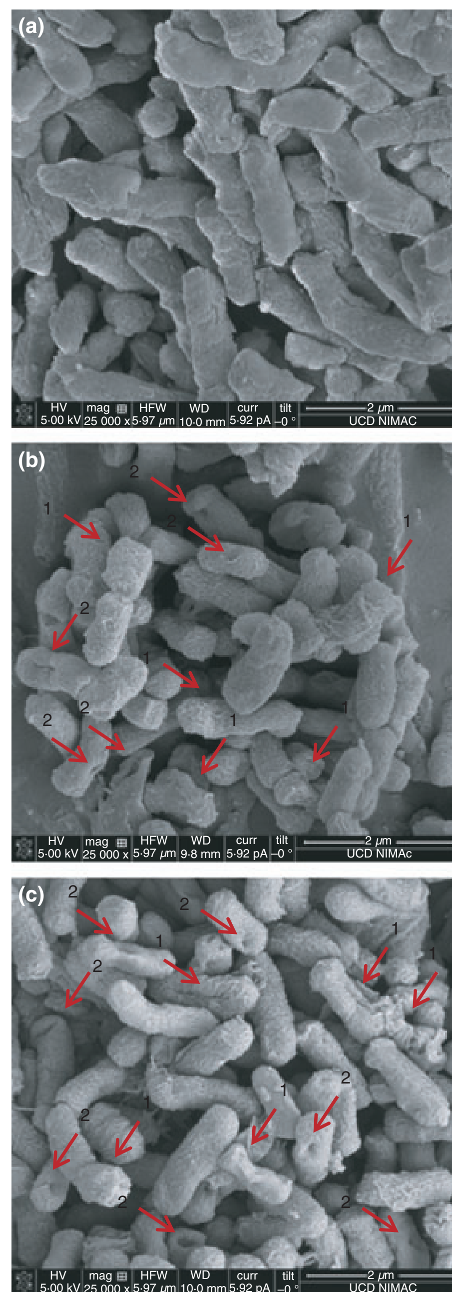

Figure 5 Scanning electron microscopy images of *Escherichia coli* ATCC 25922 treated with dielectric barrier discharge atmospheric cold plasma for 30 s and stored for 24 h: (a) untreated and after (b) direct plasma treatment and (c) indirect plasma treatment.

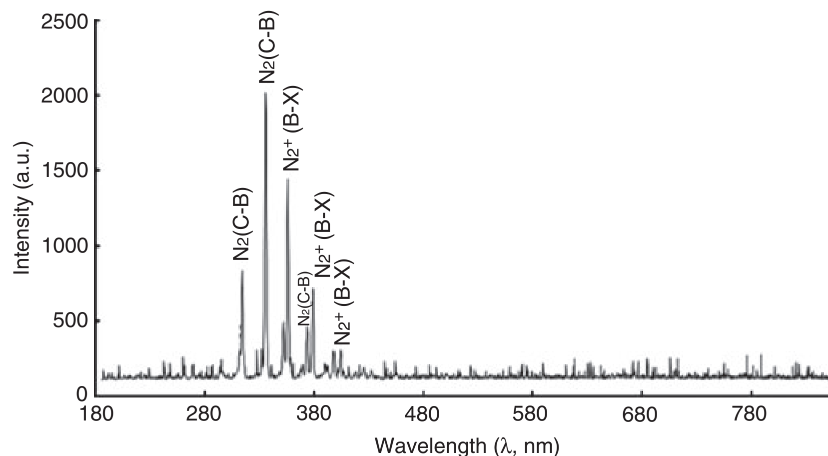

Figure 6 Emission spectrum of dielectric barrier discharge atmospheric cold plasma operating in air under atmospheric pressure.

long half-life tends to lose energy because of particle collisions, quenching its energy before detectable light emissions.

Discussion

DBD-ACP treatment was effective for the inactivation of *E. coli* in liquids. In some cases, the extrinsic parameters studied, such as treatment time, post-treatment storage time, position of the samples with respect to the plasma discharge and the liquid media used, had a significant influence on plasma inactivation efficacy. Generally, the use of direct plasma treatment was more effective for bacterial inactivation than indirect. The possible explanation for this effect could be that the samples were located directly between the electrodes under plasma discharge and were thus exposed to all generated reactive species, such as charged particles, positive and negative ions, electrons, free radicals, excited and nonexcited molecules and atoms, heat and UV photons. In contrast, when the samples were exposed to indirect plasma treatment, that is, at some distance from plasma discharge, the charged particles did not affect the sample directly during treatment. This could be attributed to the charged particles recombining before reaching the sample; therefore, only long-lived radicals had an effect on the biological sample (Laroussi 2009). However, indirect plasma treatment has also achieved effective bacterial inactivation levels. Rod *et al.* (2012), utilizing similar DBD plasma device and indirect treatment of ready to eat sliced meat, demonstrated 1·6 log bacterial reductions. Moreover, the use of indirect exposure of plasma discharge may prove beneficial for the adoption of this technology to the widest range of a possible uses in the food industry where a balance between maintaining quality characteristics of sensitive fruits and vegetables and microbial decontamination is required.

Consistent microbial inactivation was achieved with 300 and 60 s of treatment time with either direct or indirect plasma modes of exposure. However, interactive effects of post-treatment storage time and liquid media were noted.

Post-treatment storage time emerged as a critical treatment parameter for consistency of bacterial inactivation with this system. The interactions between plasma and bacteria suspended in liquids have been reported in a number of studies (Tang *et al.* 2008; Ikawa *et al.* 2010; Julak *et al.* 2012). This complex interaction is generally based on the diffusion of plasma-generated reactive species into the liquids (Oehmigen *et al.* 2010). Plasma generated from air is characterized by various chemically reactive species, with the main role given to ozone as the most long-living and most oxidative species (Klockow and Keener 2009). Gaseous ozone has high penetrability and after contact with liquids forms residual ozone and continues to effect microbial cells (Mukhopadhyay and Ramaswamy 2012). In this study, the diffusion of reactive species into liquids during post-treatment storage and the influence of this diffusion on DBD-ACP bactericidal efficacy were examined. Complete inactivation of bacterial cells during the prolonged post-treatment storage time (Fig. 2a,b) demonstrated that the storage of samples facilitated diffusion of the generated species into the liquids with accompanying antimicrobial action on the cells. The half-life of the species generated will govern whether that species will have sufficient time for diffusion to occur. Species with very short half-lives will not have sufficient time for diffusion; consequently, it is likely that the inactivation observed in this case is owing to the relatively long-lived species.

A reduced treatment time (60 s) in conjunction with 24 h of post-treatment storage time also showed an enhanced plasma inactivation effect (Fig. 3a,b), indicating the penetration of available reactive species into the

samples and leading to complete bacterial inactivation. Although significant bacterial reductions were also achieved with 60 s of direct treatment time and 1 h of post-treatment storage, large variations in the results were noted. Possibly, the composition of the chemically reactive species generated and/or their concentrations within the container are not identical at each plasma application, which may be more apparent when short treatments are applied in conjunction with a short post-treatment storage time. The concentration of ozone was monitored at all stages of sampling in this study, and it was noted that the ozone concentrations after either direct or indirect plasma treatment for 300 and 60 s did not differ significantly. The reliability of inactivation efficacy achieved with 24-h post-treatment storage is a useful observation for practical application to products or systems.

It is probable that a range of other antimicrobial species in addition to ozone may also influence plasma inactivation efficiency. Previously, RNS were reported to be mainly responsible for the formation of nitrates and nitrites in the liquid media by reacting with media components, such as organic acids and proteins (Fernandez and Thompson 2012), thus changing the media pH towards acidic range, which enhanced plasma bactericidal efficacy (Burlica *et al.* 2006; Ikawa *et al.* 2010; Liu *et al.* 2010). This effect of reduced pH was further investigated by Oehmigen *et al.* (2010), which experimentally confirmed the role of RNS in liquid acidification by ACP and clearly showed media pH dependent reductions of the number of viable microorganisms. Plasma-generated hydrogen peroxide in liquids was also reported to have an inhibitory effect on the bacterial cells (Joshi *et al.* 2011). The questions remain that with the wide range of potential reactive species that can result with varying plasma generation conditions whether they have differing intensity of antimicrobial effect, varying roles in the mode of efficacy or indeed if the effects cannot be separated and are complimentary to antimicrobial efficacy. These are the key areas for future study to ensure that the extrinsic parameters for plasma generation conditions can be optimally applied for product decontamination and antimicrobial efficacy.

To investigate the complexity that might be present in a range of products (e. g., organic acids, proteins, amino acids, which can interact with plasma reactive species) to which this type of nonthermal technology might be applied, a preliminary study, using two simple media PBS and MRD, was warranted. A strong effect of media composition which was also related to the mode of plasma exposure was noted at reduced plasma treatment times, where only 24 h of post-treatment storage was applied. Direct plasma treatment of 20 s provided complete elimination of bacteria in both media. However, with indirect plasma treatment below 45 s, the effects of media type became apparent: as treatment time was reduced from 30 to 20 s, the inactivation efficacy decreased, and no reductions in the number of viable cells were observed after 10 s of indirect plasma treatment in MRD. However, in PBS which has a simpler composition than MRD, complete bacterial inactivation using either direct or indirect plasma treatment was recorded at all of the treatment times studied (Fig. 4a,b). Conversely, for samples exposed to longer treatment time (300 s), greater antimicrobial efficiency was recorded in MRD by comparison with PBS.

A possible explanation for these trends in inactivation efficacy with respect to media composition could include effects of pH and the type and concentration of active species present at the same treatment conditions in the different media. With respect to the possible effect of pH, inactivation was correlated with a pH decrease. Additionally, the results obtained from OES point to the fact that air DBD-ACP is a significant source of RNS and this in turn could cause acidification of MRD. MRD is mainly composed of sodium chloride and low concentrations of peptone. Although the concentration of peptone is low, it may interact with plasma generated RNS, resulting in a significant reduction of MRD pH, thereby enhancing plasma inactivation efficiency in the case of extended treatment time (300 s). When samples were exposed to shorter treatment times (<60 s), the MRD pH was less affected. The relationship between treatment time and resulting sample pH on inactivation efficacy may be closely linked in the case of more complex media and should be carefully considered in any application study. When the effect of acidic pH on survival of *E. coli* was assessed independently of plasma treatment, it showed no significant effect on the rate of bacterial inactivation. This confirmed that bacterial inactivation was a result of DBD-ACP exposure, and it may be due to a possible synergistic effect of an acidic MRD environment in combination with plasma-generated reactive species. In contrast, PBS is a buffered solution mainly composed of sodium chloride and phosphates where pH changes are minimal in response to addition of either strong acid or base. The DBD-ACP bactericidal effect in PBS at very low treatment times might be due to specific reactions occurring between the plasma species and sodium chloride, as a main constituent of PBS, altering it to a more bactericidal structure. Recent investigations of ozonated saline solution for medicinal uses, which is relatively close to the chemical composition of PBS, pointed out that in the presence of ozone, hypochlorous acid, even if in trace amounts, will also be generated. Hypochlorous acid is known as an excellent bactericidal compound which can exert a deleterious reactivity with protein –SH groups, amino groups, with DNA, RNA and lipids (Bocci *et al.* 2011).

To further our understanding of the inactivation mechanism, SEM analysis was performed on *E. coli* cells treated directly/indirectly for 30 s in MRD and subsequently stored for 24 h prior to SEM preparations. SEM images demonstrated that plasma-treated cell surface were altered or dehydrated as cells exhibited a shrunken morphology, but visually no cell lysis was observed. It was previously reported that ozone-treated *E. coli* cells remain intact and cell lysis is not the major mechanism of bacterial inactivation (Patil *et al.* 2011). The possible bactericidal action of plasma is more likely based on the diffusion of plasma-generated reactive species through the cell membrane into the cell where they react and possibly damage proteins and nucleic acids leading to cell death. However, further studies are needed to get clearer insights into mode of action of ACP, the effects of ACP generated species on cellular components, and the chemistry involved in liquids mediated by those species.

The inactivation efficiency of the DBD-ACP system used in this study was affected by the mode of exposure, treatment time, post-treatment storage time and media composition. There were also interactive effects between these parameters; however, 7 log reduction of *E. coli* population was recorded in the more complex media with direct plasma exposure for 20 s in conjunction with a post-treatment storage time of 24 h. In conclusion, despite the varying parameters that influenced plasma bactericidal activity, the novel in-package DBD-ACP technology showed an efficient inactivation of high concentrations of *E. coli* in liquids under both direct and indirect treatment modes.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/2207-2013) under grant agreement number 285820. The authors wish to acknowledge Jack Connolly, National Centre for Plasma Science and Technology, for his assistance with the OES.

References

Arjunan, K.P. and Clyne, A.M. (2011) Hydroxyl radical and hydrogen peroxide are primarily responsible for dielectric barrier discharge plasma induced angiogenesis. *Plasma Process Polym* **8**, 1154–1164.

Bárdos, L. and Barankova, H. (2010) Cold atmospheric plasma: sources, processes, and applications. *Thin Solid Films* **518**, 6705–6713.

Bocci, V., Zanardi, I. and Travagli, V. (2011) Oxygen/ozone as a medical gas mixture. A critical evaluation of the various methods clarifies positive and negative aspects. *Med Gas Res* **1**, 6.

Burlica, R., Kirkpatrick, M.J. and Locke, B.R. (2006) Formation of reactive species in gliding arc discharges with liquid water. *J Electrostat* **64**, 35–43.

Chiper, A.S., Chen, W., Mejlholm, O., Dalgaard, P. and Stamate, E. (2011) Atmospheric pressure plasma produced inside a closed package by dielectric barrier discharge in Ar/CO2 for bacterial inactivation of biological samples. *Plasma Sources Sci Technol* **20**, 10.

Chiang, M.H., Wu, J.Y., Li, Y.H., Wu, S., Chen, S.H. and Chang, C.L. (2010) Inactivation of *E. coli* and *B. subtilis* by a parallel-plate dielectric barrier discharge jet. *Surf Coat Technol* **204**, 3729–3737.

Conrads, H. and Schmidt, M. (2000) Plasma generation and plasma sources. *Plasma Sources Sci Technol* **9**, 441–454.

Cooper, M., Fridman, G., Fridman, A. and Joshi, S.G. (2010) Biological responses of *Bacillus stratosphericus* to floating electrode-dielectric barrier discharge plasma treatment. *J Appl Microbiol* **109**, 2039–2048.

Du, C.M., Wang, J., Zhang, L., Li, H.X., Liu, H. and Xiong, Y. (2012) The application of a non-thermal plasma generated by gas – liquid gliding arc discharge in sterilization. *New J Phys* **14**, 013010. doi: 10.1088/1367-2630/14/1/013010.

Ehlbeck, J., Schnabel, U., Polak, M., Winter, J., Woedtke, T., Brandenburg, R., Hagen, T. and Weltmann, K.D. (2011) Low temperature atmospheric pressure plasma sources for microbial decontamination. *J Phys D: Appl Phys* **44**, 013002. doi: 10.1088/0022-3727/44/1/013002

Eto, H., Ono, Y., Ogino, A. and Nagatsu, M. (2008) Low-temperature sterilisation of wrapped materials using flexible sheet-type dielectric barrier discharge. *Appl Phys Lett* **93**, 221502. doi: 10.1063/1.3039808

Fernandez, A. and Thompson, A. (2012) The inactivation of *Salmonella* by cold atmospheric plasma treatment. *Food Res Int* **45**, 678–684.

Fernandez, A., Shearer, N., Wilson, D.R. and Thompson, A. (2012) Effect of microbial loading on the efficiency of cold atmospheric gas plasma inactivation of *Salmonella entericaserovar Typhimurium*. *Int J Food Microbiol* **152**, 175–180.

Frohling, A., Baier, M., Ehlbeck, J., Knorr, D. and Schluter, O. (2012) Atmospheric pressure plasma treatment of *Listeria innocua* and *Escherichia coli* at polysaccharide surfaces: inactivation kinetics and flow cytometric characterisation. *Innov Food Sci Emerg Technol* **13**, 142–150.

Gallagher, M.J., Vaze, J.N., Gangoli, S., Vasilets, V.N., Gustol, A.F., Milovanova, T.N., Anandan, S., Murasko, D.M. *et al.* (2007) Rapid inactivation of airborne bacteria using atmospheric pressure dielectric barrier grating discharge. *IEEE Trans Plasma Sci* **35**, 1501–1510.

Green, A. K., Guzel-Seydim, Z. B. and Seydim, A. C. (2012) Chemical and physical properties of ozone. In *Ozone in Food Processing* ed. O'Donnel, C., Tiwary, B. K., Cullen, P. J. and Rice, R. G. pp. 26–28. UK: Blackwell Publishing Ltd.

Ikawa, S., Kitano, K. and Hamaguchi, S. (2010) Effect of pH on bacterial inactivation in aqueous solutions due to

low-temperature atmospheric pressure plasma application. *Plasma Processes Polym* **7**, 33–42.

Joshi, S.G., Cooper, M., Yost, A., Paff, M., Ercan, U.K., Fridman, G., Fridman, G., Fridman, A. *et al.* (2011) Nonthermal dielectric-barrier discharge plasma-induced inactivation involves oxidative DNA damage and membrane lipid peroxidation in *Escherichia coli*. *Antimicrob Agents Chemother* **55**, 1053–1062.

Julak, J., Scholtz, V., Kotucova, S. and Janouskova, O. (2012) The persistent microbiocidal effect in water exposed to the corona discharge. *Phy Med* **28**, 230–239.

Kim, B., Yun, H., Jung, S., Jung, Y., Jung, H., Choe, W. and Jo, C. (2011) Effect of atmospheric pressure plasma on activation of pathogens inoculated onto bacon using two different gas compositions. *Food Microbiol* **28**, 9–13.

Klockow, P.A. and Keener, K.M. (2009) Safety and quality assessment of packaged spinach treated with a novel ozone-generation system. *LWT – Food Sci Technol* **42**, 1047–1053.

Kogelschatz, U. (2000) Fundamentals and applications of dielectric barrier discharges. Available at: http://www.coronalab.net/wxzl/plasma-16.pdf. [Accessed 12 March 2012].

Korachi, M., Gurol, C. and Aslan, N. (2010) Atmospheric plasma discharge sterilization effects on whole cell fatty acids profile of *Escherichia coli* and *Staphylococcus aureus*. *J Electrostat* **68**, 508–512.

Laroussi, M. (2009) Low-temperature plasmas for medicine? Plasma Science. *IEEE Trans Plasma Sci* **37**, 714–725.

Laroussi, M. and Leipold, F. (2004) Evaluation of the roles of reactive species, heat, and UV radiation in the inactivation of bacterial cells by air plasmas at atmospheric pressure. *Int J Mass Spectrom* **233**, 81–86.

Lee, H.J., Jung, H., Choe, W., Ham, J.S., Lee, J.H. and Jo, C. (2011) Inactivation of *Listeria monocytogenes* on agar and processed meat surfaces by atmospheric pressure plasma jets. *Food Microbiol* **28**, 1468–1471.

Leipold, F., Schultz-Jensen, N., Kusano, Y., Bindslev, H. and Jacobsen, T. (2011) Decontamination of objects in a sealed container by means of atmospheric pressure plasmas. *Food Control* **22**, 1296–1301.

Liu, F., Sun, P., Bai, N., Tia, Y., Zhou, H., Wei, S., Zhou, Y., Zhang, J. *et al.* (2010) Inactivation of bacteria in an aqueous environment by a direct-current, cold-atmospheric pressure air plasma microjet. *Plasma Process Polym* **7**, 231–236.

Machala, Z., Janda, M., Hensel, K., Jedlovsky, I., Lestinska, L., Foltin, V., Martisovits, V. and Morvova, M. (2007) Emission spectroscopy of atmospheric pressure plasmas for bio-medical and environmental applications. *J Mol Spectrosc* **243**, 194–201.

Miao, H. and Yun, G. (2011) The sterilisation of *Escherichia coli* by dielectric-barrier discharge plasma at atmospheric pressure. *Appl Surf Sci* **257**, 7056–7070.

Misra, N.N., Tiwari, B.K., Raghavarao, K.S.M.S. and Cullen, P. J. (2011) Nonthermal plasma inactivation of food-borne pathogens. *Food Eng Rev* **3**, 159–170.

Montie, T.C., Kelly-Wintenberg, K. and Roth, J.R. (2000) An overview of research using the one atmosphere uniform glow discharge plasma (OAUGDP) for sterilisation of surfaces and materials. *IEEE Trans Plasma Sci* **28**, 1.

Moreau, M., Orange, N. and Feuilloley, M.G.J. (2008) Non-thermal plasma technologies: new tools for bio-decontamination. *Biotechnol Adv* **26**, 610–617.

Mukhopadhyay, S. and Ramaswamy, R. (2012) Application of emerging technologies to control *Salmonella* in foods: a review. *Food Res Int* **45**, 666–677.

Oehmigen, K., Hähnel, M., Brandenburg, R., Wilke, Ch., Weltmann, K.D. and Woedtke, T. (2010) The role of acidification for antimicrobial activity of atmospheric pressure plasma in liquids. *Plasma Processes Polym* **7**, 250–257.

Patil, S., Valdramidis, V.P., Karatzas, K.A.G., Cullen, P.J. and Bourke, P. (2011) Assessing the microbial oxidative stress mechanism of ozone treatment through the responses of *Escherichia coli mutants*. *J Appl Microbiol* **111**, 136–144.

Rod, S.K., Hansen, F., Leipold, F. and Knochel, S. (2012) Cold atmospheric pressure plasma treatment of ready-to-eat meat: inactivation of *Listeria innocua* and changes in productquality. *Food Microbiol* **30**, 1–6.

Schwabedissen, A., Lacinski, P., Chen, X. and Engemann, J. (2007) PlasmaLabel – a new method to disinfect goods inside a closed package using dielectric barrier discharges. *Contrib Plasma Phys* **47**, 551–558.

Song, H.P., Kim, B., Choe, J.H., Jung, S., Moon, S.S., Choe, W. and Jo, C. (2009) Evaluation of atmospheric pressure plasma to improve the safety of sliced cheese and ham inoculated by 3-strain cocktail *Listeria monocytogenes*. *Food Microbiol* **26**, 432–436.

Tang, Y.Z., Lu, X.P., Laroussi, M. and Dobbs, F.C. (2008) Subletal and killing effects of atmospheric-pressure, non-thermal plasma on eukaryotic microalgae in aqueous media. *Plasma Process Polym* **5**, 552–558.

Tendero, C., Tixier, C., Tristant, P., Desmaison, J. and Leprince, P. (2006) Atmospheric pressure plasmas: a review. *Spectrochim Acta* **61**, 2–30.

Thanomsub, B., Anupunpisit, V., Chanphetch, S., Watcharachaipong, T., Poonkhum, R. and Srisukont, C. (2002) Effects of ozone treatment on cell growth and ultrastructural changes in bacteria. *J Gen Appl Microbiol* **48**, 193–199.

Yu, H., Perni, S., Shi, J.J., Wang, D.Z., Kong, M.G. and Shama, G. (2006) Effects of cell surface loading and phase of growth in cold atmospheric gas plasma inactivation of *Escherichia coli* K12. *J Appl Microbiol* **101**, 1323–1330.

Yun, H., Kim, B., Jung, S., Kruk, Z.A., Kim, D.B., Choe, W. and Jo, C. (2010) Inactivation of *Listeria monocytogenes* inoculated on disposable plastic tray, aluminium foil, and paper cup by atmospheric pressure plasma. *Food Control* **21**, 1182–1186.