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Investigation of a Large Gap Cold Plasma Reactor for Continuous In-package Decontamination of Fresh Strawberries and Spinach

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Investigation of a large gap cold plasma reactor for continuous in-package decontamination of fresh strawberries and spinach

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ABSTRACT

The aim of this work was to investigate the efficacy of a large gap atmospheric cold plasma (ACP) generated with an open-air high-voltage dielectric barrier discharge (DBD) pilot-scale reactor, operated in either static (batch) or continuous mode for produce decontamination and quality retention. Significant reductions in the bacterial populations inoculated on the strawberries and spinach were obtained after the static mode of ACP treatment with 2.0 and 2.2 $\log_{10}$ CFU/ml reductions for *E. coli* and 1.3 and 1.7 $\log_{10}$ CFU/ml reductions for *L. innocua*, respectively. Continuous treatment was effective against *L. innocua* inoculated on strawberries, with 3.8 $\log_{10}$ CFU/ml reductions achieved. No significant differences in colour, firmness, pH or total soluble solids (TSS) was observed between control and ACP-treated samples with the effects of treatment retained during the shelf-life period. The pilot-scale atmospheric air plasma reactor retained the strawberry quality characteristics in tandem with useful antimicrobial efficacy.

Industrial relevance: This in-package plasma technology approach is a low-power, water-free, non-thermal, post-package treatment. Generating cold plasma discharges inside food packages achieved useful antimicrobial effects on fresh produce. Depending on the bacterial type, produce and mode of ACP treatment significant reductions in the populations of pathogenic microorganisms attached to the fresh produce was achieved within 2.5 min of treatment. The principal technical advantages include contaminant control, quality retention, mitigation of re-contamination and crucially the retention of bactericidal reactive gas molecules in the food package volume, which then revert back to the original gas.

1. Introduction

Fresh produce such as spinach, lettuce, radish, alfalfa sprouts, tomatoes, peppers, cantaloupe and strawberries have been implicated in human health outbreaks caused by contamination with *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes* (EFSA & ECDC, 2018; Olaimat & Holley, 2012). *E. coli* is a Gram-negative, short rod-shaped bacteria, which is the most common facultative anaerobe found in the gastrointestinal tract of humans and other mammals and warm-blooded animals (McClure, 2005). Enterohemorrhagic *E. coli* O157:H7 is a major foodborne pathogen responsible for the severe illnesses in humans (Lim, Yoon, & Hovde, 2010). In 2017, in the EU, *E. coli* (STEC) resulted in 6073 confirmed cases of infections and in 20 deaths (EFSA & ECDC, 2018). *L. monocytogenes* is a Gram-positive rod-shaped, facultative anaerobe, which is the agent of the disease listeriosis (Stefanovic, Reid, Nadon, & Grant, 2010). Deterioration of fresh produce as a consequence of microbiological spoilage may also constitute a hazard for consumers through the possible presence of microbial (myco)toxins (Rawat, 2015). The causative agents of microbiological spoilage in fruits and vegetables are highly variable. A range of environmental factors, such as storage temperature and produce pH, will influence microbial community diversity and microbial resistance. These can impact the efficacy of decontamination procedures, and as a consequence the microbiological quality and stability of the produce with respect to shelf-life

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(Gallagher & Mahajan, 2011; Leff & Fierer, 2013). Other factors, which enhance the propagation of pathogenic and spoilage microorganisms on fresh produce include a high water content, damage during harvesting, transport and type of processing (Spadaro & Gullino, 2004). Current trends of reduced use of agrochemicals may also lead to an increase in the numbers of pathogenic fungi present on fresh produce, therefore, leading to increased mycotoxin production (Van Boxstael et al., 2013). Therefore, the use of optimised minimal processing technology is necessary in order to retain nutritional quality as well as to maintain microbiological safety of perishable high-value produce.

Atmospheric cold plasma (ACP) has demonstrated a high potential for the reduction of microbial loads on fresh fruits and vegetables with good retention of produce quality attributes. ACP has a non-uniform distribution of energy among the constituent species, where multiple chemical reactions and reactive species are generated (Niemira, 2012; Scholtz, Pazlarová, Soušková, Khun, & Julák, 2015). The major reactive agents that play a role in inactivation of microbial targets, independently or in synergy, include reactive oxygen species (ROS) (singlet oxygen, superoxide anion, ozone) and reactive nitrogen species (RNS) (atomic nitrogen, excited nitrogen, nitric oxide); if humidity is present, hydroxyl anions and radicals or hydrogen peroxide are also generated (Scholtz et al., 2015). The composition and abundance of chemical species is often determined by the source used for generation of the plasma. The commonly used forms of ACP in terms of a high potential for industrial applications are dielectric barrier discharge (DBD) and plasma jets. The major advantages of the DBDs include the ease of the discharge ignition and adaptability to suit various produce commodities and the possibility of treatment of produce inside sealed-packaging material where the elimination of post-processing produce contamination can be achieved. Our previous research generated cold plasma discharges inside food packages at bench-scale, leading to rapid inactivation (within 2 min) of foodborne pathogens on the surface of different types of produce (Ziuzina, Patil, Cullen, Keener, & Bourke, 2014) and against bacterial biofilms and associated cells internalised in fresh produce tissue (Ziuzina, Han, Cullen, & Bourke, 2015). Furthermore, sporicidal effects were demonstrated (Los et al., 2018; Patil et al., 2014). Misra et al. (2014) reported a 2-$\log_{10}$ CFU/g reduction of natural microbiota of produce, which was achieved while retaining produce quality. However, to date, a single package unit approach was used to characterise the efficacy of the post- and in-package plasma process and the evidence on the use of ACP for larger-scale operation remains limited.

The aim of this work was to evaluate the effects of the pilot-scale SAFEBAG in package plasma system under conditions representative of industrial practices. The system was tested under static operational mode – to represent packages of fresh produce at the post-sealing stage of a production line, as well as under continuous operational mode – to represent processing of larger numbers of packaged fresh produce. The target microorganisms selected were *E. coli* and *L. innocua* and the produce was strawberries and spinach leaves. The effects of treatment on the quality and nutritional profiles, including colour, firmness, pH, total soluble solids (TSS) and changes in the sample metabolites were evaluated. The aims of enhanced microbiological safety and extension of produce shelf-life without compromising product quality remained critical to the study design. Therefore, the impact of treatment on total microbiota and quality parameters of un-inoculated strawberry samples was studied for immediate and retained effects of treatment during a storage study.

2. Materials and methods

2.1. Plasma system description

The prototype SAFEBAG system is depicted on Fig. 1. The system employed a DBD reactor operating in open air. It consists of two parallel 1 m-long electrodes providing space for an adjustable discharge gap (up to 4.5 cm) allowing for several flexible packages (depending on the package size) to be treated simultaneously in static or continuous mode (Fig. 2), i.e., when a conveyor belt carries the bags through the plasma discharge. The control panel allows for control of applied voltage (0–100 kV) and is provided with an interactive meter displaying readings of input voltage (RMS) and discharge current. A Bergoz current probe placed in the circuit allows for readings of discharge current waveforms via connection of an oscilloscope to the BNC socket.

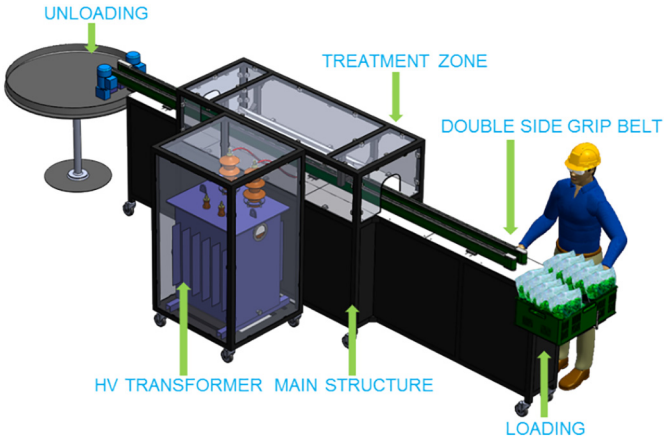

Fig. 1. The SAFEBAG Prototype plasma system and system's main components.

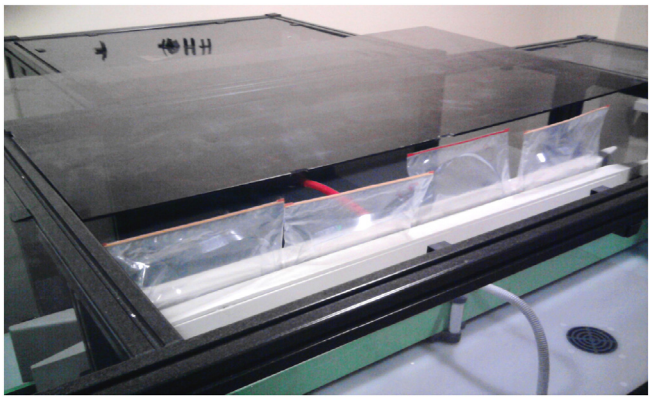

Fig. 2. Prototype SAFEBAG system treats several flexible packages simultaneously, in static mode or continuously.

Two side-grip belts displace sealed bags filled with fresh-cut produce into the treatment zone, where they pass through the discharge gap. Use of high voltages facilitates excitation and ionisation of the gas contained within the package, resulting in the generation of significant amounts of reactive oxygen and nitrogen species (RONS) that convey a bactericidal effect on the fresh product. The speed of the belts can control the duration of the treatment, after which the bags are released to the unloading platform. Several operation parameters characterized the input and output performance of the plasma system; maximum input voltage of 233 V (rms), maximum peak applied voltage of 115 kV, maximum consumed power (rms average) of 900 W, minimum discharge gap of 1 cm, discharge current (rms average) of 2.2–5.0 A and an expected ozone concentration range (for air RH = 50% in 3-l volume packages at 2.5-min plasma exposure) of 1600–2000 ppm (by volume).

2.2. Plasma diagnostics

2.2.1. Electrical and optical measurements

The current waveforms were measured using the Bergoz probe available on the SAFEBAG Prototype and connected to an oscilloscope (Agilent Infinivision 2000). An average discharge current of 2.2 A (rms)

is measured by the current meter available on the display panel of the SAFEBAG prototype. The optical emission of the DBD discharge generated by the SAFEBAG prototype was observed using an Ocean Optics spectrometer HR2000 + with a resolution of 0.8 nm. The optical signal was observed through collimators situated at 0.5 m from the edge of the electrodes, viewing along the discharge gap.

2.2.2. Temporal evolution of ozone and water vapour density in post-discharge gas

The post-discharge temporal evolution of key chemical species within SAFEBAG packages was assessed using GASTEC detectors in the post-discharge time-frame (5, 7, 10, 13, 20, 22, 24 and 27 min). Values were averaged over three experiments. Commercially available zip-lock bags were used (size 23 × 27 cm, volume 3 l). Four bags were exposed at each experiment (the positioning of the packages along the conveyor can be seen in Figs. 2 and 4). Experiments were performed using a gap of 1 cm between electrodes leading to an average discharge current of 2.1 A, for a static plasma exposure time of 2.5 min. The ozone, water and H_2O_2 concentrations were measured using GASTEC detectors (type GAS18M, GAS30 and GAS6 respectively) in the post-discharge. The gas (air) humidity levels were measured before the discharge using both GASTEC detectors and a commercially available humidity meter (Testo Instruments, UK). An RH = 37–41% was recorded for the initial filling air at a room temperature of 17–21 °C.

2.3. Microbiological studies

2.3.1. Bacterial strains and inocula preparation

Two challenge microorganisms were used. *Escherichia coli* NCTC 12900 was obtained from National Collection of Type Cultures of the Health Protection Agency (HPA, UK) and *Listeria innocua* NCTC 11299 was obtained from the microbiology stock culture of the School of Food Science and Environmental Health of the Technological University Dublin. The working inoculum was prepared as followed: cells of overnight cultures were harvested by centrifugation, washed twice and re-suspended in sterile phosphate buffered solution (PBS, Oxoid LTD, UK), resulting in the suspension with final cell concentration of 8–9 $\log_{10}$ CFU/ml. The concentration of inoculum was confirmed by plating appropriate dilutions on TSA, followed by incubation at 37 °C for 24 h and 48 h for *E. coli* and *L. innocua*, respectively.

2.3.2. Preparation of produce and pathogen inoculation

The influence of produce geometric features and surface characteristics on the efficacy of the system were evaluated. Strawberries (Fragaria ananasa, var. Elsanta) and spinach (Class 1, Origin: Spain) were purchased from the local supermarket and stored at 4 °C until use. The weight of each strawberry and spinach leaf was in the range of 20–40 g and 0.5–1.5 g, respectively. The same produce cultivar was used for each microbiological and quality experiment.

For inoculation purposes, strawberries were placed with the blossom end down on sterile petri dishes; spinach leaves were labelled and placed on sterile thin foil. The samples were spot-inoculated with bacteria using 100 µl of a culture. The droplets were deposited in several different locations, ensuring that the inoculum did not flow to the side of the samples. Inoculated samples were dried for 1 h in laminar flow safety cabinet to allow the attachment of bacteria on the surface of produce prior to the ACP treatment.

2.4. Experimental design

To assess the effect of treatment on pathogen reduction in large samples, strawberries (100–130 g) were sealed within polyethylene terephthalate (PET) bags (~25 × 25 cm). Prior to sealing, inoculated and labelled spinach leaves were mixed with un-inoculated leaves to make up a final sample mass of 20 g. All samples were packaged in air. Samples were treated for 2.5 min at 100 kV setting using either static mode or continuous mode on a conveyor belt. In static mode, the produce packages are in stationary position within the plasma discharge zone for a defined time, essentially reflecting a batch process. In continuous mode the produce packages move along the treatment plasma discharge treatment zone of the prototype. During the static mode, 2 bags containing produce were treated simultaneously, while for continuous operation the treatment zone was filled with the bags. All samples were stored for 24 h at 4 °C post treatment. To assess the effects of treatment on the produce background microbiota, uninoculated samples were subjected to the same treatment and post treatment storage conditions. To evaluate any possible effect of extended storage on bacterial growth, inoculated but untreated samples were stored for 24 h under identical temperature conditions. All experiments were performed in duplicate and replicated twice.

2.4.1. Shelf-life study

Strawberry, as a high value but perishable berry, was the model selected to challenge the preservation effects of plasma treatment throughout prolonged storage, assessing microbiological and quality indices. Control strawberries were subjected to 2.5 min of continuous treatment and stored at either 4 °C or 10 °C for 0 (control), 1, 3, 7 and 9 days in sealed bags. A parallel storage abuse temperature condition study exposed strawberries stored at 4 °C to a room temperature for 24 h after day 3, where-after product was returned to refrigerated temperature of 4 °C for further evaluation at days 4 and 7. All experiments were performed in duplicate and replicated twice and results are reported as $\log_{10}$ CFU/sample.

2.5. Microbiological quality analysis

For microbiological analysis, inoculated untreated control samples (to estimate initial attached bacterial population), inoculated untreated samples stored for 24 h (to assess the effect of storage on microbial growth), un-inoculated untreated control samples (to determine initial background microbiota), and either inoculated or un-inoculated ACP treated samples were analysed. The samples were aseptically transferred into sterile stomacher bags (BA6041, Seward LTD, UK) containing 10 ml of sterile maximum recovery diluent (MRD) and hand rubbed for 2–3 min. The resulting suspension was serially diluted in MRD. Surviving *E. coli* and *L. innocua* populations were determined by the agar overlay method. Briefly, aliquots of an appropriate dilution were surface plated on TSA, incubated for 2–4 h, and overlayed with the appropriate selective media: Sorbitol MacConkey agar (SMAC, ScharlauChemie, Spain) supplemented with Cefixime-Tellurite (CT, Oxoid LTD, England) for *E. coli* and polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM, ScharlauChemie, Spain) supplemented with PALCAM Listeria Selective Supplement (Oxoid LTD, England) for *L. innocua*. Plates were then incubated for 24–48 h at 37 °C. Surviving background microbiota of the un-inoculated samples was evaluated using non-selective media TSA for estimation of total aerobic mesophilic bacteria and Potato Dextrose agar (PDA, Scharlau Chemie, Spain) for estimation of yeasts and moulds, with further incubation of agar plates at 37 °C and 25 °C, for 48 h and 5 days, respectively. The limit of detection for bacterial recovery on food samples was 1.0 $\log_{10}$ CFU/sample.

2.6. Nutritional and chemical quality analysis

2.6.1. Colour measurement

Produce colour was quantified using an L^*-a^*-b^* colorimeter (using Colour Quest XE Hunter Lab, Northants, U.K.) for control and treated samples. The colour measurement was performed on each strawberry (along four symmetrical sections) and average values reported. The instrument was calibrated using white (L^* = 93.97, a^* = 0.88 and b^* = 1.21) and green (L^* = 56.23, a^* = 21.85, b^* = 8.31) standard

tiles. The hue angle was calculated as $h^* = \tan^{-1}\left(\frac{b^*}{a^*}\right)$ and chroma as $C^* = \sqrt{(b^{*2} + a^{*2})}$.

2.6.2. pH measurement

The pH of the strawberries was determined by using a handheld pH-meter with spear electrode (Eutech Instruments, Thermo Fisher Scientific Inc., Netherlands). The pH of control and treated groups was measured before packaging and after treatment on daily basis in triplicate for two strawberries at each sampling time.

2.6.3. Firmness

The firmness of control and treated strawberry samples was analysed using an Instron texture analyser (Instron 4302 Universal Testing Machine, Canton MA, USA). The texturometer was mounted with a 500 N load cell and equipped with a 2 mm flat head stainless steel cylindrical probe. Strawberry samples were punctured at a probe speed of 12 mm/min to a depth of 10 mm. A single whole strawberry was placed on the stage for each measurement. The maximum force (N) required to puncture the sample was used as an indication of firmness. Data was analysed using the Bluehill software (Instron, MA, USA). The firmness of three strawberries from each package was measured individually and an average firmness value was reported. Experiments were conducted in duplicate.

2.6.4. Total soluble solids (TSS)

Soluble solids were measured using a hand-held refractometer (Bellingham and Stanley Ltd., UK). Refractive index was recorded and converted to °Brix. Measurements were performed at room temperature. Distilled water was used to clean the refractometer prism after each analysis.

2.6.5. Fourier transform infrared (FTIR) spectroscopy

2.6.5.1. Sample preparation. The control and treated group of strawberries were chopped and frozen for 24 h at −80 °C in a cryogenic fridge (Model 906, Thermo Scientific Forma-86 Ultralow Freezer). Subsequently, they were freeze-dried for a period of 24 h and then manually ground into powder using a mortar and pestle. A 3% w/w dilution of the ground samples was prepared by mixing 9 mg of the sample with 281 mg of dry potassium bromide (KBr, Sigma-Aldrich, Ireland). Pellets were prepared by exerting a pressure of 100 kg/cm^2 for approximately 1 min in a pellet press (Specac, United Kingdom).

2.6.5.2. FTIR analysis. The IR spectra were recorded using a Nicolet Avatar 360 FTIR E.S.P. (Thermo Scientific, Waltham, MA, USA) over the frequency range 4000–400 cm^{-1} by co-adding 64 interferograms. Two scans of each pellet were collected in transmittance units at 1 cm^{-1} resolution at room temperature using OMNIC software (version ESP 5.2). These spectra were subtracted against background air spectrum. After every scan, a new reference air background spectrum was taken. To account for variations that could result from pellet thickness and uniformity, different pellets were prepared from the same sample and their spectra pooled (spectra were nearly identical). The spectral data was corrected for artefacts and undesirable scatter effect by multiplicative scatter correction (MSC), using methods described in earlier publications (Misra, Pankaj, Frias, Keener, & Cullen, 2015; Misra, Sullivan, & Cullen, 2015). Finally, second derivative spectra were used to improve the infrared band resolution and thus, enhance the discrimination of vibrators contributing to the shape of raw FTIR spectra.

2.6.5.3. Principal component analysis (PCA) and hierarchical clustering on PCs (HC-PC). PCA was performed on the second derivative spectrum to visualise the high dimensional spectral data. The PCA loadings plot was used to detect the frequency range where maximum changes were detected. Hierarchical clustering on principal components (HC-PC) of the spectral data matrix was also performed using the R statistical software (http://www.r-project.org/) with *FactoMineR* package (Lê, Josse, & Husson, 2008). The HC-PC allows detecting sample clusters in an unsupervised manner.

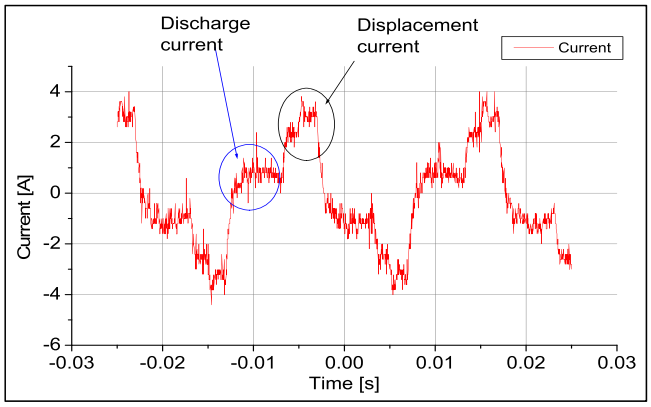

Fig. 3. The typical current waveforms. The discharge current is superimposed over the displacement current in the electrodes' circuit.

2.7. Statistical analysis

Analysis of variance (ANOVA) was performed using SPSS 21.0 (SPSS Inc., IBM). The surviving populations of *E. coli* and *L. innocua* following ACP treatment were compared. Means of the untreated controls (0 h and 24 h) and ACP treated samples were compared within the produce group according to the method of Fisher's Least Significant Difference-LSD at the 0.05 level.

3. Results and discussion

3.1. Plasma parameters

3.1.1. Electrical and optical measurements

The current waveforms typical evolution is shown in Fig. 3 The discharge had a glow-filamentary mode and their pattern can be used to appreciate the discharge operation during different treatments (use of different gaps, voltages and number of bags and their load). The discharge current and displacement current are superimposed as the transformer was a bipolar type. The two optical emission spectra (Fig. 4) correspond to average rms discharge currents of 1.4 A (blue) and 2.2 A (red), respectively, showing an increase of light emission intensity with increase in discharge current. The spectra are similar to

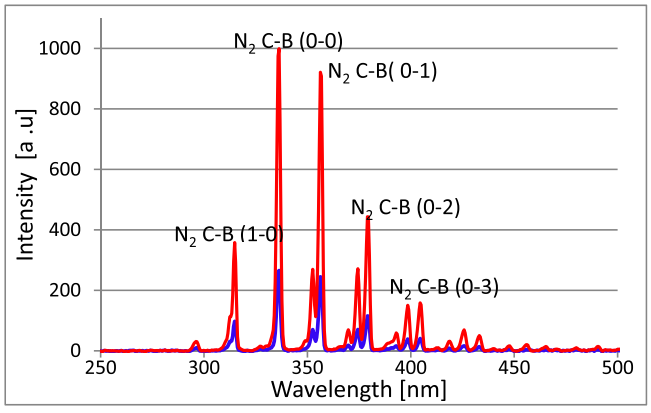

Fig. 4. The OES spectra for the discharge on SAFEBAG Prototype at discharge currents of 1.4 A (rms) and 2.2 A (rms). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

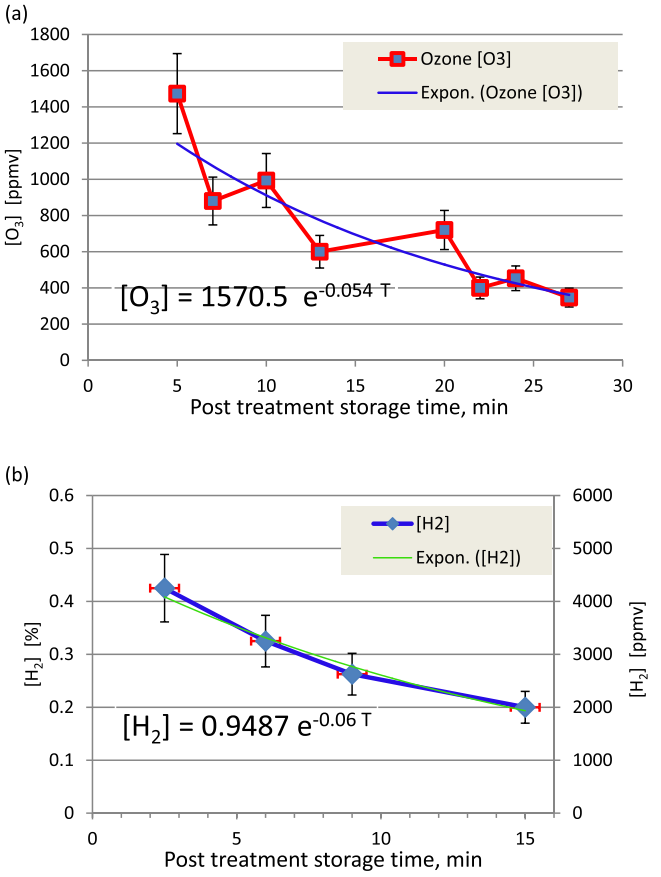

Fig. 5. Post-discharge concentration of a) ozone and b) H_2 in the SAFEBAG. The bars show the relative errors from an average of three experiments.

those obtained in laboratory experiments for air DBD discharges at large gaps (2 cm) and shows the characteristic spectrum of air with nitrogen bands emission, mainly the second positive system (SPS) and some weak emissions from the first positive system (FPS), data not shown. No emission from the N_2^+ ions bands (391 nm) of the first negative system (FNS) were observed, indicating low electron excitation energies (well below 20 eV).

3.1.2. Post-discharge gas composition inside the package

Both evolutions of O_3 and H_2 have similar time decrease rates in the post-discharge, due to gas leakage from the bags (Fig. 5a and b, respectively). The decrease is exponential with a rate of: Exp ($-0.06 \times$ T [min]). Much larger values, closer to initial values in the post-discharge, are expected for commercially sealed bags where leakage is lower. The amount of H_2 measured may include H_2O_2 and HNO_x, as readings of chemical detectors may be influenced by other hydrogen-rich compounds.

From Table 1, by comparing the amount of H_2O before and after plasma treatment, we can estimate the amount of water dissociated by the discharge to about 30%. The presence of H_2 or other hydrogen compounds like HNO_x (x = 1, 4) indicates that the SAFEBAG Prototype can generate inside the packages the humid air plasma chemistry leading to high bactericidal efficiency due to the combined effect of several long-lived strong bactericidal species (O_3, HNO_x, H_2O_2).

Table 1
The concentration of water before plasma treatment and in the post-treatment (H_2O, and H_2 and possibly HNO_x) levels from GASTEC detectors.

Temp	H_2O content (initial)			Post-discharge	
	RH	Conversion		[H_2O]	[H_2]
°C	%	ppmv	mg/L	mg/L	ppmv
21.6	33	8513	6.28	4.2	4250
21.4	37	9437	6.96	5	3800
17.3	41	8089	6	4	4000

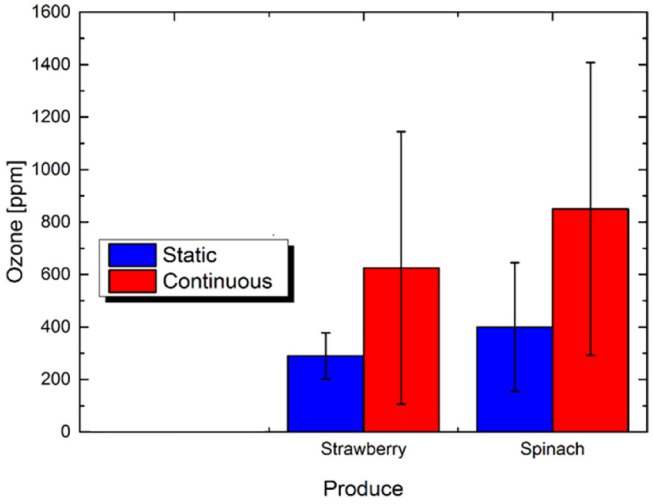

Fig. 6. Ozone concentrations (ppm) inside the packages for static and continuous mode of operation (2.5 min), when loaded with different produce.

3.1.3. Ozone concentrations

Ozone is one of the key factors contributing to antimicrobial efficacy of in package ACP. The overall ozone concentrations detected inside packages containing either strawberries or spinach after ACP treatment were within the range of 200–800 ppm (Fig. 6). Higher ozone concentrations were generated during the treatment of spinach leaves. The levels of ozone generated inside the bags also depended on the plasma operational mode with higher concentrations recorded for continuous treatment than for static, regardless of the type of produce treated inside the bag. The influence of produce type on generation of ozone was previously reported, where produce surface characteristics possibly played an important role in dissolution rates of ozone generated inside the package with lower ozone levels recorded for strawberry samples than for tomatoes (Ziuzina et al., 2014). In addition, the variability in ozone concentration can be attributed to humidity introduced by the produce, besides the use of atmospheric air for induction of the plasma and the associated dynamic plasma chemistry.

3.2. Microbiological quality

Microbiological quality of fresh produce is one of the most important factors determining the performance of a minimal processing technology. Two model microorganisms (*E. coli* and *L. innocua*) representing those pathogens that are frequently associated with fresh produce related outbreaks but also those that can persist in fresh produce storage conditions, in addition to possessing different structural characteristics were selected. The choice of produce was based on the produce structural and surface features: strawberries have a spherical geometry and convolutions at the surface, while spinach leaves possess large surface to volume ratio and laminar geometry.

3.2.1. Challenge micro-organisms

The influence of ACP treatment on bacteria inoculated on strawberries and spinach are presented in Fig. 7. In general, similar inactivation levels of *E. coli* were obtained with respect to different types of produce, however, the effect of the mode of treatment on the recovery of cells was apparent (Fig. 7a). Higher antimicrobial effects were

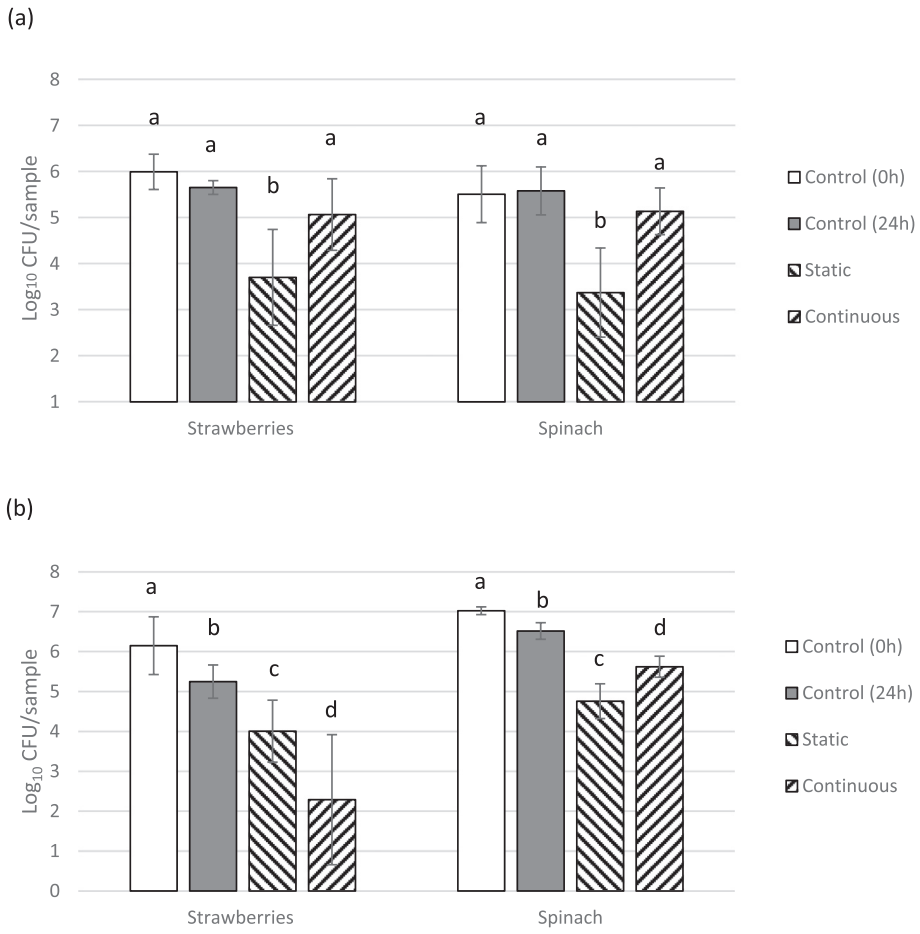

Fig. 7. Effect of static and continuous mode of ACP treatment (2.5 min) and post treatment storage for 24 h at 4 °C on inactivation of *E. coli* (a) and *L. innocua* (b) on strawberries and spinach leaves. Limit of detection: 1 $\log_{10}$ CFU/sample; Vertical bars represent standard deviation. Different letters indicate a significant difference in population levels where mean values were compared within the same produce type.

achieved for the static mode than for the continuous mode of treatment. Significant reductions ($p < 0.05$) of *E. coli* by 2.0 and 2.2 $\log_{10}$ CFU/sample were achieved on strawberries and spinach leaves, respectively, after 2.5 min of static mode of treatment as compared to the corresponding untreated 24 h controls. Continuous treatment had minimal impact on *E. coli* populations (0.5 log) with respect to both types of produce evaluated. Thus, the previously reported (Ziuzina et al., 2014) influence of produce type and inherent surface characteristics on decontamination efficacy of plasma treatment for inactivation of pathogens was not an observable factor here.

Fig. 7b presents the effects of ACP treatments on the numbers of *L. innocua* inoculated on produce. The antimicrobial effect of static treatment was influenced by the bacterial type with higher resistance observed for *L. innocua* than for *E. coli*; reductions in populations of *L. innocua* by 1.3 and 1.7 $\log_{10}$ CFU/samples on strawberries and spinach were achieved respectively, which were still significant when compared to microbial numbers on the untreated and stored for 24 h controls. In accordance with other reports (Ziuzina et al., 2014; Mai-Procnow et al., 2016) the difference in bacterial response to treatments could be due to bacterial cell wall characteristics, which impacts the prevalent mechanism of inactivation, where thicker cell wall of the gram positive bacteria may present a barrier to the diffusion of plasma reactive species through the cell wall, impacting antimicrobial efficacy of treatment. Interestingly, with continuous treatment and when inoculated on both types of produce *Listeria* populations were more sensitive than populations of *E. coli* with 2.9 and 0.9 log cycle reductions achieved on strawberries and spinach, respectively. This suggests that besides the bacterial cell wall characteristics, other factors can play a role in the resistance to ACP. For instance, Han et al. (2015) reported different reaction mechanisms between plasma reactive species and cellular components with a lower inactivation rates observed for gram-negative *E.coli* than for gram-positive *S. aureus* when treated in form of a cell suspension. Another study reported that gram-negative *P. aeruginosa* became more resistant to ACP when grown in co-culture than as a mono-species biofilm (Mai-Prochnow et al. 2016). Therefore, further investigations are required to elucidate mechanisms involved in bacterial protection against plasma reactive species generated during different processing modes as well as to assess the bacterial interaction with the host plant. In terms of the mode of treatment, *Listeria* inoculated on spinach had higher resistance to continuous treatment than to static, as was observed for *E. coli*.

3.2.2. Background microbiota

Static ACP significantly reduced populations of mesophilic bacteria of strawberries by 1.8 $\log_{10}$ CFU/sample, whereas only 0.8 $\log_{10}$ reduction of mesophiles were achieved with the continuous mode. Both treatment modes reduced the populations of strawberry yeasts and moulds by an average of 1.2 $\log_{10}$ CFU/sample (Fig. 8a).

There was a smaller impact of ACP treatment on background microbiota for spinach (Fig. 8b) with higher antimicrobial effects achieved with the static operational mode than with the continuous. Thus, the static treatment reduced populations of mesophilic bacteria and yeasts/moulds by 1.1 and 0.6 $\log_{10}$ CFU/sample, respectively, whereas continuous mode reduced populations of mesophiles by 0.3 log and no reductions were observed for yeasts and moulds. Besides the influence of the mode of treatment, this study also demonstrated that microbiological inactivation efficacy of ACP treatment was influenced by the type of produce. An increase in the produce total surface area as in the case of the spinach leaves as well as the leaves overlapping during the processing could contribute to attenuated antimicrobial efficacy of the treatment, even when in-package post treatment storage time was employed. The diversity of product associated microbiome and

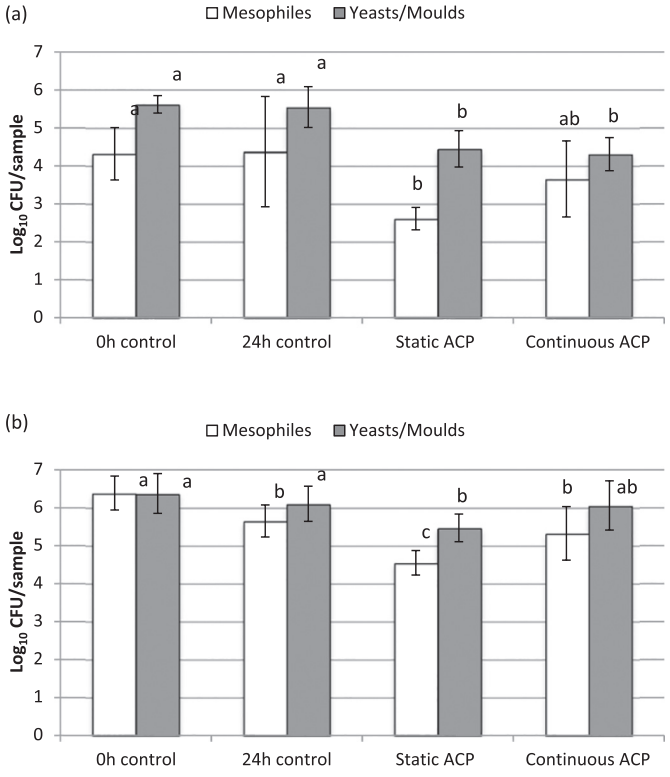

Fig. 8. Effect of static and continuous mode of ACP treatment (2.5 min) and storage time of 24 h at 4 °C on background microbiota of (a) strawberries and (b) spinach leaves. Limit of detection: 1 $\log_{10}$ CFU/sample. Vertical bars represent standard deviation. Different letters indicate a significant difference in population levels, where mean values were compared within the same microbial type.

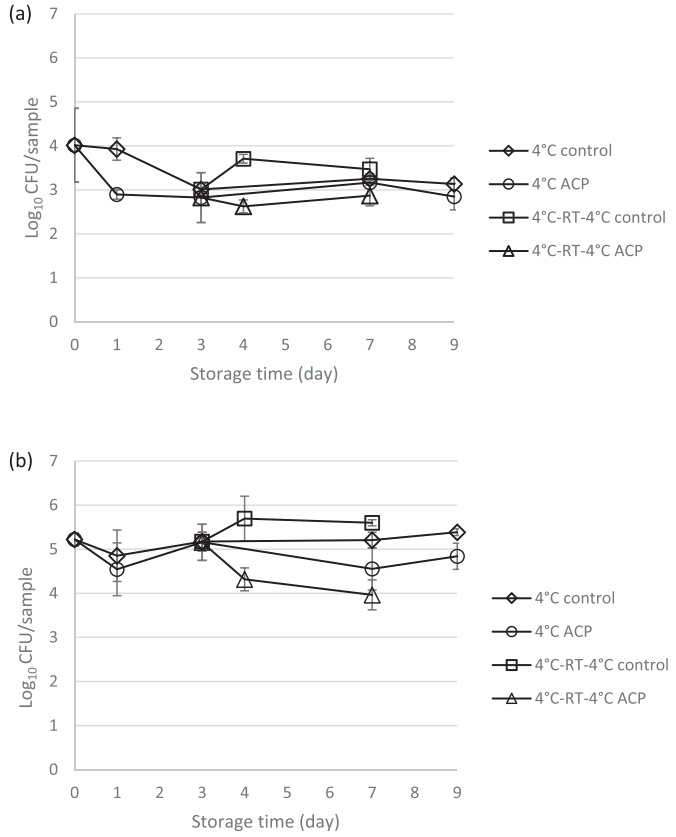

Fig. 9. Effect of continuous mode of ACP treatment (2.5 min) on strawberries (a) aerobic mesophilic counts and (b) yeast and moulds during 9 days storage at 4 °C and 4 °C interrupted by storage for 24 h at room temperature (4 °C-RT-4 °C) storage.

population ratios may also impact the overall efficacy of ACP in a product dependent manner.

Cold plasma contains ions, free electrons, UV light and reactive species which cause damage to cell membranes and intracellular components of prokaryotic and eukaryotic microorganisms. Generating cold plasma discharges inside food packages can achieve rapid bactericidal effects through the retention of bactericidal species over longer periods during the produce storage. However, in accordance with the current results, different produce commodities will require application of specific treatment parameters.

3.2.3. Strawberries storage study

Storage conditions of 4 °C were used to reproduce the typical environment employed in industry. Storage at 10 °C was utilised in order to demonstrate the efficacy of ACP treatment on produce microbial communities stored at temperature abuse conditions. A parallel shelf-life study was carried out utilising strawberries, where storage at 4 °C on day 3 was interrupted with 24 h of storage at room temperature in order to challenge the overall long term efficacy of ACP treatment and reproduce possible changes in the environment as temperature of storage during transportation and prior to retail display are highly varied (Lopez-Velasco, Davis, Boyer, Williams, & Ponder, 2010).

3.2.3.1. Effect of storage and abuse conditions. Continuous treatment in conjunction with storage at 4 °C significantly reduced populations of mesophiles from 4.0 $\log_{10}$ CFU/sample of the untreated controls to 2.9 $\log_{10}$ CFU/sample on day 1 ($p < 0.05$) (Fig. 9a). However, for the remainder of the storage period, no significant differences were retained. In the case of the control samples, a decrease in bacterial populations by 1.0 log unit was observed on day 3, which was maintained up to day 9.

In contrast, in the temperature abuse study by day 4, after introducing a 24 h storage at room temperature conditions, the population of mesophilic bacteria present on the control strawberries significantly increased from 3.0 to 3.7 $\log_{10}$ CFU/sample ($p < 0.05$). However, this storage temperature abuse did not lead to significant bacterial growth on the ACP treated samples, resulting in significant difference between ACP treated and the corresponding controls on days 4 and 7 ($p < 0.05$). In this case, reported reductions could be directly attributed to bactericidal action of ACP.

The impact of continuous treatment on strawberries yeasts and moulds populations was more prominent after one week storage, where a significant difference between untreated controls and ACP treated groups was recorded ($p < 0.05$). An introduction of 24 h storage at room temperature resulted in a significant increase in yeast and moulds in the control groups. Again, significantly lower populations were recorded for ACP treated samples compared to untreated controls (Fig. 9b).

3.2.3.2. Effect of ACP on microbial populations of strawberries stored at 10 °C. Fig. 10a shows that the impact of ACP treatment became evident by day 7 where bacterial numbers were significantly decreased by 1.0 and 1.4 log units, respectively, by comparison with day 0 controls ($p < 0.05$) and the bacterial population surviving on day 9 was significantly lower compared with control populations on any of the days tested. Similar persistent effects of treatment were observed in the case of yeasts and moulds (Fig. 10b). ACP treated sample populations decreased to 4.5 $\log_{10}$ CFU/sample, whereas populations of the controls continued to grow reaching 5.4 $\log_{10}$ CFU/sample by day 9 of storage.

These results clearly demonstrate that with relatively short, in-package, continuous ACP treatment it was possible to reduce microbial

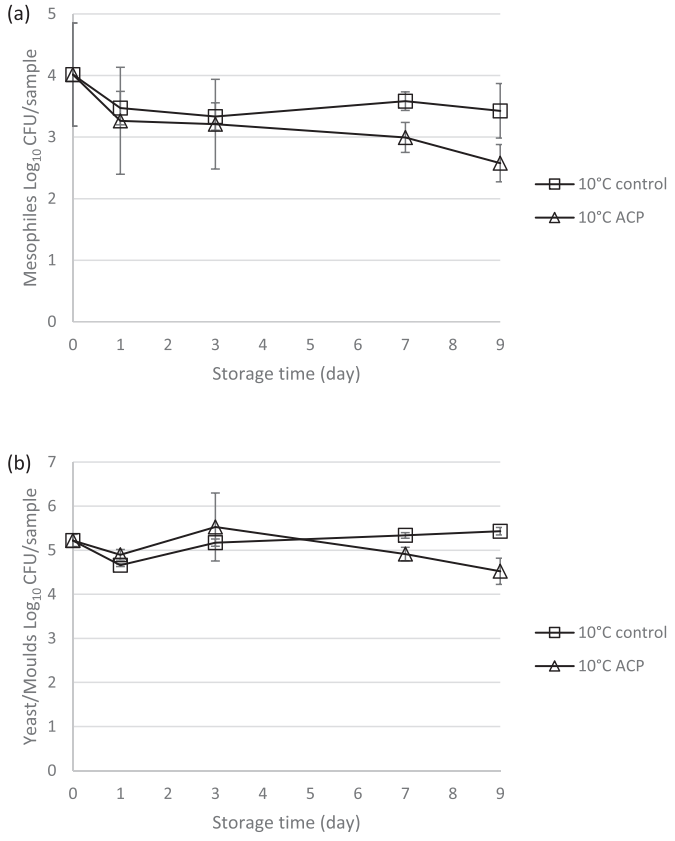

Fig. 10. Effect of continuous mode of ACP treatment (2.5 min) on strawberries (a) aerobic mesophilic counts and (b) yeast and moulds during 9 days storage at 10 °C.

load from the surface of strawberries up to Day 9 of storage at refrigerated conditions, below the levels present on the untreated controls. However, the protective impact of ACP was more prominent when 24 h temperature abuse at room temperature was introduced on day 3 of storage at 4 °C.

3.3. Quality indices

3.3.1. Colour

Colour is considered a critical attribute, which visually characterises the freshness of most fruits and vegetables and plays a key role in food choice, preference and acceptability (Martin-Diana et al., 2007). Fig. 11 presents the change in the colour of strawberries after 2.5 min of static treatment and 24 h post treatment storage. The control strawberry samples were found to be different in terms of colour on day 1, as compared to day 0. While a minor decrease in the redness and chroma of plasma treated strawberries compared to control was recorded by the instrument on day 1, this difference was statistically insignificant ($p < 0.05$). The results were reproducible as observed from repetition of the experiments on separate days (Trt 1, and Trt 2 in Fig. 11). The insignificant change in colour of plasma treated strawberries was also observed in our earlier study (Misra et al., 2014). Fig. 12 depicts the impact of continuous treatment in conjunction with the extended storage for 7 days on the colour of strawberries. A change in the colour parameters of strawberries was noticeable on day 7 in both control and treated strawberries. A decrease in lightness parameter was observed, which was more prominent in treated berries compared to control samples. This was most likely an outcome of the fungal growth, which was observed on both control and the treated samples on day 7.

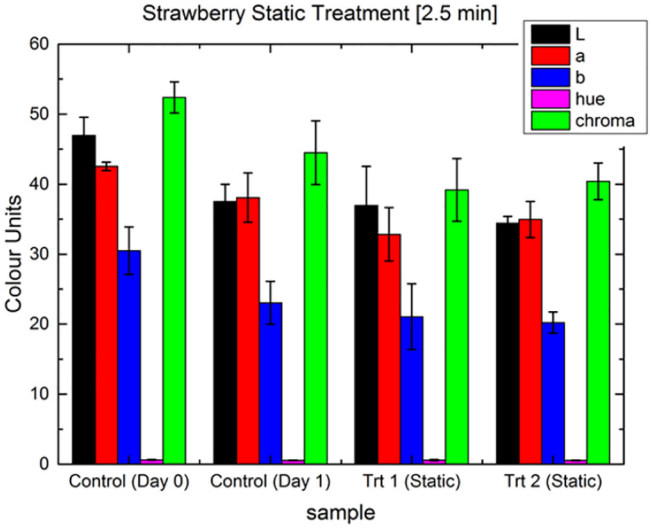

Fig. 11. Change in colour of strawberries following treatments under static mode. Trt: treated samples. Trt 1 and Trt 2 refer to two independent treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

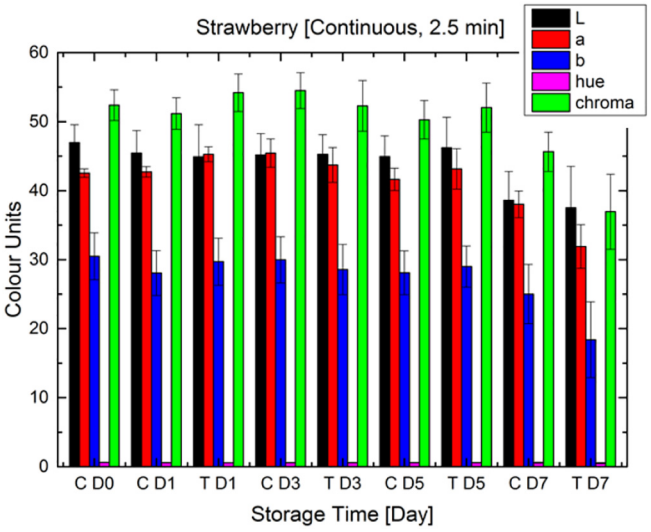

Fig. 12. Change in colour of strawberries during storage, following treatments under continuous mode. C: Control and T: ACP treated samples. D: Day of analysis post storage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.2. Firmness

No significant difference ($p > 0.05$) between firmness of day 0 control, day 1 control (i.e. after in-package storage for 24 h) and treated (static mode) strawberries was observed (Fig. 13a). The results were reproducible on performing the experiment on separate days (Trt1 and Trt2), and these results are also in agreement with observations form an earlier study (Misra et al., 2014). In contrast, the firmness of strawberries decreased over the storage period (Fig. 13b). However, the difference between the firmness of the control and treated group of strawberries was insignificant ($p > 0.05$) on any day of storage, except day 7, where the plasma treated strawberries required a lower peak puncture force compared to the control. It is worthwhile mentioning that firmness loss is generally a challenge in the case of cut fruits and vegetables where the exposed tissue surface could result in cell leakage; however, this is not the case with whole intact fruits, as is the case in this study.

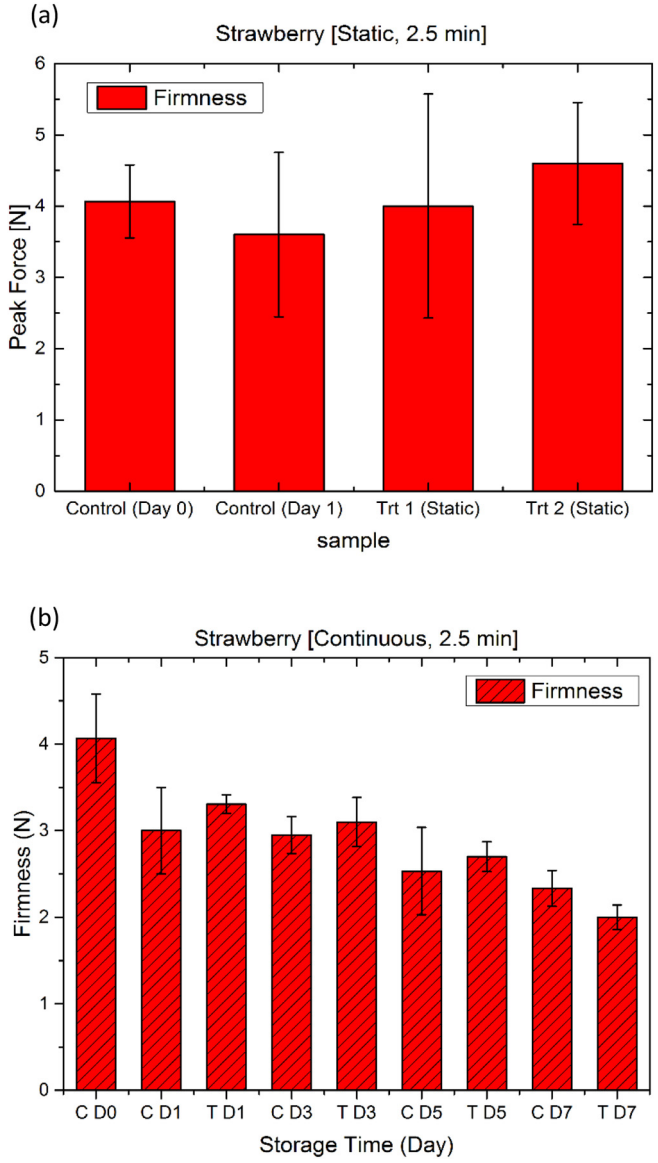

Fig. 13. (a) Peak force (N) required to puncture the control and treated (static mode) strawberries. Trt 1 and Trt 2 refer to two independent treatments. (b) Firmness of control and plasma treated strawberries over a storage period of 7 days.

3.3.3. The change in pH and TSS

Total soluble solids (TSS) play an important role in affecting fruit quality, consumer acceptability and processing. Following 24 h storage (day 1), there was no significant change in the pH or TSS of the control samples or samples treated under static mode (Table 2). The pH and TSS of the samples treated under continuous mode over the storage period of 7 days can be observed in Fig. 14 (a and b, respectively). Clearly, the control and plasma treated samples did not show any significant difference ($p > 0.05$) in their pH or TSS levels on any of the

Table 2
TSS and pH of control and treated under static mode strawberries.

Sample	pH	TSS
Control (day 0)	3.66 ± 0.20	6.70 ± 0.44
Control (day 1)	3.51 ± 0.08	6.50 ± 0.40
ACP 1 (static)	3.65 ± 0.13	6.00 ± 1.00
ACP 2 (static)	3.53 ± 0.17	6.50 ± 0.50

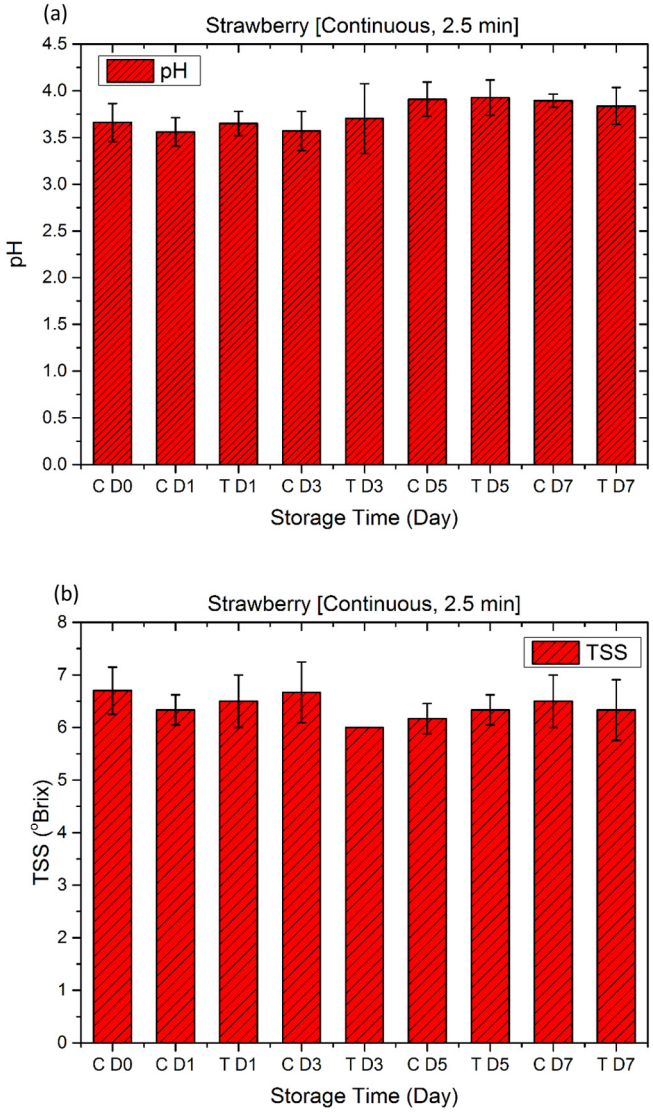

Fig. 14. The change in (a) pH and (b) TSS of control strawberries and strawberries treated under continuous mode over a storage period of 7 days. C: Control and T: treated samples.

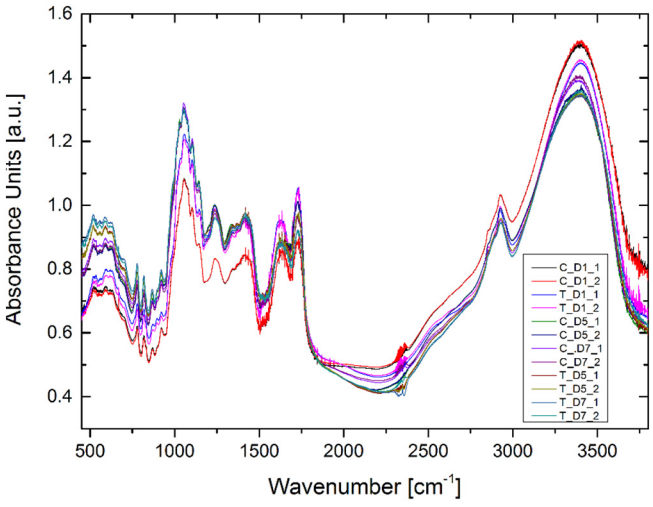

Fig. 15. Overlaid raw FTIR spectrum of control and treated group of strawberries.

days evaluated.

3.4. FTIR spectroscopy

The infrared spectrum in the region 450–3800 cm^{-1} frequency is presented in Fig. 15. The wide band extending across the wavelengths between 2700 and 3600 cm^{-1} originates from the presence of water molecules and alkyl C-H bond stretching and does not provide useful information regarding any potential changes. The N-H stretch vibrations in the 3300–3400 cm^{-1} region originating from amino group of proteins also overlap in this frequency range. The major constituents in dried strawberry include sugars, mainly fructose and glucose, followed by fibre and protein (Giampieri et al., 2012). The signals in 620–655 cm^{-1} region originate from C-CH_3 vibrations, while those of 1410–1480 cm^{-1} originate from C-H bend in sugars (Misra, Pankaj, et al., 2015). The spectra exhibit evidence of the presence of pectin by the appearance of the CO stretching vibration band at approximately 1725 cm^{-1} (Suutarinen, Änäkäinen, & Autio, 1998). Nevertheless, the overlap from CO stretching vibration of the esters in this region cannot be ruled out. The wide band in the range 1350–1450 cm^{-1} was assigned to O-H deformation from structural carbohydrates. The band in the region of 1150 to 1170 cm^{-1} originates from C-O vibrations in carbohydrates. The bands between 1730 and 1755 cm^{-1} and 1205–1265 cm^{-1} originated from C = O stretch and C-O-C stretch respectively and were most likely an outcome of the fatty substances. The carbonyl vibrations of the protein backbone were also noticeable between 1700 cm^{-1} and 1600 cm^{-1} (amide I region) in a second derivative spectrum (not shown).

We applied PCA to the spectral data to reveal any grouping between the samples (Fig. 16). The first two principal components (PCs) accounted for > 93% of the total variance and thus contained most of the information of the original data, which is associated with the chemical groups of samples. The within sample variability, due to technical repetitions, is small compared to the between treatments variability as observed from the minimal separation between duplicate measurement points. Results from PCA suggested that the treated and control samples were distinct on day 1 (i.e. after 24 h of post-treatment storage). However, the samples from day 2 onwards were closely spaced, indicating the likelihood of convergence of chemical compositions due to recovery from the physiological stress induced by the plasma treatments.

To further confirm the hypothesis of convergence of chemical compositions of control and treated samples and discover any inherent clustering patterns in the dataset, an unsupervised clustering algorithm of the spectral data in the principal co-ordinates space was performed. The unsupervised clustering method suggested three distinct clusters (Fig. 17). The strawberry treated samples were distinct from the control on day 1. This is partly in agreement with our results obtained with the lab scale in-pack plasma system for treatment of strawberries (Misra et al., 2014). However, there was no significant difference among the samples from day 2, as observed from the single cluster for the control and treated samples. Detection of such natural clustering strongly suggested that there are no chemical changes in the chemical profile of strawberries following non-thermal plasma treatments during long term storage that could be detected from FTIR spectroscopy.

ACP technology is finding increasing attention in the food sector with potential for a wide range of decontamination applications. The prime advantages of in-package cold plasma treatment include post packaging treatment, which mitigates against re-contamination and the fact that the bactericidal molecules, which can be generated in air or modified atmosphere revert back to the original gas within few hours of storage. An atmospheric air generated plasma contains ions, free electrons, UV light and a range of ROS and RNS which cause damage to cell membranes and intracellular components of prokaryotic and eukaryotic cells. It was demonstrated that generating cold plasma discharges inside food packages can achieve rapid bactericidal effects through the retention of bactericidal species over longer periods during the produce storage. However, based on the current results and our results from previous publications, it is likely that different produce commodities will require application of different treatment parameters to improve microbiological safety and retain quality of fresh produce. This work also demonstrated that complex interactions between type of bacteria, produce surface characteristics and mode of treatment should be taken into consideration to attain the maximum advantages with this technology.

4. Conclusion

The current work demonstrated that with the pilot-scale large gap ACP system for in package treatment, significant reductions of pathogenic as well as spoilage microorganisms could be achieved and that the in-package approach did not adversely affect product quality properties. Greater reductions were achieved for *E. coli* on strawberries and spinach with application of static treatment, whereas for *L. innocua* inoculated on strawberries, higher reductions were achieved with continuous treatment. The shelf-life study demonstrated that with continuous ACP treatment it was possible to reduce microbial populations of strawberries throughout prolonged storage time at standard

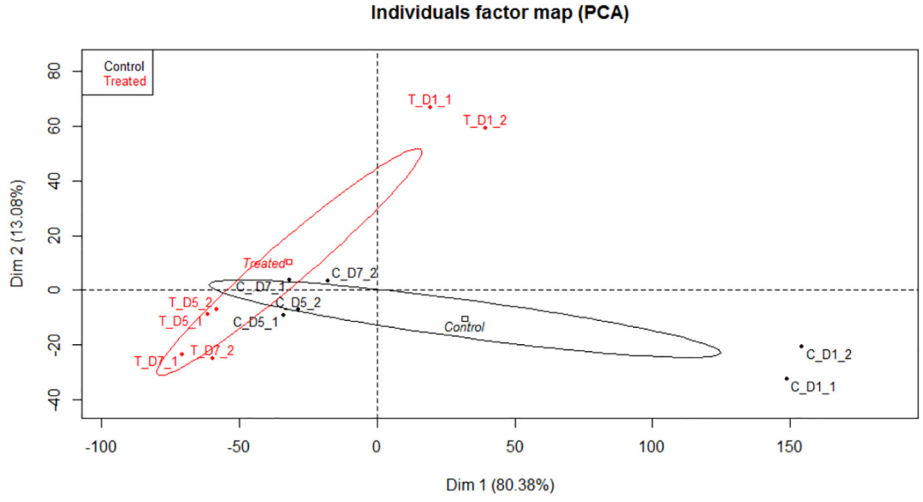

Fig. 16. Principal Component Analysis of FTIR spectral data. C: Control, T = Treated, D1, D2, D5, D7 refer to days of storage. The last numeric value for each marker label indicates the replicate number (1 or 2).

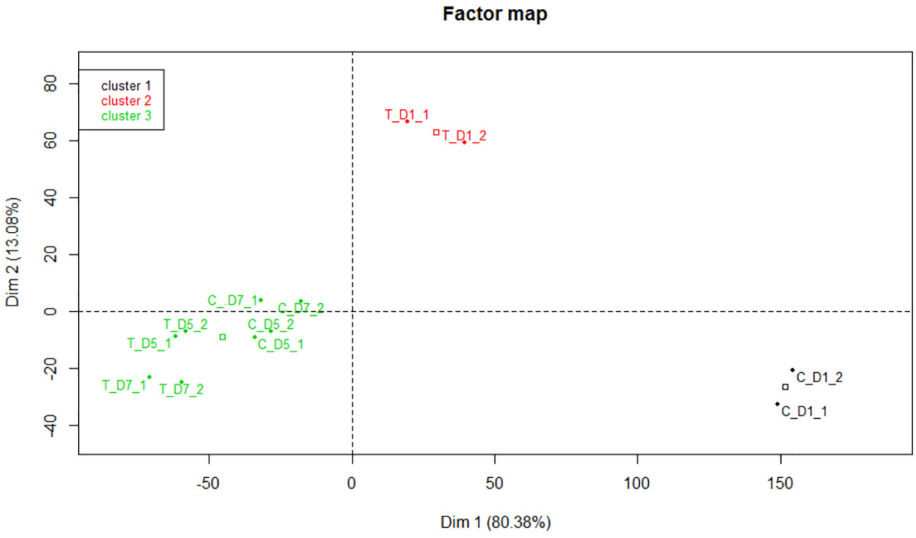

Fig. 17. Clusters formed on the principal coordinate space by the clustering algorithm. C: Control, T = Treated, D1, D2, D5, D7 refer to days of storage. The last numeric value for each marker label indicates the replicate number (1 or 2).

4 °C and temperature abuse conditions while retaining quality profile. However, continuous treatment on the high voltage ACP prototype as a novel approach requires further optimisation of parameters such as type of electrodes, dielectric properties and conveyer belt design.

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Data availability statement

All data generated or analysed during this study are included in this published article and are available on request.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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