Food Chemistry 284 (2019) 303–311

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Strategy to achieve a 5-log *Salmonella* inactivation in tender coconut water using high voltage atmospheric cold plasma (HVACP)

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ARTICLE INFO

Keywords:
Cold plasma
Non-thermal
Coconut
Electrical discharge
Ascorbic acid
Citric acid
Minerals

ABSTRACT

This study examined high voltage atmospheric cold plasma (HVACP) technology as a non-thermal intervention for inactivating *Salmonella enterica* serovar Typhimurium LT2 (ST2) in tender coconut water (TCW). Treatment with HVACP in air at 90 kV for 120 s inactivated 1.30 $\log_{10}$ of ST2. Development of a TCW stimulant suggested an interfering role of magnesium and phosphate salts with HVACP inactivation. Generation of reactive gas species, viz. ozone and hydrogen peroxides were found to be responsible for microbial inactivation. The addition of 400 ppm citric acid to the TCW effectively reduced ST2 by 5 $\log_{10}$ during HVACP treatment. Under these conditions, higher cellular leakage and morphological damage were observed in ST2. Minimal physico-chemical changes in TCW were observed with HVACP treatment, except for an 84.35% ascorbic acid loss (added externally). These results demonstrate a potential pathway for developing highly effective cold plasma treatments to preserve fruit and vegetable juices.

1. Introduction

The increasing market demand for minimally processed, safe and high-quality food products has paved the way for development of novel non-thermal technologies for food processing. Atmospheric pressure cold plasma is an emerging process intervention for preservation of food products with advantages of being a low-cost technology that is non-toxic, leaves no known residues, and causes minimal damage to foods. Thus, it shows promise in replacing or at least, complementing conventional pasteurization technologies (Pankaj, Wan, & Keener, 2018). There are various methods of cold plasma production, viz. dielectric barrier discharge (DBD), plasma jets, coronas, and microwave discharges. Individual researchers have preferences toward specific plasma devices based on the application(s) of interest. It is worthwhile mentioning that the reactive gas species generated in plasma devices are device dependent. Thus, success of one device in one application does not guarantee success of a different plasma device in that same application. High voltage atmospheric cold plasma devices have been demonstrated to be very effective in a variety of food applications. Examples include plasma decontamination of raw and dried produce, solid foods like cheese, ham, eggshells, bacon and liquid foods like milk and juices (e.g. apple, pomegranate, orange and grape) (Pankaj et al., 2018; Xu, Garner, Tao, & Keener, 2017).

The antimicrobial nature of cold plasma treatment has been demonstrated by several researchers in the past (Liao et al., 2017). The microbial inactivation mechanisms which have been reported and suggested includes generation of reactive gas species, viz. reactive oxygen species (ROS), reactive nitrogen species (RNS), and minor contributions from ultraviolet light. This cocktail of reactive species enables the inactivation of microbes on food surfaces, and could further diffuse into liquid media, thereby effecting the acidification and microbial inactivation in liquids (Gaunt, Beggs, & Georghiou, 2006; Oehmigen et al., 2010). The use of gases like He, He/O_2, Ar, Ar/O_2, N_2/O_2, N_2/O_2/CO_2 and air has been shown to be effective in microbial inactivation with plasma (Misra, Keener, Bourke, Mosnier, & Cullen, 2014; Surowsky, Fröhling, Gottschalk, Schlüter, & Knorr, 2014).

The demand for processing and preservation of tender coconut is on the rise due to its natural rehydrating properties, nutritive composition, unique flavour and rising awareness among consumers regarding the deleterious health effects of artificial carbonated drinks. The nutritive composition of tender coconut water renders its high sensitivity towards microbial growth and thus, a very short shelf-life. Thermal processing is effective in killing microbes, but changes the nutritive and flavour characteristics of tender coconut water. Thus, the industry is in

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https://doi.org/10.1016/j.foodchem.2019.01.084
Received 26 June 2018; Received in revised form 7 December 2018; Accepted 8 January 2019
Available online 19 January 2019

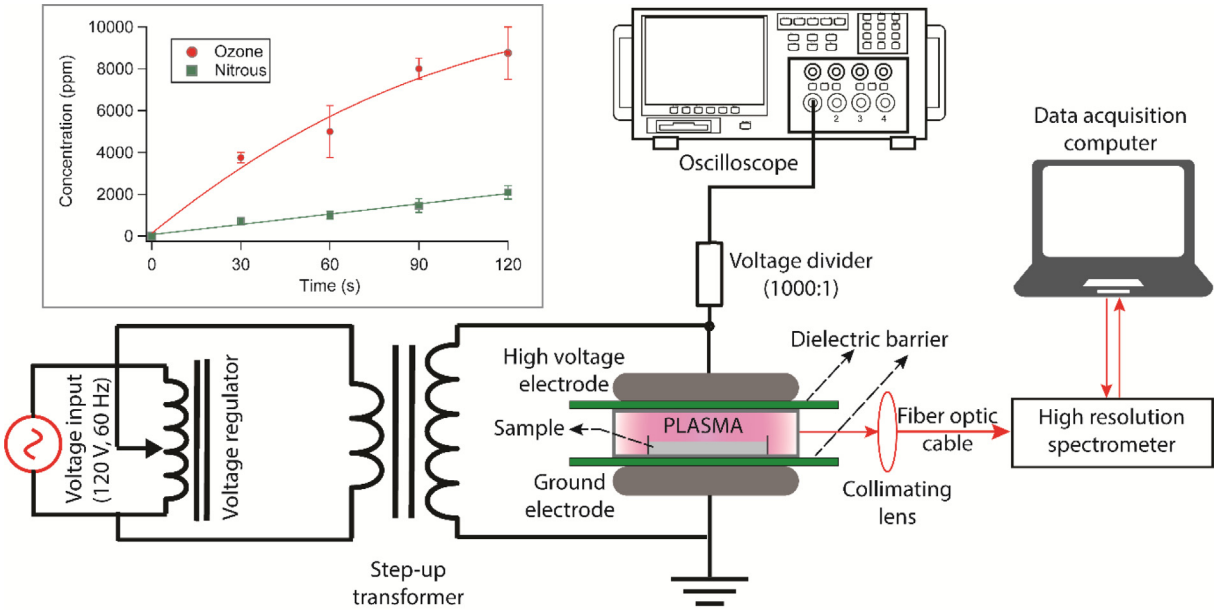

Fig. 1. Experimental set-up for high voltage atmospheric cold plasma treatment (HVACP) and optical emission spectroscopy (OES) of the plasma. Inset: Time evolution of concentration of ozone and nitrogen oxides in an empty package treated with HVACP in air at 90 kV from 0 s to 120 s. The solid lines are sigmoidal fits to guide the eye.

search of non-thermal strategies to process tender coconut water including the use of microfiltration and dense phase carbon dioxide technology (Damar, Balaban, & Sims, 2009; Mahnot, Kalita, Mahanta, & Chaudhuri, 2014), where only inherent microbes in tender coconut are removed or inactivated.

FDA has suggested that for fruit juice processing a 5 $\log_{10}$ reduction process for *Salmonella, Listeria* and *Escherichia* is necessary to be followed by processors for addressing safety issues. *Salmonella* infection has long been associated with products made from coconuts owing to contamination from improper soil health in coconut plantations and post-harvest sites. Gabriel et al. (2016) have suggested that *Salmonella* can serve as the reference microorganism for microbial inactivation efficacy evaluation of cold plasma in tender coconut water. In their study, Gabriel et al. (2016) employed a jet plasma for *Salmonella enterica* inactivation in tender coconut water. However, the jet plasma treatment was allowed to reach a maximum of 4 $\log_{10}$ population reduction after an extended treatment time of up to 25 min.

The hypothesis that motivated this work is that the composition of tender coconut water has a protective effect on bacteria against cold plasma, which can be overcome by suitably modifying the liquid chemistry. In the present study, dry air was used as the working gas for inactivating *Salmonella enterica* serovar Typhimurium LT2 (ST2) using high voltage atmospheric cold plasma (HVACP) produced using a dielectric barrier discharge. The effects of specific chemical components of tender coconut water on plasma induced microbial reduction were deduced to optimize the process for achieving a 5 $\log_{10}$ reduction in *Salmonella* sp. population. The quality changes in tender coconut water following HVACP treatment were also evaluated.

2. Materials and methods

2.1. Materials

Tender coconuts were brought from a near-by market in West Lafayette Indiana, USA as whole nuts. The nuts were cracked open and the water was collected in glass bottles. For each treatment three samples were collected and the experimental measurements were carried out in triplicates. The ingredients, namely D-glucose, D-fructose and -malic acid were obtained from Sigma, USA were of analytical grade; sodium bicarbonate, potassium chloride, magnesium chloride hexahydrate, calcium chloride dihydrate were obtained from Fisher Scientific, USA were of ACS grade; sodium phosphate dibasic anhydrous and L-ascorbic acid were obtained from Mallinckrodt, U.K were of analytical grade.; egg albumen solids and soybean oil were procured from Zoye, USA; and analytical grade citric acid was obtained from EMD, USA.

2.2. Inoculum preparation, inoculation and recovery method

Salmonella enterica serovar Typhimurium LT2, ATCC 14028 was grown in tryptic soy broth (BD Difco™, MD, USA) at 37 °C while shaking for 24 h in an incubator to reach the stationary phase. For preparation of inoculated samples, to every 100 ml of mixed solutions or tender coconut water, 125 µl of the *S. enterica* media was added and shaken well to obtain a homogenous mixture. A 25 ml volume of the tender coconut water or mix solution was taken for plasma treatment studies. Microbial recoveries were carried out after HVACP treatment for 120 s followed by a 24 h refrigerated storage period using serial dilution technique (dilutions ranged from 0 to 10^5) with 0.1% peptone water and microbial recoveries using drop plate technique on xylose lysine deoxycholate (XLD) agar (BD Difco™, MD, USA). Briefly, for the drop plate method 10 µl of the diluted or non-diluted samples were spotted and the plates were incubated at 37 °C for 24 h to count the visible colonies.

2.3. Experimental set-up for cold plasma treatments

The High Voltage Atmospheric Cold Plasma (HVACP) was generated using a dielectric barrier discharge (DBD) set-up. The system consisted of a high voltage transformer (Phenix BK130, Phenix Technologies, MD, USA) with an input voltage of 120 V at 60 Hz on the primary, a voltage regulator (0–100%, output voltage controlled within 0–120 kV), and two aluminium electrodes (15 cm diameter) between which the plasma was generated. The two electrodes were separated by using dielectric barriers, separated by a gap of 5 cm. The dielectric barriers constituted of high density polypropylene sheets (IKEA, Sweden) between which a polypropylene box (16.8 cm × 26.9 cm × 4 cm) used as the sample holding container was placed. This set-up was used for all experiments (Fig. 1). The voltage across the electrodes was recorded using a 1000:1

voltage divider (proprietary technology of Phenix Technologies) connected to an Oscilloscope (InfiniiVision, Agilent Technologies Inc., USA).

The cold plasma treatment was carried out using dry air (procured from Indiana Oxygen, Lafayette, IN USA) as the working gas, which was filled into the box at a flow rate of 0.6 L/min at for 120 s the boxes were covered using high barrier Cryovac polymeric films (BH4670T, Sealed Air, USA) and the bags were sealed using a heat sealer (Food Saver, USA). The liquid samples were placed in the discharge region in polystyrene containers. The plasma treatment voltage was fixed at 90 kV and the all experiments were carried out at room temperature. The power drawn by the system during operation was monitored using a wall power meter (P3 International, Kill A Watt, EZ Energy Monitor) and found to be 186 ± 4 W.

2.4. Optimizing HVACP to achieve a 5-log reduction of S. enterica

2.4.1. Experiment 1 – time of treatment

Initially the treatment time was varied from 30 s to 120 s to have a significant reduction at 90 kV voltage with a 25 ml volume of tender coconut water with air as the working gas. After treatment with plasma, the samples were analysed for bacterial populations, immediately after treatment, as well as post-treatment storage for 24 h in a refrigerator.

2.4.2. Experiment 2 – development of tender coconut water simulant (TCWS)

Different solutions were prepared using distilled water along with the addition of specific amounts of sugars, minerals, protein, fats and acids to develop a coconut water stimulant. The composition was set according to previously published data for coconut water composition (Richter, de Jesus, Muñoz, Lago, & Angnes, L., 2005; Yong, Ge, Ng, & Tan, 2009). Several different simulants were prepared to understand the effect of components on HVACP treatments for *Salmonella* reduction. These solutions are listed in Table 1. Changes in pH and conductivity were noted before and after treatment for all the samples. Three independent replicates were carried out and the mean and standard deviations were reported. However, for the measurement of the changes in pH and conductivity, non-inoculated samples were treated.

2.4.3. Experiment 3 – effect of citric acid and L-ascorbic acid on S. enterica populations in HVACP treated tender coconut water

Different concentration of citric acid viz. 20 ppm, 200 ppm and 2000 ppm were added to tender coconut water and the samples were treated with HVACP in air for 60 s and 120 s for all three concentrations. In addition, for coconut water fortified with citric acid at 2000 ppm, another set of samples were treated for 30 s. This was done to assess whether the highest citric acid concentration enables reducing the plasma treatment time. Additionally, a comparison was made with 400 ppm citric acid in tender coconut water for microbial reduction using air (Table 1). Further, varying concentrations of L-ascorbic acid, i.e. 20 ppm, 200 ppm, 400 ppm and 2000 ppm were added to tender coconut water and treated using HVACP with air at 90 kV for 120 s.

2.4.4. Analysis of physico-chemical properties of tender coconut water post-HVACP treatment with air plasma

Tender coconut water samples were analysed for changes in physiochemical properties with and without addition of citric acid, before and after HVACP treatment in air at 90 kV for 120 s. All the properties were measured post-treatment after 24 h of storage under refrigeration. The pH was measured using a pH meter (Spectrum Technologies, USA), conductivity was measured using a conductivity meter (Extech, EC400, USA), total soluble solids was measured using a pocket refractometer (Atago, USA), the hydrogen peroxide content was tentatively estimated using chromogenic MQuant™ peroxide test strips (0.5–25 mg/l) (EMD Millipore, USA), light transmission was measured

Table 1
Effect of various components on microbial reductions with HVACP treatment with air plasma at 90 kV for 120 s.

#	Samples	Sample code	Initial pH	Final pH	Initial population (log_{10} cfu/ml)	Final population (log_{10} cfu/ml)
1.	Distilled water	–	6.91 ± 0.01^{a}	3.35 ± 0.01^{b}	9.23 ± 0.12^{x}	N.C.^{y}
2.	2.5 g glucose + 2.5 g fructose in 100 ml water	(S)	6.62 ± 0.06^{a}	3.35 ± 0.06^{b}	7.18 ± 0.01^{x}	N.C.^{y}
3.	200 mg KCl + 27.35 mg $CaCl_2$ in 100 ml water	(M)	7.15 ± 0.05^{a}	3.42 ± 0.08^{b}	6.79 ± 0.08^{x}	N.C.^{y}
4.	0.12 g egg albumin in100 ml water	(P)	6.20 ± 0.02^{a}	3.97 ± 0.07^{b}	6.93 ± 0.07^{x}	N.C.^{y}
5.	0.07 g soybean oil in 100 ml water	(F)	6.55 ± 0.04^{a}	3.34 ± 0.07^{b}	6.74 ± 0.12^{x}	N.C.^{y}
6.	2.5 g glucose + 2.5 g fructose + 200 mg KCl + 27.35 mg $CaCl_2$ + 0.12 g egg albumin + 0.07 g soybean oil in 100 ml water	(A6.5)	6.55 ± 0.08^{a}	4.31 ± 0.03^{b}	6.74 ± 0.08^{x}	N.C.^{y}
7.	2.5 g glucose + 2.5 g fructose + 200 mg KCl + 27.35 mg $CaCl_2$ + 0.12 g egg albumin + 0.07 g soybean oil in 100 ml water (buffered with 1% Na_2CO_3)	(A7.5)	7.50 ± 00^{a}	6.93 ± 0.11^{b}	6.73 ± 0.24^{x}	N.C.^{y}
8.	2.5 g glucose + 2.5 g fructose + 200 mg KCl + 27.35 mg $CaCl_2$ + 0.12 g egg albumin + 0.07 g soybean oil in 100 ml water (buffered with 1% Na_2CO_3)	(A8.5)	8.50 ± 00^{a}	7.02 ± 0.15^{b}	6.57 ± 0.04^{x}	N.C.^{y}
9.	2.5 g glucose + 2.5 g fructose + 200 mg KCl + 27.35 mg $CaCl_2$ + 37 mg Na_2HPO_4 + 30 mg $MgCl_2$ + 0.12 g egg albumin + 0.07 g soybean oil in 100 ml water (buffered with 1% Na_2CO_3)	(TCWS1)	7.50 ± 00^{a}	7.13 ± 0.12^{b}	7.47 ± 0.16^{x}	4.99 ± 0.18^{y}
10.	2.5 g glucose + 2.5 g fructose + 200 mg KCl + 27.35 mg $CaCl_2$ + 37 mg Na_2HPO_4 + 30 mg $MgCl_2$ + 0.12 g egg albumin + 0.07 g soybean oil in + 94.4 mg Malic acid + 2.4 mg citric acid + 2.4 mg ascorbic acid in100 ml water (buffered with 1% Na_2CO_3)	(TCWS2)	6.50 ± 0.00^{a}	5.82 ± 0.14^{b}	6.84 ± 0.06^{x}	5.35 ± 0.26^{y}
11.	Coconut water	–	6.00 ± 0.50^{a}	5.6 ± 0.35^{b}	7.04 ± 0.06^{x}	5.74 ± 0.40^{y}
12.	Coconut water + 400 ppm citric acid	–	5.35 ± 0.03^{a}	5.27 ± 0.02^{ab}	7.04 ± 0.06^{x}	N.C.^{y}

Number of samples evaluated (n) = 3; N.C. = no viable counts.
Values with different letters in superscript (a, b for pH and ×, y for microbial populations, respectively, before and after treatment) are significantly different at $P < 0.05$.

spectrophotometrically at 610 nm (Hitachi U 1100 Spectrometer, USA), as suggested by Jackson, Gordon, Wizzard, McCook, and Rolle (2004), the total titratable acidity was measured by titrating 5 ml of the samples with 0.1 N sodium hydroxide solution and the results were expressed as malic acid equivalents. The hunter L^* (lightness), a^* (greenness/redness) and b^* (blueness/yellowness) colour values were measured using a colorimeter (LabScan XE, USA). Ascorbic acid was added externally at a concentration of 400 ppm along with citric acid and the concentration was estimated using 2,6-dichlorophenol-indephenol dye titration method (Ranganna, 1986).

2.5. Ozone and nitrous gas measurement

The ozone and nitrous gas measurements were carried out using ozone and nitrogen oxides detection tubes obtained from pre-calibrated Dräger® gas detection tubes, Germany. The tubes change colour on contact with specific gas to be sampled. Immediately after the HVACP treatments, 2 ml and 4 ml of gas samples were accurately pulled out of packages using a hypodermic syringe for ozone and nitrous gas estimation, respectively. The pulled gases were passed through the specific gas detection tubes using a gas pump (Dräger-Tube Pump accuro®, Germany). The measurements were carried out for air as the working gas, at time interval from 30 s to 120 s. Further measurements were also taken for HVACP treatment when liquid samples were inside the box at 120 s for air as the working gas.

2.6. Optical emission spectroscopy (OES)

OES was carried out to detect the reactive gas species during the time of high voltage plasma generation. For the emission measurements, a custom fabricated optical fibre (Ocean Optics, USA) with a core diameter of 400 µm was lined in parallel to the box in which the plasma was generated. The entrance of the fibre was held 15 cm away from the package. The fibre was attached to a collimating lens (Ocean Optics, USA) of 5 mm diameter, which transferred the characteristic light emission on plasma generation to a computer controlled HR2000+ high resolution spectrometer operated through the OceanView software (Ocean Optics, USA). The grating spectrometer had a 10 µm slit width with an optical resolution of 0.88 nm. The spectrometer was pre-calibrated by the manufacturer using a mercury-argon atomic line source. The dark current measurements were recorded by the software between each successive measurement and subtracted from the measurements. When the plasma was generated on voltage application the emission spectrum were acquired with an integration time of 5 s and an average of 6 scans was used for reporting purposes. The experimental setup is as shown in Fig. 1.

Emission spectrum of the discharge was recorded for both, empty packages, as well as packages with tender coconut water. The characteristic molecular and atomic transitions associated with the spectral bands and lines were identified using published reports and NIST (National Institute of Standards and Technology) database (NIST, 2018).

2.7. Cell membrane integrity

The integrity of the bacterial cell membranes was assessed according to the protocol described by Lu, Patil, Keener, Cullen, and Bourke (2014). The integrity was examined by determination of the release of UV absorbing materials at 260 nm and 280 nm. For cell leakage, three bacteria inoculated samples were considered viz., TCWS2 but without egg protein, TCWS2 treated with air plasma (TCWS2 + Air), TCWS2 with addition of 400 ppm citric acid, and TCWS2 with addition of 400 ppm of citric acid that was air plasma treated (TCWS2 + CA + Air). The samples were centrifuged at 13,200*g* for 10 min in a centrifuge (Avanti J-26XP, Beckman Coulter, Inc. USA) and 200 µl from the supernatant was carefully transferred into microtitre plates and measured at 260 nm and 280 nm using a microplate reader (Epoch, BioTeck, USA).

2.8. Scanning electron microscopy (SEM)

The effect of HVACP treatment with air on ST2 was carried out by using the tender coconut water stimulant (TCWS2) with added 400 ppm citric acid as the liquid media. Non-treated samples were taken as the control. One ml of both, inoculated controls and treated samples, were primarily fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h at 4 °C and few drops of the fixed cells were transferred to coverslips coated with 0.1% poly-L-lysine. These samples were then post-fixed in 1% osmium tetroxide and dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90% and 100%). Finally, the slips were transferred into hexamethyldisilazane and air dried. Dried specimens were coated with platinum in a Cressington 208HR sputter coater (Ted Pella, Inc, USA) and imaged in a FEI Nova NanoSEM 200 (FEI Ltd, USA) at 5 kV and the images were taken at 20,000× magnification.

2.9. Transmission electron microscopy (TEM)

For TEM, control TCWS2 inoculated with *S. enterica* cells and HVACP treated TCWS2 with 400 ppm citric acid inoculated with *S. enterica* were compared. The sample preparation for TEM was also the same as for the SEM protocol, with the exception of post-fixing with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and the samples were negative stained with aqueous 2% uranyl acetate. Images were acquired on a Philips CM100 microscope (FEI Ltd, USA) equipped with a LaB6 electron source and operated at 100 kV.

2.10. Statistical analysis

All the experiments were carried out in triplicate and the data were presented as mean ± standard deviation. Statistical methods for data involved analysis of variance, and the significance of differences between paired treatments using *t*-test for multiple sample comparison. Duncan multiple range test was carried out for HVACP led inactivation in presence of citric acid and ascorbic acid, as well as for the cell leakage data. The significance level was tested at an alpha level of 0.05 (*P*-value) using SPSS software ver. 20 (IBM® SPSS® Statistics 20). Identification of the peaks and their locations in optical emission spectrum was carried out in MATLAB (The Mathworks, MA, USA).

3. Results and discussion

3.1. Ozone and nitrous gas generation

Ozone and nitrous gas measurements were carried out immediately after HVACP treatment for air as the working gas. Ozone predominantly forms via a three-body collision reaction in the air plasma (involving an intermediate species), while the generation of nitrogen oxide (NO) and nitrogen dioxide (NO_2) species from nitrogen is interlinked with the ozone formation, via the following reactions (Misra, Pankaj, Frias, Keener, & Cullen, 2015; Moiseev et al., 2014)-

$$N + O_2 \rightarrow NO + O \quad (1)$$

$$N + NO \rightarrow N_2 + O \quad (2)$$

$$O_2 + O + M \rightarrow O_3 + M \quad (3)$$

$$NO + O_3 \rightarrow NO_2 + O_2 \quad (4)$$

An increase in ozone and nitrous gas ($NO + NO_2$) concentrations was noted with the increase in treatment time, as shown in Fig. 1 (inset). After 120 s of air plasma, empty packages had an ozone and nitrous gas concentration of 8750 ± 1250 ppm and 2080 ± 320 ppm respectively. Further, when the tender coconut water was present inside

the package, the ozone and nitrous gases concentration were 4170 ± 720 ppm and 580 ± 60 ppm, respectively at the end of 120 s. Thus, it was inferred that in the presence of tender coconut water in the package, the reactive oxygen and nitrogen species (RONS) diffused into the liquid, thereby showing relatively lower concentrations in the gas phase. However, a minor contribution of the decreased overall gas available in the package from introducing the coconut water cannot be overruled. The solubility of ozone and nitrous gases have also been documented by researchers (Lee et al., 2016). Ozone being a strong broad spectrum antimicrobial agent (Khadre, Yousef, & Kim, 2001), its generation in HVACP renders the antimicrobial activity. Research suggests that nitrous gases and their derivatives, majorly peroxynitrite ($ONOO^-$) generated in liquids during plasma treatment are effective antimicrobial agents (Oehmigen et al., 2010). Therefore, both ozone and nitrous gas species were attributed to the bactericidal effects in the current work. It is well- known that water in the discharge space results in the formation of hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2), from the action of the diffusing reactive oxygen species in the gas phase, which are known antimicrobial agents (Misra & Jo, 2017).

3.2. Optical emission spectroscopy (OES)

The humid air plasma chemistry is known to be quite complex, involving thousands of reactions and dozens of species. Emission spectroscopy of plasma allows the study of the nature of excited species in plasma. The time resolved difference of the emission spectra for air, with and without coconut water sample, during the air plasma treatment is shown in Fig. 2. From the emission spectra for air plasma with or without sample the presence of strong emissions in the wavelength range of 315–405 nm were recorded. These were identified as transitions from nitrogen second positive system, N_2(C-B) and first negative system, N_2^+ (B-X).

The spectrum presented here for nitrogen transition is similar to the spectrum presented by different authors (Connolly et al., 2013; Misra, Keener, Bourke, & Cullen, 2015). The band heads of the $N_2(C^3\Pi_u \rightarrow B^3\Pi_g)$ second positive system were recorded around 336.9 nm, 357.3 nm, 380.0 nm and 405.4 nm, while the spectral emission of the nitrogen monopositive ion $N_2(B^2\sum_u^+ \rightarrow X^2\sum_g^+)$ was recorded at 390.6 nm and 427 nm with relatively low intensities. The intense spectral signatures are testimonial to the occurrence of energetic collisions of electrons with molecular nitrogen in air. The electron energy density function (EEDF) in plasma is an important characteristic governing the plasma chemistry. The EEDF is related to the rate of ionization by electron collisions, the gas temperature, and the effectiveness of energy transfer to the gas molecules. The N_2 emission band head at 390.6 nm is an outcome of the direct electron excitation from the ground level as a response to the high-energy electrons of the EEDF, whereas the N_2 emission at 336.9 nm is sensitive to the low-energy electrons. The relatively low intensity of N_2 emission at 390.6 nm as compared to emission at 336.9 nm emphasizes the low degree of ionization, and hence also the low temperature of the plasma source. The hydroxyl molecular band, $A_2(\Sigma^+ \rightarrow X^2\Pi)$ was recorded at a very low intensity with a band-head at 308.7 nm. The oxygen molecular band around 762 nm was also recorded, which is associated with the vibrations of the $O_2(b_1\Sigma_g^+ \rightarrow X_3\Sigma_g^-)(0, 0)$ magnetic dipole transition. The band at 873 nm was suspected to have originated from the 3s ^{4}p-3p^{4}D^0 transition in atomic nitrogen. Intensities of peaks of the OES spectra for air when coconut water was present in the package was lower compared to the corresponding peaks when the package was empty, as evident from the positive peaks after the difference (Fig. 2). This is dominantly due to the solubilisation of reactive gas species in tender coconut water, with a likely minor contribution from the comparatively lower total gas available from introduction of the samples. The reactive gas species generated in air plasma, and their solubilization products are well-proven to possess antibacterial effects (Arjunan, Obrusník, Jones, Zajíčková, & Ptasinska, 2016). As a final note, it is worthwhile noting that the plasma emission in UV region is very limited, and therefore, the antibacterial action of the plasma source is dominated by the reactive oxygen and nitrogen species.

3.3. Optimizing HVACP to achieve the 5-log reduction of S. enterica

3.3.1. Treatment time for Salmonella reduction

Inoculated tender coconut water when treated with air plasma led to a 1.30 ± 0.30 log reduction for a treatment time of 120 s and after storage under refrigeration for 24 h. The comparison of *Salmonella* reduction in tender coconut water immediately after treatment and post 24 h storage in refrigeration are shown in Fig. 3(A). The figure shows that lesser microbial reduction was observed when microbial recoveries were carried out immediately than post storage for 24 h. Considering the observed maximum reduction, all further experiments were carried out at the fixed plasma treatment condition of 90 kV for 120 s with air as the working gas and a post-treatment storage for 24 h under refrigerated conditions. The generation of ozone, nitrous gases and the reactive gas species of oxygen and nitrogen are attributed towards the microbial reductions in the experiments as mentioned in previous section. Furthermore, as the samples were directly kept within the inter-electrode space, an electroporation effect from the high electric field may also be responsible for microbial load reduction (Zimmermann et al., 2012). However, confirmation of this hypothesis will require

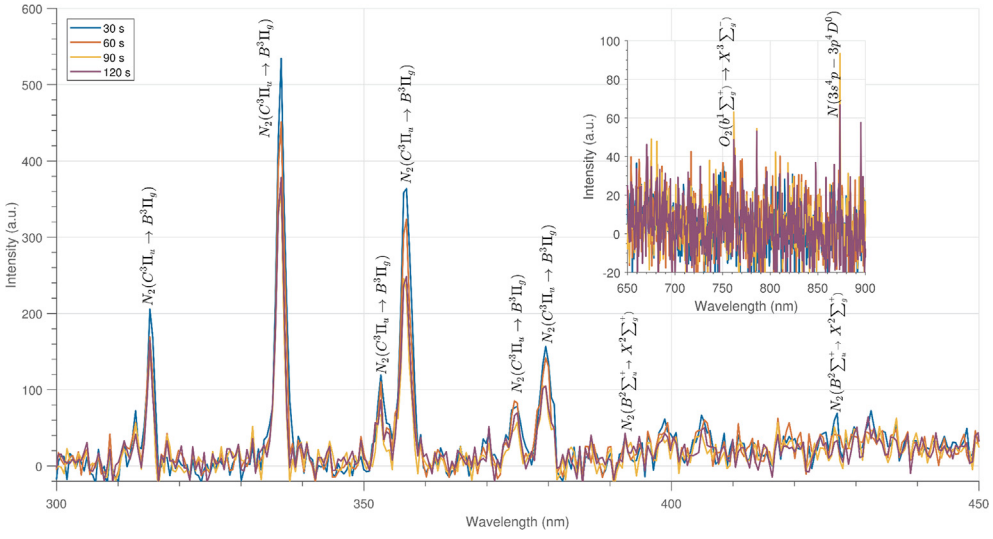

Fig. 2. Time resolved differential optical emission spectrum for HVACP treatment in empty package (without tender coconut water) and package with tender coconut water sample. Inset shows the relevant peaks in the red and near infrared region of the emission spectrum. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

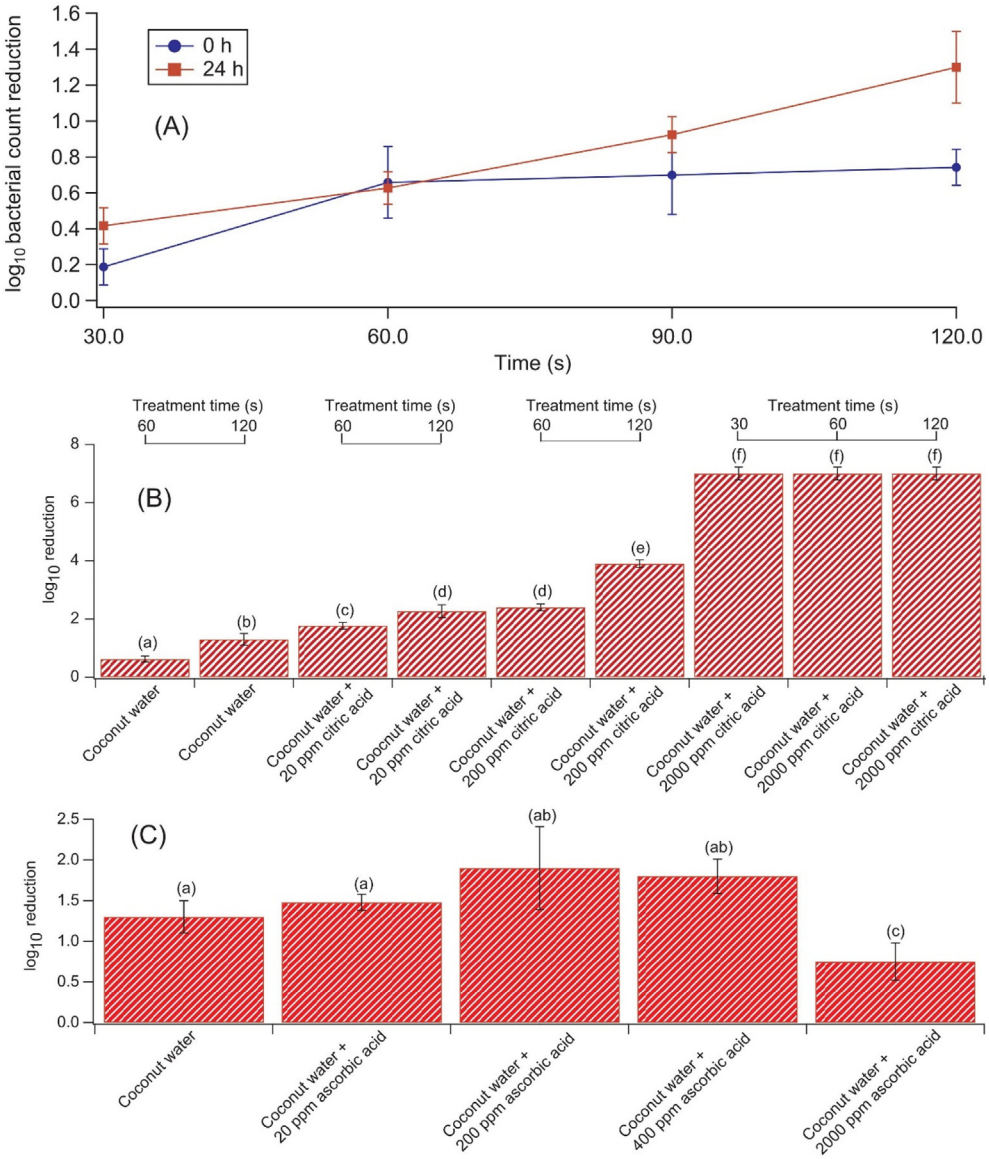

Fig. 3. (A) Reduction of *S. enterica* in coconut water as a function of treatment time using air plasma at 90 kV after 0 h and 24 h storage under refrigeration. (B) *S. enterica* reduction at different treatment times with varying citric acid concentration, and (C) with varying concentration of ascorbic acid using HVACP at 90 kV for 120 s using air as the working gas. The different alphabets a, b, c etc. denote significantly different values at $P < 0.05$.

dedicated and extensive experimental work.

3.3.2. Development of tender coconut water simulant and understanding the reduction of S. enterica population through HVACP treatment

HVACP treated tender coconut water showed a 1.3 ± 0.30 log reduction at 120 s plasma treatment time and a pH reduction from 6.00 ± 0.5 to 5.6 ± 0.35. The simulated tender coconut water mix was prepared to evaluate the effect of specific ingredients which might interact with the plasma treatment. Specific solutions of the components under study are as shown in Table 1. These solutions were treated with HVACP in air at 90 kV for 120 s and the measurements were taken 24 h post refrigerated storage similar to the treatment provided for tender coconut water. Initially, on checking the effect of HVACP treatment on inoculated distilled water, a complete reduction of *Salmonella* within 120 s treatment followed by a change in pH of distilled water from 6.91 ± 0.01 to 3.35 ± 0.01 was observed. Further, solution mix of only sugars (S) or mineral solution (M) of potassium chloride and calcium chloride or only egg albumin protein (P) or only soybean oil (F) in water or a mix of sugar, protein, potassium chloride and calcium chloride (A6.5) also showed similar acidification and complete microbial reduction after HVACP treatment. Acidification in plasma treated liquids is due to the generation of reactive gas species of nitrogen which generate nitrous acids causing a subsequent pH decrease (Oehmigen et al., 2010). Such a drastic decrease in pH was not observed for tender coconut water treatment; thus, initially it was inferred that lesser acidification might be the probable cause for limited microbial reduction. Subsequently, the solutions with different components were buffered with 1% sodium bicarbonate and the pH was adjusted to 7.50 ± 0.00 (A7.5) and 8.50 ± 0.00 (A8.5) to add more buffering so as to reduce the effect of acidulation. Thereafter, the solutions were treated with plasma again causing a complete microbial reduction but with minimum change in pH of the solutions, as the pH fell to 6.93 ± 0.11 and 7.02 ± 0.15 from the initial values of 7.5 and 8.5 respectively. Thus, it was inferred that acidification alone was not the major cause for microbial reduction after plasma treatment, but also the generated reactive gas species were majorly responsible for the microbial deaths. The link between reactive gas species and the microbial inactivation is well-established in literature (Gaunt et al., 2006;

Guo, Huang, & Wang, 2015; Laroussi & Leipold, 2004; Naitali, Kamgang-Youbi, Herry, Bellon-Fontaine, & Brisset, 2010), which is in line with the results of this study.

The addition of sodium phosphate dibasic and magnesium chloride to the simulated mixture containing other components and along with buffering from sodium carbonate resulting in a pH of 7.5 ± 00 (TCWS1) posed hindrance to microbial reduction from HVACP treatments. The plasma treatment in TCWS1 decreased the initial microbial load from 7.47 ± 0.16 to 4.99 ± 0.18 log CFU/ml. Next, to the same solution, when other acid components like malic acid, ascorbic acid and citric acid were added and the pH of the solution adjusted to 6.5 ± 0.00, the HVACP treatment resulted in a microbial reduction form 6.84 ± 0.06 to 5.35 ± 0.26 log CFU/ml. Therefore, TCWS2 mix was considered as the appropriate simulant that exhibited similar microbial reduction response under HVACP treatment as compared to tender coconut water. Additionally, it could be inferred that the presence of phosphate and magnesium ions could be a reason for lower microbial reduction in TCWS1 and TCWS2. Phosphates and magnesium being important nutrients, these may have facilitated in providing the bacterial cells a point of recovery from disruption and cellular damage due to plasma treatment. Phosphates and magnesium ions are both responsible for cellular maintenance including synthesis of ATP, nucleic acids, phospholipids, proteins and acting as cofactors for various biosynthetic pathways and enzymatic reactions (Groisman et al., 2013; Juhna, Birzniece, & Rubulis, 2007). Thus, the absence of these two micronutrients in distilled water as well as solutions S, M, P, F, A6.5, A7.5 and A8.5 might have impaired the bacterial cells to not have any recovery from the damage of cellular components upon plasma treatments as no repair mechanisms related to these nutrients were in place. The presence of phosphate and magnesium ions in coconut water has been confirmed by Richter et al. (2005). Thus, these two nutrients interfere with plasma inactivation mechanisms.

3.3.3. Effect of citric acid concentration on S. enterica population and HVACP process time

From Fig. 3(B) it can be observed that with an increase in citric acid concentration, an increase in microbial reduction can be established. It may also be observed that with an increased concentration of citric acid i.e. at 2000 ppm, more than 5 $\log_{10}$ reduction of *S. enterica* was achieved in 30 s rather than 120 s of treatment time. As another observation, the 400 pm citric acid addition to tender coconut water was sufficient to achieve a 5 $\log_{10}$ reduction of *S. enetrica* with HVACP, thereby meeting the approved performance standard for an effective antimicrobial process as given by FDA (2001).

Citric acid did not have any antimicrobial activity at the concentrations studied, rather along with HVACP treatment a synergistically increased antimicrobial activity was found. One of the reasons of this increased antimicrobial effect could be associated with the hydrogen peroxide generated during HVACP treatment as recorded in Table 2. This data suggests that hydrogen peroxide was more stable in HVACP treatment when citric acid was present in the system. Other theories that could explain the observations are- (i) due to electroporation, citric acid more readily diffused into the microbial cells through the plasma membrane causing acidification of the cytoplasm and thereby stopping cellular growth (Lambert & Stratford, 1999); (ii) the possible generation of peroxy form of citric acid upon HVACP treatment, due to reaction with hydrogen peroxide causing generation of peroxyorganic acid/organic acid systems which is known to have a better antibacterial efficacy than that of the parent organic acid (Marriott, Schilling, & Gravani, 2018). On the other hand, synergistic effect of hydrogen peroxide and organic acids as antimicrobials have also been observed (Martin & Maris, 2012). With ascorbic acid addition in tender coconut water, large reduction in microbes were not seen; rather, a concentration of 2000 ppm resulted in significantly lower reduction as compared to tender coconut water no added ascorbic acid (Fig. 3(C)). Ascorbic acid being an antioxidant, the generated free radicals in HVACP treatment might have been neutralized thereby hindering the antimicrobial efficacy of plasma treatment. A concentration of 400 ppm ascorbic acid when added to coconut water and treated with HVACP, decreased the ascorbic acid content by 84.35% (see, Table 2) suggesting that the ascorbic acid might have been used up in neutralizing the free radicals.

3.3.4. Effect on physicochemical properties of tender coconut water following HVACP treatment in air

Table 2 shows the changes in various physico-chemical parameters of tender coconut water upon treatment with HVACP in air at 90 kV for 120 s, with and without added citric acid. It was observed that a significant change in pH occurred for tender coconut water after air plasma treatment. However, the pH change for tender coconut water with added citric acid although had a lesser pH than normal tender coconut water, the observed change after plasma treatment was not significant ($P > 0.05$). A minor increase in the electrical conductivity was observed for tender coconut water, irrespective of citric acid addition. The solubilisation of the reactive gas species could explain the increased electrical conductivity. Such increase in conductivity was also observed in plasma treated grape juice (Pankaj, Wan, Colonna, & Keener, 2017).

The ascorbic acid content of tender coconut water seemed unaffected by plasma treatment in air. However, on addition of 400 ppm of ascorbic acid to coconut water and subjecting it to HVACP treatment, a decrease of 84.35% of ascorbic acid occurred. Reduction of ascorbic acid in strawberries, orange and apple juices following exposure to cold plasma has been reported in literature (Misra et al., 2015; Pankaj et al., 2018). Ascorbic acid being a free radical scavenger, the decrease may be due to neutralization of free radicals generated during plasma treatment and conversion to dehydroascorbic acid. This suggests that when ascorbic acid is to be used as a preservative for plasma treated products it must be added after plasma treatment. The plasma treatment was found to result in generation of hydrogen peroxide at concentrations of 10 mg/l in tender coconut water, both with and without addition of citric acid. Generation of hydrogen peroxide in liquids on exposure to cold plasma has also been demonstrated in the past by Liu et al. (2015). The hydrogen peroxide generated from plasma discharge is attributed as one of the reasons for the antimicrobial action of HVACP technology (Gaunt et al., 2006).

The total soluble solids and clarity of tender coconut water did not show any significant changes after plasma treatment as compared to the controls for both, tender coconut water and tender coconut water with citric acid. A minor decrease in total titratable acidity was observed following plasma treatment of the tender coconut water (with or without citric acid), which could be attributed to the likely production of hydroxyl radical in the aqueous phase (Pankaj et al., 2017). It is worthwhile mentioning that the observed decrease in acidities were statistically insignificant ($P > 0.05$).

Although no perceived colour changes in the samples were observed after plasma treatment but with the instrumental measurements an increase in L^* values for plasma treated samples was recorded. This is likely to be due to the formation of a few aggregates of the solids in the coconut water following plasma treatment. The nature of these materials is expected to be proteinaceous or a mixture of proteins and oligosaccharides which could originate from polysaccharide breakdown products. However, this proposition needs to be confirmed through further studies. Breakdown of polysaccharides in prebiotic orange juice treatment following cold plasma treatments was recently reported (Almeida et al., 2015). While the a^* values for air plasma treated coconut water did not have a significant change, the addition of citric acid prior to plasma treatment shifted the colour towards red region. The b^* values for tender coconut water on air plasma treatment showed an increase for samples with or without citric acid.

Table 2
Changes in physiochemical parameters on HVACP treatment of coconut water with air plasma at 90 kV for 120 s.

#	Parameter	Sample	Initial values	Post-plasma values
1.	pH	Coconut water	6.29 ± 0.06^{a}	6.00 ± 0.03^{b}
		Coconut water + 400 ppm citric acid	5.35 ± 0.03^{a}	5.27 ± 0.02^{ab}
2.	Conductivity (mS)	Coconut water	8.15 ± 0.05^{a}	8.13 ± 0.02^{a}
		Coconut water + 400 ppm citric acid	7.76 ± 0.00^{a}	8.17 ± 0.12^{b}
3.	Total soluble solids (°Brix)	Coconut water	7.50 ± 0.01^{a}	7.50 ± 0.01^{a}
		Coconut water + 400 ppm citric acid	7.60 ± 0.01^{a}	7.60 ± 0.01^{a}
4.	Ascorbic acid (mg/100 ml)	Coconut water	5.87 ± 1.27^{a}	5.87 ± 0.73^{a}
		Coconut water + 400 ppm citric acid + 400 ppm ascorbic acid	48.50 ± 0.73^{a}	7.59 ± 1.12^{b}
5.	H_2O_2 content (mg/1000 ml)	Coconut water	0 ± 0.00^{a}	10 ± 0.00^{b}
		Coconut water + 400 ppm citric acid	0 ± 0.00^{a}	10 ± 0.00^{b}
6.	Transmission (%)	Coconut water	88.70 ± 0.62^{a}	89.00 ± 0.30^{a}
		Coconut water + 400 ppm citric acid	88.90 ± 0.25^{a}	89.5 ± 0.04^{a}
7.	Total titratable acidity (%)	Coconut water	0.155 ± 0.003^{a}	0.140 ± 0.003^{b}
		Coconut water + 400 ppm citric acid	0.218 ± 0.003^{a}	0.210 ± 0.010^{a}
8.	L^* value	Coconut water	16.53 ± 0.05^{a}	16.80 ± 0.03^{b}
		Coconut water + 400 ppm citric acid	12.50 ± 0.04^{a}	15.73 ± 0.07^{b}
9.	a^* value	Coconut water	−0.37 ± 0.12^{a}	−0.40 ± 0.07^{a}
		Coconut water + 400 ppm citric acid	−0.37 ± 0.07^{a}	−0.19 ± 0.09^{b}
10.	b^* value	Coconut water	4.72 ± 0.10^{a}	5.16 ± 0.04^{b}
		Coconut water + 400 ppm citric acid	2.88 ± 0.15^{a}	4.94 ± 0.08^{b}

Number of samples evaluated (n) = 3.
Values with different letters are significantly different at $P < 0.05$ before and after treatment.

3.4. Cell membrane integrity

Fig. 4(A) presents the data pertinent to release of UV absorbing intracellular components which includes the nucleic acids absorbing at 260 nm, and the proteins absorbing at 280 nm. A significant increase in release of intracellular components was not observed for plasma treated simulated tender coconut water *vis-à-vis* untreated samples. This was likely to be due to the formation of reversible pores suggesting a repair mechanism in-place in the cells; thus, the release of intracellular material was still controlled. A significant increase in release of intracellular components was however, observed on addition of citric acid, as recorded from the light absorption. The release of intracellular materials from bacterial cells after plasma treatment has also been observed by Lu et al. (2014). In an earlier study using helium and oxygen radio-frequency plasma, it was observed that even *E. coli* cells had severe cytoplasmic deformations due to membrane disruption and leakage of bacterial chromosome following exposure to plasma (Hong et al., 2009).

3.5. Scanning electron microscopy and transmission electron microscopy

Fig. 4(B) and (C) show the untreated *S. enterica* cells under SEM and TEM respectively. Under SEM it was observed that the untreated cells remained intact while plasma treated cells were deformed with notable shrinkage (Fig. 4(D)). Under TEM, debris of disintegrated cells could be observed, which confirmed the inactivation and cell lysis in the bacteria (Fig. 4(E)). This observation is in line with previous reports, wherein the cellular disintegration was observed after cold plasma treatment (Han et al., 2016; Surowsky et al., 2014; Xu et al., 2017). It is to be clarified that some granular or crystal structures were observed on the flagella of the cells under SEM, and these were due to the presence of mineral crystals that did not wash away during the sample preparation. A detailed review of the mechanisms underlying bacterial inactivation was recently presented by Liao et al. (2017). In conclusion, the electron microscopic studies revealed that the plasma treatment resulted in a loss of viability of the bacterial cells.

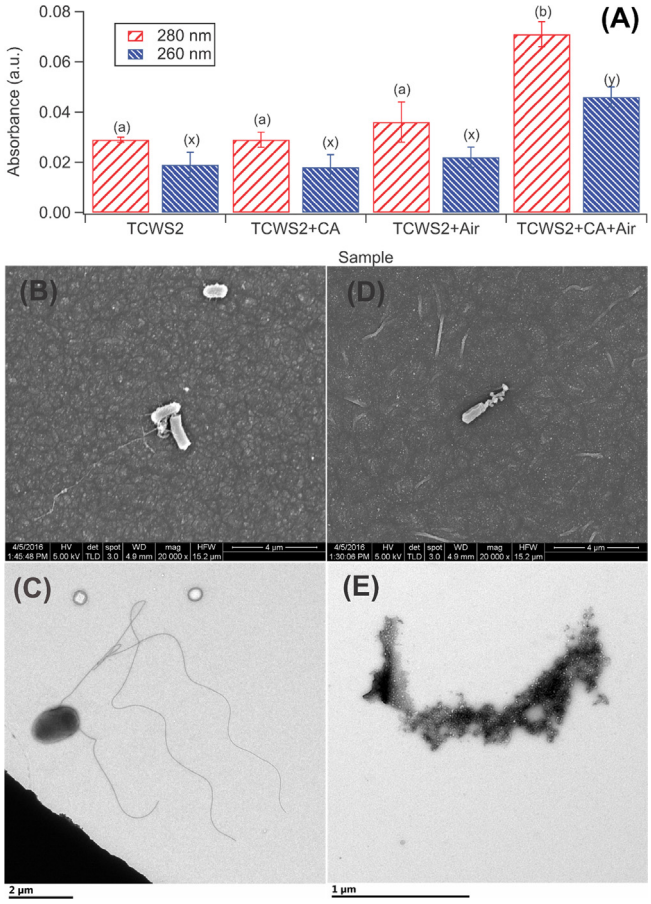

Fig. 4. (A) Cell leakage at 280 nm and 260 nm of *S. enterica* in simulated mix (TCWS2) with and without citric acid after HVACP treatment at 90 kV for 120 s. The different alphabets, (a, b) and (x, y) denote significantly different values at $P < 0.05$ for absorbance at 280 nm and 260 nm, respectively. (B) and (D) shows the morphological characteristics of *S. enterica* observed under SEM respectively before treatment with air plasma, (C) and (E) shows the morphological changes of *S. enterica* after HVACP treatment in air.

4. Conclusion

The study shows that non-thermal HVACP treatment at 90 kV for 120 s followed by 24 h refrigerated storage was effective in achieving 5-

log_{10} reduction of *S. enterica* in tender coconut water when 400 ppm of citric acid was added before treatment. The results fulfil the 5-log_{10} reduction criteria approved for juice processing (FDA, 2001). The development of a simulated tender coconut water mix helped to show that the acidification was not the major reason for microbial inactivation, but rather that the reactive gas species were responsible for microbial reductions. Further generation of hydrogen peroxide and a supposed generation of other compounds or antimicrobial systems might be a possible cause for higher reduction due to citric acid addition. The presence of magnesium and phosphate in tender coconut water were supposed to be the reasons for lower microbial efficiency in tender coconut water. Presence of other ingredients like sugars, protein and oil did not affect the microbial reduction due to plasma treatment. Furthermore, electron microscopy showed that plasma treatment caused cell destruction, which was again confirmed by cell integrity data which showed more leakage on HVACP treatment in presence of citric acid. Increased citric acid concentration facilitated a rapid plasma treatment process time. The physico-chemical parameters did not have any drastic impact on plasma treatment with air as a working gas, whereas ascorbic when added externally was found to have depleted after the plasma treatment. More experiments and analysis need to be carried out to unravel the specific action and interaction of citric acid with HVACP that allows achievement of significant microbial reductions, which is an important finding of this work. In addition, further work is also needed to verify the possibility of the bacteria entering a viable but non-culturable (VBNC) state using metabolic or molecular assays. In summary, however, the results allow the conclusion that HVACP technology would be an effective non-thermal processing intervention for microbial inactivation in tender coconut water.

Acknowledgements

Nikhil acknowledges the United States-India Education Foundation for Fulbright-Nehru doctoral and professional research grant (IIE Grantee ID 15151385) that allowed him to work at the Department of Food Science, Purdue University. Authors acknowledge Jeanett L. Jensen, Zifan Wan, Lei Shelly Xu and Ximena Yepez for their support and assistance.

Conflict of interest

Authors declare no conflict of interests.

Declaration of interest

Patent applied for with the application no. PCT/US2017/03269.

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