Korean J. Food Sci. An.
Vol. 33, No. 3, pp. 317~324(2013)
DOI http://dx.do.org/10.5851/kosfa.2013.33.3.317

ARTICLE

Inactivation of *Campylobacter jejuni* using Radio-frequency Atmospheric Pressure Plasma on Agar Plates and Chicken Hams

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Abstract

Radio-frequency driven atmospheric pressure plasma using argon gas was studied in the inactivation of *Campylobacter jejuni* in order to investigate its applicability. First, the inactivation study was conducted on an agar surface. *C. jejuni* NCTC11168 was reduced by more than 7 Log CFU after an 88 s treatment. Another strain, ATCC49943, was studied; however, the inactivation was less efficient, with a 5 Log CFU reduction after a 2 min treatment. Then, chicken breast ham was studied at the10^6 CFU inoculation level. The inactivation efficiency was much lower for both strains compared to that on the agar plates. *C. jejuni* NCTC11168 and ATCC49943 were reduced by 3 Log CFU after a 6 min treatment and by 1.5 Log CFU after a 10 min treatment, respectively. The scanning electron microscopy analysis indicated that *C. jejuni* cells were deformed or transformed into coccoid form under the plasma treatment. During the plasma treatment, the temperature of the samples did not rise above 43°C, suggesting that heat did not contribute to the inactivation. Meanwhile, water activity significantly decreased after a 10 min treatment ($p<0.05$). This study conveyed that radio-frequency atmospheric pressure plasma can effectively inactivate *C. jejuni* with strain-specific variation.

Key words: *Campylobacter jejuni*, radio-frequency atmospheric pressure plasma, inactivation, chicken breast ham, scanning electron microscopy

Introduction

Recently, interest in the non-thermal inactivation of microorganisms causing food-borne illnesses has increased. Non-thermal sterilization has an advantage over traditional thermal sterilization in that it can inactivate pathogens without deteriorating the sensory qualities of the food such as taste and flavor. Many non-thermal applications such as pulsed electric fields and high hydrostatic pressures have been studied and developed to satisfy the high demands for fresh foods (Senorans *et al*., 2003). In the same context, interest in non-thermal plasma technology has recently increased to study the feasibility of this technique for the inactivation of food-borne pathogenic bacteria.

A plasma is an ionized gas, the 'fourth state of matter'. As energy increases, the state of the material changes from solid to liquid, then to gas, and then to plasma. Gas such as argon or oxygen can be ionized using electrical energy and the ionization produces ionized gas, electrons, neutrons, free radicals, and UV (Tendero *et al*., 2006; von Keudell *et al*., 2010). Plasma, especially the ions, can break chemical bonds as well as etch and damage biomolecules by the so-called 'chemical sputtering' (Moreau *et al*., 2008; von Keudell *et al*., 2010). Its oxidizing power and UV can also contribute to the damage process. Plasma has many applications such as wound healing, surface modification, and the sterilization of heat-sensitive medical instruments (Laroussi, 2005). It has been recently demonstrated that plasma can effectively inactivate various food-borne pathogenic bacteria such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Campylobacter, Salmonella,* and *Staphylococcus aureus* (Critzer *et al*., 2007; Dirks *et al*., 2012; Niemira and Sites, 2008; Sureshkumar *et al*., 2010; Yun *et al*., 2010). For example, *E. coli* O157:H7 was reduced by 5 Log CFU and 3 Log CFU on agar plates and apples, respectively, after a 1-2 min treatment using a one atmosphere uniform glow discharge plasma (Critzer *et al*., 2007). Radio-frequency (RF) plasma reduced *S. aureus* by 6 Log CFU using a N_2-O_2 mixture (Sureshkumar *et al*., 2010).

Campylobacter jejuni is one of the major food-borne pathogenic bacteria worldwide (Scallan *et al*., 2011; Young and Mansfield, 2005). This species is highly associated

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with poultry such as chicken, turkey, and duck (Humphrey *et al*., 2007). It causes diarrhea, abdominal cramps, and fever in humans even though most patients recover from the symptoms without any treatment (Young and Mansfield, 2005). Non-thermal dielectric barrier discharge plasma has been shown to reduce *C. jejuni* and *S. enterica* on the surface of chicken breast and thigh by 1-3 Log CFU (Dirks *et al*., 2012). In this study, we examined the ability of radio-frequency (RF) non-thermal plasma to inactivate *C. jejuni* on agar plates and chicken breast hams.

Materials and Methods

C. jejuni strains, growth conditions, and preparation of inocula

C. jejuni NCTC11168 and ATCC49943 purchased from the American Type Culture Collection (USA) were used in this study. Bacterial strains in glycerol stocks at -80°C were retrieved and grown on tryptic soy agar supplemented with 5% (v/v) sheep blood (TSAB) at 37°C under microaerobic conditions (6-12% (v/v) O_2, 5-8% (v/v) CO_2) generated using AnaeroPack-MicroAero (Mitsubishi Gas Chemical Co., Japan). After 32-48 h of incubation, the cultures were suspended in tryptic soy broth at an optical density (OD) of 0.1-0.2 at 600 nm. An aliquot (100 µL) of each culture was spread on TSAB plates and grown for an additional 14-15 h under the same conditions. The culture was suspended in tryptic soy broth at OD 0.1-0.2 (10^8-10^9 CFU/mL) using cell scrapers and then used as an inoculum.

Plasma apparatus and treatment conditions

An RF-driven atmospheric pressure plasma unit was used in this study (Fig. 1). This unit has an in-line plasma treatment system, and the length of plasma discharge is 160 mm. Argon was used as a gas for plasma generation at a fixed flow rate of 20,000 sccm with 200 W power. The argon gas was flushed into a horizontal electrode at which the plasma was generated. The plasma generated was then pushed downward in ambient air. The apparatus was equipped with a sample pedestal (15 cm×15 cm) on which samples were placed for exposure to the plasma treatment. The distance between the plasma discharge and the samples was approximately 2.0-2.5 cm. A cooling pump was operated at 15°C to circulate a coolant to prevent the discharge unit from being overheated. For uniform plasma exposure, the sample pedestal moved back and forth in a range of 6.0 cm at 1.5 cm/s in operation.

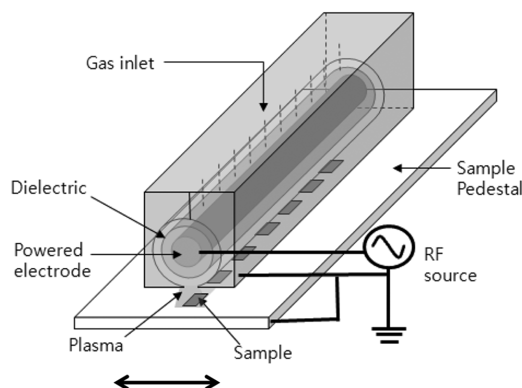

Fig. 1. Diagram of radio-frequency driven atmospheric pressure plasma used in this study.

Plasma treatment on the agar plates

Aliquots (100 µL) of the prepared inoculum were gently spread (10^7-10^8 CFU) with a pipette tip on plate count agar (BD, USA) in a 3.5 cm diameter circle. Then, the plates were semi-dried (15 min-1 h) in air. The plates were exposed to the plasma treatment for 0 s, 12 s, 24 s, 40 s, 1 min, 1 min 28 s, 2 min, or 5 min in duplicates. After the plasma treatment of the samples, the bacterial cells were suspended in 10 mL of sterile 0.85% NaCl solution from the agar plates using cell scrapers, serially diluted in 0.85% NaCl solution by 10-fold to 10^{-5}, and then the suspension and each dilution were spread on TSAB plates. The plates were incubated at 37°C under microaerobic conditions for approximately 48 h, and the colonies grown on the plates were enumerated to determine the number of viable cells. By this method, around 100% of the viable cells were recovered from the agar plates.

Plasma treatment on the chicken breast hams

Chicken breast sliced hams (1 mm thick) were purchased from the markets and cut into 1 cm×1 cm pieces. The ham samples were placed on small petri dishes and inoculated with aliquots (10 µL) of the inoculum at 10^6-10^7 CFU. The inoculum was gently spread over the surface of the ham samples with a pipette tip. After incubation at room temperature for approximately 5 min, the samples were exposed to the plasma treatment for 2 min, 4 min, 6 min, 8 min, or 10 min in duplicates. After the plasma treatment, the samples were vigorously agitated in 5 mL of sterile 0.85% NaCl solution by vortex for 1 min. Then, the supernatants were serially diluted in 0.85% NaCl solution by 10-fold to 10^{-5}. The supernatants and each dilution were spread on TSAB supplemented with cefoperazone (32 µg/mL), vancomycin (10 µg/mL), and amphotericin B (10 µg/mL) for the selective growth of *Campylobacter*. The

plates were incubated at 37°C under microaerobic conditions for 64-72 h, and the colonies grown on the plates were enumerated. A negative control group of ham samples without any inoculation yielded no colonies on the plates.

Scanning electron microscopy (SEM) pictures

To prepare samples for SEM, *C. jejuni* cells on agar plates or on chicken breast hams were treated with plasma for 2 min. The cells on agar plates were suspended in phosphate-buffered saline and pelleted by centrifugation. The pelleted cells from the agar plates or the cells on the chicken breast hams were fixed in a 2.5% paraformaldehyde-glutaraldehyde mixture buffered with 0.1 M phosphate (pH 7.2) for 2 h. Then, the samples were post fixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in graded ethanol, substituted by isoamyl acetate, and dried at the critical point in CO_2. Finally, the samples were sputtered with gold in a sputter coater (SC502, Polaron) and observed using the scanning electron microscope, HITACHI S4300N (HITACHI, Japan), installed at the Korea Research Institute of Bioscience & Biotechnology (KRIBB).

Temperature monitoring of the agar plates and chicken breast hams under plasma treatment

To monitor the temperature on the agar plates and the chicken breast hams during plasma treatment, the temperature probe of a thermometer (50S K/J thermometer, Fluke, USA) was fixed onto the surface of the agar plates or stuck into the chicken breast sliced hams placed on the petri dish from the side. During the plasma treatment, the temperatures of the agar plate and the chicken breast hams were recorded every 8 s for 5 min and for 10 min, respectively.

Measurement of the water activity and water content of the ham samples

A previous method (AOAC, 2005) was followed to measure the water activity and the water content. To simulate the plasma treatment conditions, 10 μL of tryptic soy broth was added to the chicken breast ham samples. After the plasma treatment, the water content was analyzed by drying samples at 105°C in a drying oven for 4 h. The water content (%, w/w) was calculated as the ratio of weight loss after drying to the initial weight of the samples before drying. The water activity was measured by an Aw analyzer (ms1 Set aw, Novasina, Switzerland).

Statistical analysis

Differences in CFU between any two different treatment groups were analyzed by a t-test using Microsoft Office Excel 2007. P values less than 0.05 were considered significant. The data of the water activity and the water content were subjected to analysis of variance (ANOVA) using a SAS statistical program (SAS Institute Inc., USA). Multiple comparisons of the treatment groups were performed using Duncan's multiple range test for any significant differences.

Results

To determine if RF atmospheric pressure plasma can effectively inactivate *C. jejuni*, the plasma treatment was first tested on agar plates at an initial CFU of 10^7-10^8 (Fig. 2). The CFU of *C. jejuni* NCTC11168 was significantly reduced by approximately 2 Log CFU (p<0.05)

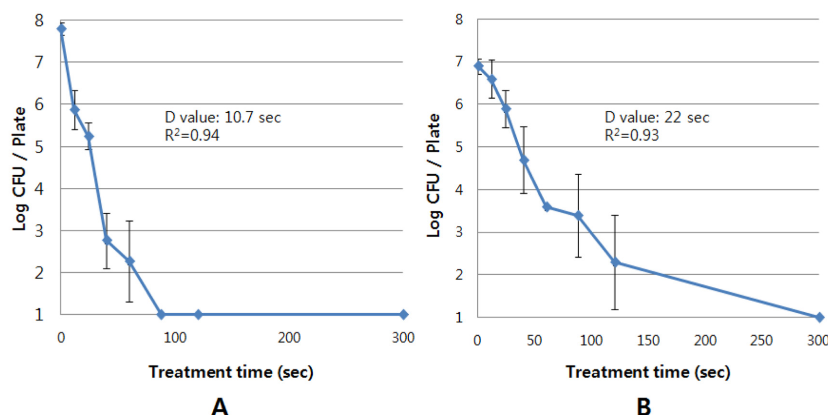

Fig. 2. Inactivation of (A) *C. jejuni* NCTC11168 and (B) *C. jejuni* ATCC49943 on agar plates with RF atmospheric pressure plasma. Data presented are based on 2-3 independent experiments. Detection limit was 1 Log CFU and any data below the detection limit were plotted at 1 Log CFU. Error bar represents standard deviation. Decimal reduction time (D value) was calculated based on dynamic range of inactivation (0-60 s for NCTC11168; 0-88 s for ATCC49943). R^2 represents correlation coefficient.

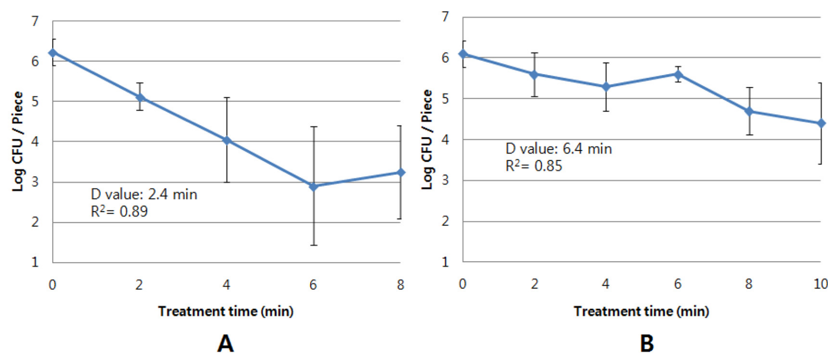

Fig. 3. Inactivation of (A) *C. jejuni* NCTC11168 and (B) *C. jejuni* ATCC49943 on chicken breast ham samples with RF atmospheric pressure plasma. Data presented are based on 2 independent experiments. Error bar represents standard deviation. R^2 represents correlation coefficient.

after an initial 12 s treatment (Fig. 2A). The inactivation continued with time and the CFU level fell below the detection limit after an 88 s treatment. Based on the inactivation kinetics, the decimal reduction time (D value) of the NCTC11168 strain was calculated as 10.7 s. To study if any strain-specific variation exists in inactivation, another *C. jejuni* strain, ATCC49943, was studied under the same conditions. In this strain, significant reduction (p< 0.05) occurred at 24 s instead of at 12 s (Fig. 2B). Although inactivation continued with time, inactivation was less efficient for ATCC49943 compared to that for NCTC11168 (Fig. 2). CFU counts of ATCC49943 were significantly higher than those of NCTC11168 at 12, 24, 40, 88, and 120 s (p<0.05) and the CFU level was still above the detection limit at the 2 min time point. Viable cells were non-detectable only at the 5 min. Overall, the inactivation slope of strain ATCC49943 was not as steep as that of strain NCTC11168 and its D value was 22 s in contrast to the D value of 11 s of NCTC11168.

Because plasma treatment effectively inactivated both *C. jejuni* strains on agar plates, chicken breast ham samples purchased from markets were studied to evaluate inactivation efficacy (Fig. 3). The inactivation rate was much slower and less efficient on the ham samples compared to that on the agar plates. With an initial inoculation level at 10^6-10^7 CFU, the plasma treatment reduced NCTC11168 by 1 Log CFU every 2 min until the 6 min time point, resulting in a 3 Log reduction overall (Fig. 3A). Another 2 min treatment did not further inactivate the strain, suggesting a tailing effect. In the ham samples, inactivation was also less efficient for ATCC49943 than for NCTC11168, similar to the agar plates. Only a 1.5 Log reduction occurred after an 8-10 min treatment of ATCC 49943 (Fig. 3B). The D values of NCTC11168 and ATCC 49943 in the ham samples were 2.4 min and 6.4 min, respectively. These D values are approximately 10-20 fold longer than those for the agar plates.

SEM analysis was conducted on *C. jejuni* NCTC11168 cells before and after the plasma treatment to study if the plasma treatment can affect the morphology of *C. jejuni* cells (Fig. 4). The *C. jejuni* cells had a typical spiral shape on both the agar plates (Fig. 4A) and the ham samples (Fig. 4C) before the plasma treatment. After the plasma treatment, however, its typical spiral shape was lost in many of the cells. They were deformed or transformed into a coccoid form in many of the cells on both the agar plates (Fig. 4B) and the ham samples (Fig. 4D). Despite the deformation, membrane damage was not apparent after the plasma treatment.

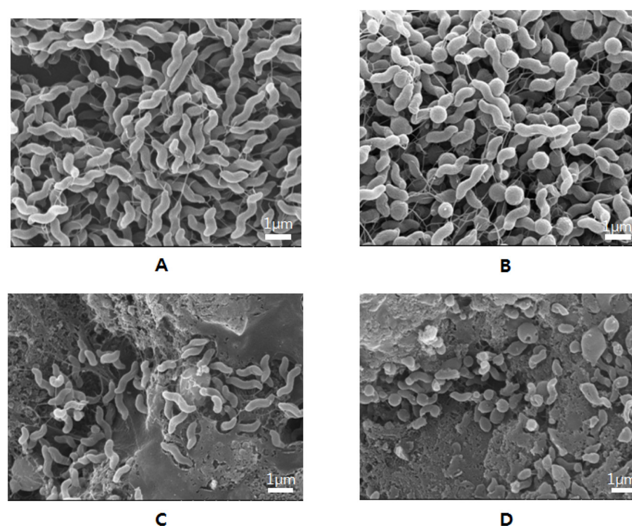

Fig. 4. SEM pictures of *C. jejuni* NCTC11168 either before or after plasma treatment on agar plates and chicken breast hams: (A) Before plasma treatment on agar plate, (B) After plasma treatment on agar plate, (C) Before plasma treatment on chicken breast ham, (D) After plasma treatment on chicken breast ham. Scale bar was inserted at right bottom corner.

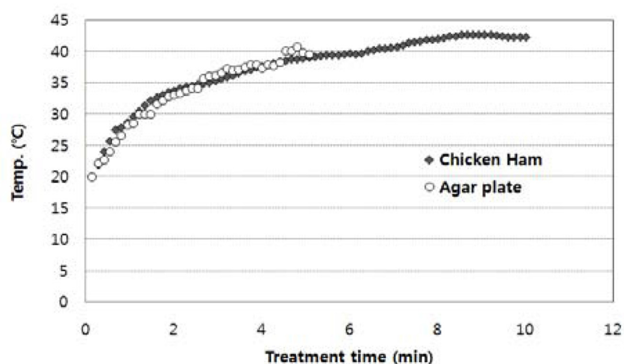

Fig. 5. Temperature monitoring of agar plates and chicken breast hams under plasma treatment. Temperature was recorded every 8 s in agar plates and chicken breast hams for 5 min and 10 min, respectively.

Temperature was monitored on the agar plates and the ham samples during the plasma treatment to confirm that the treatment is non-thermal (Fig. 5). The plasma treatment was not completely non-thermal, and the temperature steadily increased for the first 2 min. Then, it started to level off and did not further increase. The agar and ham samples reached only 41-43°C during the plasma treatment, suggesting that the inactivation of the *C. jejuni* cells was not related to the heat generated from the plasma.

It is known that plasma treatment has a desiccation effect (von Keudell *et al*., 2010). Therefore, the water activity and water content of the ham samples were measured to determine if our plasma treatment had any desiccation effect on the ham samples. The water activity was constant with a range of 0.934-0.935 at 0, 2, 4, or 6 min of treatment with no statistically significant difference ($p>0.05$) among those treatment groups. However, the 8 min and 10 min treatment significantly decreased the water activity to 0.929 and 0.919, respectively ($p<0.05$). On the other hand, the water content gradually decreased as the treatment time increased. The water content significantly decreased from 67.9% to 65.3% after the 4 min treatment and to 59.6% after the 10 min treatment ($p<0.05$). These data suggest that the plasma treatment had a desiccation effect on the ham samples.

Discussion

Although interest in plasma application to inactivate food-borne pathogenic bacteria has grown in recent years, the information regarding the effect of plasma treatment on *C. jejuni* is still very limited. In one recent study (Dirks *et al*., 2012), the *C. jejuni* contamination level was reduced by 2-3 Log CFU on chicken breast and thigh samples using a dielectric barrier discharge plasma after a 2-3 min treatment, which seems to be better than the performance of the RF plasma (0.5-1.5 Log reduction after the same time period) used in our study. However, it is still very difficult to draw conclusions because many of the conditions were different between the two studies such as the samples (raw chicken breast and thigh vs. chicken breast ham), initial inoculum levels (10^3-10^4 CFU vs. 10^6-10^7 CFU), and the *C. jejuni* strains used (RM2002 vs. NCTC11168 and ATCC49943).

Such a large difference in the sterilization efficiency between the agar plates and the ham samples suggests that the sample surface condition is a critical factor in determining the sterilization efficiency of a plasma treatment. For example, Perni *et al*. (2008b) studied the inactivation of *E. coli* and *L. monocytogenes* and found that the inactivation of the pathogens on the cut surfaces of mango and cantaloupe was much slower than that on the membrane filters using a cold atmospheric plasma. When the pericarp was compared to the cut surface in mangos, the inactivation efficiency for *E. coli* was much lower on the cut surface compared to that on the pericarp (Perni *et al*., 2008a, b). The authors found that the bacterial cells had migrated into the interior through the cut surface, suggesting that the migrated cells inside the mango tissue could be protected from the plasma treatment. In our study, a population of *C. jejuni* cells could have migrated into the crevices in the ham samples and the plasma treatment might not have reached the population. Our hypothesis is supported by the rough surface and the crevices of the ham samples, as demonstrated in the SEM analysis (Fig. 4). The possible migration of the *C. jejuni* cells may explain the tailing phenomenon observed for NCTC11168 on the ham samples. A tailing phenomenon was not observed for the agar plates that have a flat surface without crevices. It was previously reported by Critzer *et al*. (2007) and Fernández *et al*. (2013) who studied *Salmonella* Enteritidis and *Salmonella* Typhimurium, respectively, on fresh produce such as cantaloupe, lettuce, strawberry, and potato of irregular surfaces compared to on agar or membrane filters of flat surfaces.

Based on the SEM analysis, the plasma treatment affected the cell morphology of the *C. jejuni* cells, changing their form from a typical spiral form to a coccoid form. The coccoid form in *C. jejuni* is often associated with its degeneration resulting from unfavorable conditions or environments such as aged culture, exposure to air, and heat or cold stresses (Buck *et al*., 1983; He and Chen, 2010; Jang *et al*., 2007; Moran and Upton, 1987b; Park, 2002;

Tangwatcharin *et al*., 2006). Superoxide dismutase, a superoxide anion scavenging enzyme, can minimize the conversion of a spiral form to a coccoid form in air (Moran and Upton, 1987a). It suggests that reactive oxygen species can be involved in the conversion to a coccoid form. In this study, reactive oxygen species such as OH radicals may have been produced on the wet sample surface using the argon gas under atmospheric pressure in the plasma treatment (Srivastava and Wang, 2011). If so, these species could have played a role in the conversion of the spiral form to the coccoid form. However, cells with the coccoid form are not necessarily dead in *C. jejuni* (He and Chen, 2010). It suggests that it is still unclear if those coccoidal cells are dead in the plasma treatment. Aside from the conversion to coccoid form, there was no apparent damage on the cell membranes of *C. jejuni* in the SEM analysis. This result is in contrast to the results from other bacteria showing severe membrane damages, such as *E. coli*, *Lactobacillus acidophilus*, *Streptococcus mutans* (Korachi *et al*., 2010; Yang *et al*., 2011). Such a damaged cell membrane usually causes DNA or protein leakage (Deng *et al*., 2010; Yang *et al*., 2011). We attempted to detect DNA and protein leakage by measuring the absorbance of the supernatant at 260 nm and 280 nm, respectively, after spinning down the suspended cells following the plasma treatment. However, we could not detect any significant increase in the DNA or protein concentrations in the plasma treatment group compared to those of the control group (data not shown), suggesting that no significant amount of DNA or protein leaked from *C. jejuni* cells after the plasma treatment. This result corresponds to the absence of obvious damage on the cell membranes of *C. jejuni* as observed in the SEM pictures.

The reason for a significant difference in the sensitivity to the plasma treatment between NCTC11168 and ATCC 49943 remains elusive. A previous study suggests that the surface morphology in a mold, *Aspergillus niger*, may affect the sensitivity to plasma treatment (von Keudell *et al*., 2010). Genetic diversity in *C. jejuni* strains is high, and extensive variation exists in the surface structures such as capsules and lipooligosaccharides (Dorrell *et al*., 2001; Parker *et al*., 2006; Wareing *et al*., 2002). Thus, variation in the surface structure may exist between NCTC11168 and ATCC49943. Not surprisingly, the genome of ATCC 49943 does not appear to be quite similar to that of NCTC 11168 (Hofreuter *et al*., 2006; Hofreuter *et al*., 2008).

Our data clearly show that the RF atmospheric pressure plasma has a desiccation effect on food samples. However, in this study, it should not have contributed to the inactivation of *C. jejuni* because the water activity did not significantly change up to the 6 or 8 min treatment. In addition, regarding plasma treatment on agar surface, no signs of desiccation were observed for at least 1 min treatment. It also suggests that desiccation does not account for most of the inactivation on the agar surface.

Nevertheless, this study still suggests that the desiccation effect by prolonged plasma treatment of meat samples may be undesirable because the decreases of water activity and water content could result in the increase of hardness on texture parameters (Jin *et al.*, 2011; Serra *et al.*, 2005). More studies are necessary to understand the effect of non-thermal plasma treatment on the sensory qualities of food products because few studies have been performed to our knowledge. In addition to the desiccation effect, there are several other potential limitations to overcome in the application of plasma for meat samples. For example, plasma can produce reactive oxygen species and its possible negative effects, such as lipid oxidation and off flavor, have yet to be studied. Other product quality issues such as discoloration have to be carefully studied and managed for industrial application. In fact, Rød *et al.* (2012) reported that the lipid oxidation occurred in ready-to-eat meat after plasma treatment even though it was still within the acceptable range for sensory qualities. According to Kim *et al.* (2011), a significantly higher level of lipid oxidation was observed in plasma-treated bacons compared to controls after 7 d storage. These studies suggest that the lipid oxidation may be an issue for plasma treatment of meat products and warrants for further studies. Overall, this study shows that non-thermal RF atmospheric pressure plasma has a potential application in inactivating *C. jejuni* on food samples, but additional studies are necessary to evaluate the effects of non-thermal plasma technology on the qualities of meat products.

Acknowledgements

This research was funded by Korea Food Research Institute. We thank FEMTO Science Co. for providing an RF atmospheric pressure plasma equipment.

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(Received 2012.11.26/Revised 2013.4.2/Accepted 2013.4.28)