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Evaluation of pathogen inactivation on sliced cheese induced by encapsulated atmospheric pressure dielectric barrier discharge plasma

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ABSTRACT

Pathogen inactivation induced by atmospheric pressure dielectric barrier discharge (DBD) (250 W, 15 kHz, air discharge) produced in a rectangular plastic container and the effect of post-treatment storage time on inactivation were evaluated using agar plates and cheese slices. When agar plates were treated with plasma, populations of *Escherichia coli*, *Salmonella* Typhimurium, and *Listeria monocytogenes* showed 3.57, 6.69, and 6.53 decimal reductions at 60 s, 45 s, and 7 min, respectively. When the pathogens tested were inoculated on cheese slices, 2.67, 3.10, and 1.65 decimal reductions were achieved at the same respective treatment times. The post-treatment storage duration following plasma treatment potently affected further reduction in pathogen populations. Therefore, the newly developed encapsulated DBD-plasma system for use in a container can be applied to improve the safety of sliced cheese, and increasing post-treatment storage time can greatly enhance the system's pathogen-inactivation efficiency.

1. Introduction

Concerns regarding food safety, especially with regard to the presence of bacterial pathogens in food, have continued to increase over the years; a trend that is associated with the increase in the number of recorded outbreaks of food-borne illnesses (FDA, 2008). Available data indicate that *Salmonella* and *Escherichia coli* O157:H7 are the main pathogens found in contaminated foods (FAO/WHO, 2008). Another bacterium found in food is *Listeria monocytogenes*, which can cause the serious disease listeriosis in humans (Leipold et al., 2011). With the increase in food safety concerns, the demand for designing innovative sterilization technology appropriate for use on food has grown.

The use of an ionized gas, also known as plasma, has emerged as non-thermal sterilization technology (Fernández et al., 2013; Laroussi, 2002). Plasma consists of various species including photons, electrons, positive and negative ions, free radicals, and neutral atoms, as well as reactive species with sufficient electrical energy to break covalent bonds and induce numerous chemical reactions (Laroussi, 2002; Moisan et al., 2002). Antimicrobial agents in plasma are generally divided into three groups: ultraviolet (UV) photons, charged particles, and reactive species such as superoxide, hydroxyl radicals, nitric oxide, ozone and others (Deng et al., 2006; Fernández et al., 2013; Fröhling et al., 2012). Among those substances reactive oxygen species (ROS) produced by plasma have been widely reported to play a critical role in microbial inactivation (Deng et al., 2006; Lee et al., 2012).

Using APP at high power produced greater antimicrobial effects on sliced cheese and ham than using APP at a low power (Song et al., 2009). Kim et al. (2011) used the same plasma source as Song and colleagues to decontaminate pathogens on bacon and observed that using helium mixed with oxygen as the process gas was more effective in sterilizing food than using helium alone. Fridman et al. (2007) compared the antimicrobial effects of direct and indirect plasma treatments and concluded that the effect of charged particles on plasma plays the essential role.

The previous studies discussed so far have attempted to identify an efficient plasma system that is optimally suited for sterilizing

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foodborne pathogens, but all systems developed cannot be applied in the case of sealed foods because plasma penetrates packaging materials poorly. Food products are typically stored in containers, bags, cans, or bottles. Thus, designing a plasma device that generates APP within a sealed environment would greatly benefit the food industry because this would avoid the problem of cross-contamination after packaging. A microwave plasma system connected with a closed bottle was used to investigate the bactericidal effect of plasma on total aerobic bacteria of fresh pork (Fröhling et al., 2012). Maisch et al. (2012) tested the antimicrobial effect on pork-skin *S. aureus* of APP plasma, which was contained in a plastic box with a lid on one side. However, these methods are limited by changes in their quality or bactericidal effects. In this study, the objective was to evaluate the inactivation of pathogens on agar plates and cheese by using a newly developed system, a DBD encapsulated in a container.

2. Materials and methods

2.1. Sample preparation and sterilization

For use in the microbial-inactivation test, cheese slices were purchased from a local market in Daejeon, Korea. Cheese samples were first sterilized by means of electron-beam irradiation (35 kGy) generated using a 2.5-MeV linear electron beam RF accelerator (EB-Tech, Daejeon, Korea), then cut into 15 × 15 × 2 mm sections (2.2 g) before inoculating the pieces with pathogens. As samples for inactivation test on agar plate (50 mm in diameter and 10 mm in depth), tryptic soy agar (TSA) plates, nutrient agar (NA) plates, and plates with TSA containing 0.6% yeast extract were also prepared and used for plasma treatment (all kinds of agars were purchased from Difco Laboratories, Detroit, USA).

2.2. Microorganisms and inoculation

E. coli (KCTC 1682), *Salmonella* Typhimurium (KCTC 1925), and *L. monocytogenes* (KCTC 3569) were cultivated in tryptic soy broth (Difco), nutrient broth (Difco), and tryptic soy broth containing 0.6% yeast extract, respectively, at 37 °C for 48 h. The cultures were centrifuged at 3100 rpm for 15 min at 4 °C in a refrigerated centrifuge (UNION 32R, Hanil Science Industrial Co. Ltd., Korea) and then the pellets were washed twice using sterile saline solution (0.85%). The pellets were re-suspended in the same saline solution to obtain final viable cell densities of 10^8–10^9 CFU/mL. The prepared agar plates and cheese slices were inoculated with 10 and 50 μL of this microbial solution, respectively. To enable the microorganisms to attach to the cheese slices, the samples were placed on a clean bench for 15 min at room temperature.

2.3. Treatment with DBD

As shown in Fig. 1, an encapsulated DBD plasma source was prepared using a rectangular, parallelepiped plastic container (137 × 104 × 53 mm). Fig. 1(a) shows the schematic of the DBD actuator that was made of copper electrodes and a polytetrafluoroethylene (PTFE) sheet, and this actuator was attached to inner walls of the container as illustrated in Fig. 1(b). To investigate the inactivation effect of pathogens, the samples of agar plates and cheese slices were separately placed at the bottom of the container. A bipolar square-waveform voltage at 15 kHz was applied to one electrode while the other electrode was grounded and the plasma was generated at 250-W input power inside the container. Because a thin rubber was installed at an interface between the lid and the main container, we could easily connect electrodes located inside the container to the power supply without modifying the container.

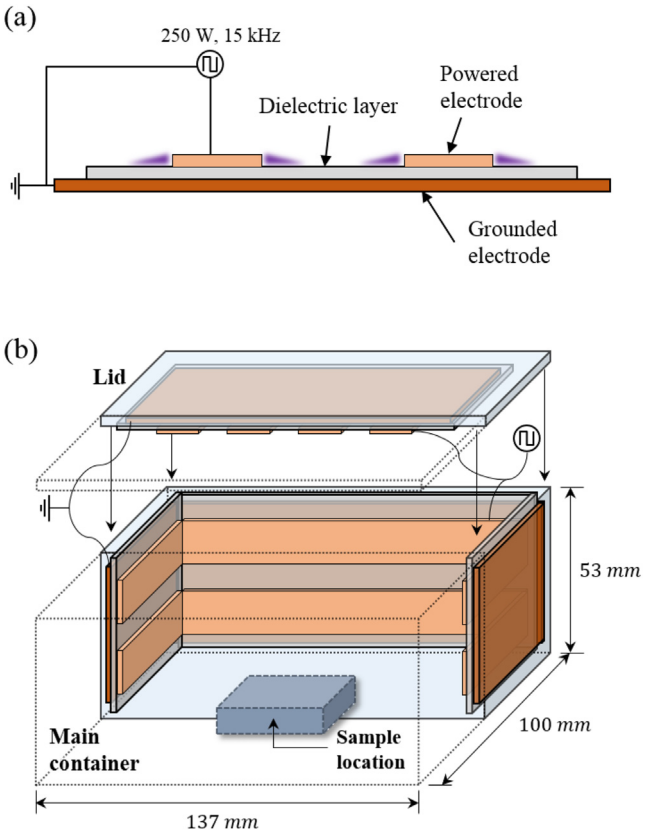

Fig. 1. (a) Detailed illustration of DBD actuator, and (b) Schematic diagram of the full experimental system for plasma treatment.

The high voltage was able to be delivered from the power supply to our DBD apparatus through conductive wires. The control sample was prepared as the same condition without plasma treatment (0 min). In addition, to investigate the effect of post-treatment storage duration on sterilization, the samples were maintained within the container with the lid closed for the specified lengths of time after plasma treatments: post-treatment times were, for the agar samples, 1, 2, and 3 min for *E. coli* and *S.* Typhimurium and 1, 3, and 5 min for *L. monocytogenes*; for the cheese samples, all pathogens were stored for 1, 3, and 5 min after plasma treatment. These post-treatment times were selected after conducting several preliminary tests.

2.4. Microbial analysis

After the plasma treatment, the cheese slices were blended using 20 mL of sterile saline solution (0.85%) on the same day and then serially diluted in sterile saline solution. The media used for *E. coli*, *S.* Typhimurium, and *L. monocytogenes* were TSA, NA, and TSA containing 0.6% yeast extract, respectively. Each diluent (0.1 mL) was spread on the appropriate medium and the agar plates were incubated at 37 °C for 48 h. The plasma-treated agar plates that were inoculated with the pathogens were also incubated at the same conditions. Microbial counts were determined and expressed as Log CFU/g or Log CFU/mL.

2.5. Statistical analysis

The present study was conducted 3 independent trails (replicates) with 2 observations per each trail. Statistical analysis was performed using one-way analysis of variance (ANOVA), and

significant differences between mean values were determined using the Duncan's multiple comparison test in SAS software (SAS, Release 9.2, SAS Institute Inc., Cary, NC); $P < 0.05$ was considered statistically significant. Mean values and standard errors of the mean (SEM) are reported.

3. Results and discussion

3.1. Effect of plasma treatment time on bacterial inactivation

When agar plates were exposed to plasma, the initial counts (10^7–10^9 CFU/g) of *E. coli*, *S.* Typhimurium, and *L. monocytogenes* decreased substantially, and no viable cells of these pathogens were detected after treatments lasting for 90 s, 60 s, and 10 min, respectively (Fig. 2). Thus, more time was required to inactivate *L. monocytogenes* than to inactivate *E. coli* and *S.* Typhimurium by using plasma. This may be because *L. monocytogenes* is a Gram-positive bacterium, whereas *E. coli* and *S.* Typhimurium are Gram-negative: Gram-negative bacteria possess a unique outer membrane in their cell envelope and could thus be more vulnerable than Gram-positive bacteria, which have a thick peptidoglycan structure on the outside of the cell that is resistant to chemical changes. Because of this peptidoglycan structure, the APP-induced inactivation rate of Gram-positive bacteria was measured to be lower than that of Gram-negative bacteria (Deng et al., 2006; Laroussi, 2002; Montie et al., 2000). Laroussi (2002) reported that the outer membrane of Gram-negative bacteria exhibited structural damage following exposure to cold plasma, whereas Gram-positive bacteria did not show the same degree of morphological changes. APP has also previously been shown to cause greater inactivation of Gram-negative than Gram-positive bacteria (Laroussi, 2002; Lee et al., 2012; Montie et al., 2000).

The inactivation effect of plasma in a container on the pathogens inoculated onto cheese slices is shown in Fig. 3. The populations of *E. coli*, *S.* Typhimurium, and *L. monocytogenes* on the cheese slices were diminished markedly and were measured to be 2.88, 3.11, and 2.26 decimal reductions after treatment for 15 min, respectively.

The plasma used here showed greater inactivation of pathogens on agar plates than on cheese slices. The interactions between plasma and samples can be influenced by various factors including surface roughness, adsorption of diffusing plasma, and moisture (Ataide et al., 2003; Lee et al., 2011; Deng et al., 2006), and some or all of these factors may affect the survival of pathogens. Inactivation of *L. monocytogenes* on sliced ham was greater than that on chicken breast, which was considerably rougher than the ham (Lee et al., 2011).

Song et al. (2009) applied APP at 75, 100, 125, and 150 W to a 3-strain cocktail of *L. monocytogenes* inoculated on sliced cheese and calculated the D-values to be 71.43, 62.50, 19.65, and 17.27 s from the survival curve, respectively. Recently, Lee et al. (2012) determined that when plasma was applied together with He/O_2 for 1, 5, 10, and 15 min on sliced cheese, *E. coli* counts were reduced by 0.05, 0.87, 1.89, and 1.97 Log CFU/mL and those of *S. aureus* were reduced by 0.08, 0.31, 0.59, and 0.91 Log CFU/mL, respectively, although the sliced cheese was damaged after 10 and 15 min of APP treatment. However, in this study, no damage of cheese slices was detectable to the naked eye after treatment for 10 min (data not shown). Moreover, the plasma system used in this study did not include any input gas except air.

3.2. Effect of post-treatment duration on bacterial inactivation

The initial counts of *E. coli*, *S.* Typhimurium, and *L. monocytogenes* on plasma-treated agar plates were 3–4 Log CFU/g and these counts were considerably decreased with an increase in post-treatment duration (Fig. 4). Furthermore, no viable

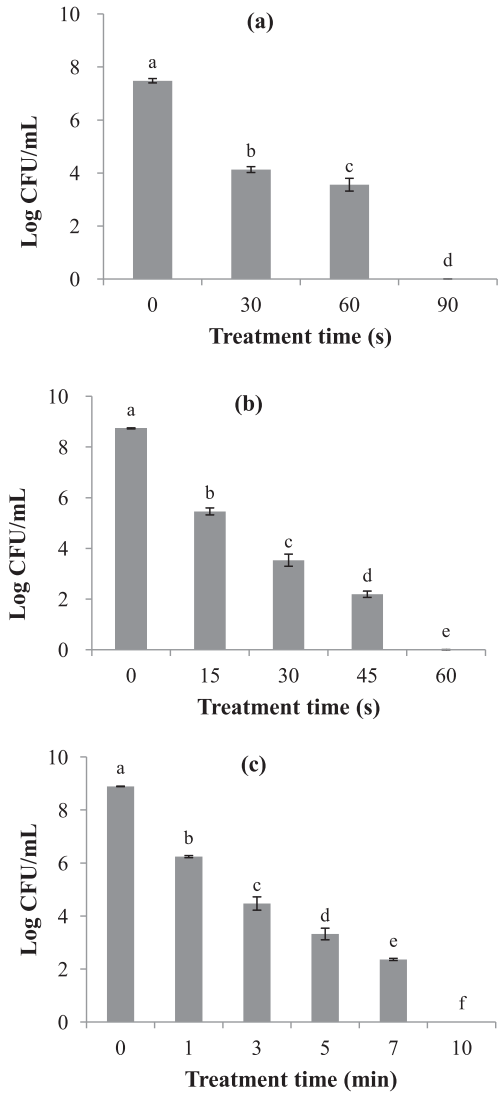

Fig. 2. Inactivation of pathogens inoculated on agar plates by plasma. (a) *Escherichia coli*; (b) *Salmonella* Typhimurium; and (c) *Listeria monocytogenes*. [a–e]Different letters among the treatments differ significantly ($p < 0.05$).

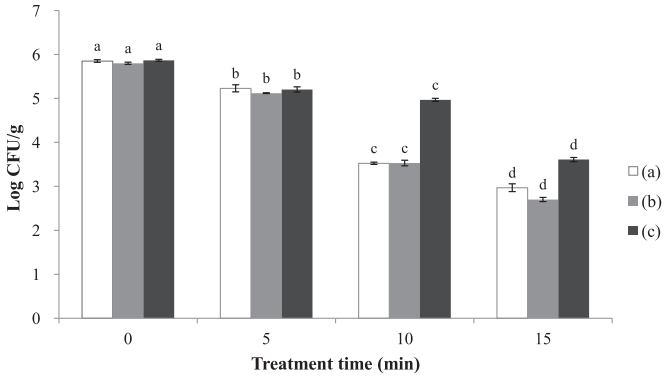

Fig. 3. Inactivation of pathogens inoculated on cheese slices by plasma. (a) *Escherichia coli*; (b) *Salmonella* Typhimurium; and (c) *Listeria monocytogenes*. [a–d]Different letters among the treatments within the same samples differ significantly ($p < 0.05$).

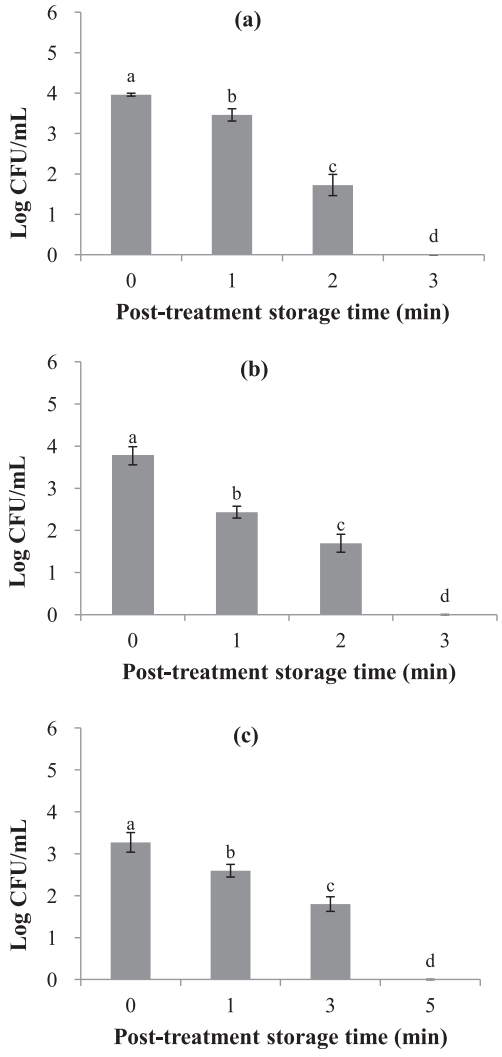

Fig. 4. Effect of post-treatment storage duration after plasma treatment on pathogen numbers on agar plates. (a) *Escherichia coli*, after plasma treatment for 30 s; (b) *Salmonella* Typhimurium, after plasma treatment for 30 s; (c) *Listeria monocytogenes*, after plasma treatment for 5 min. [a–d]Different letters among the treatments differ significantly ($p < 0.05$).

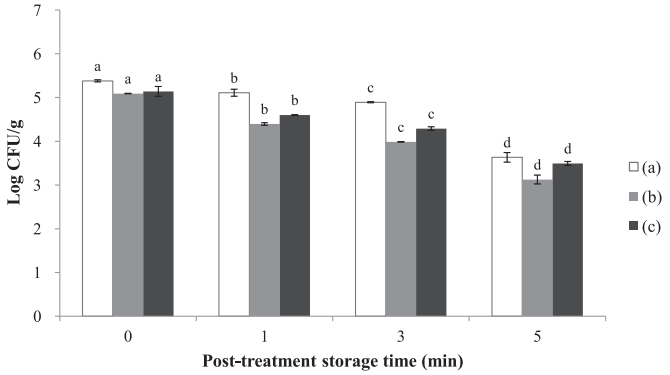

Fig. 5. Effect of post-treatment storage duration after plasma treatment on pathogen numbers on cheese slices. (a) *Escherichia coli*, after plasma treatment for 5 min; (b) *Salmonella* Typhimurium, after plasma treatment for 5 min; (c) *Listeria monocytogenes*, after plasma treatment for 10 min. [a–d]Different letters among the treatments within the same samples differ significantly ($p < 0.05$).

cells were detected after a post-treatment duration of 3 min in plates inoculated with *E. coli* and *S.* Typhimurium and 5 min in plates inoculated with *L. monocytogenes* (Fig. 5).

Post-treatment duration refers to the sealed-type plasma being switched off after a period of treatment, a process also called indirect plasma treatment. This state of the plasma has emerged as a key treatment parameter that determines consistency in bacterial inactivation, because the remaining reactive species are trapped inside the sealed container during the post-treatment duration (Fröhling et al., 2012; Leipold et al., 2011; Rød et al., 2012; Ziuzina et al., 2012). When DBD is switched off and then switched on, residual ozone is present in sealed bags (Rød et al., 2012; Schwabedissen et al., 2007). Moreover, Leipold et al. (2011) showed that ozone was preserved for approximately 10 min when a sealed bag was exposed to DBD for 30 s. ROS including ozone can attach to unsaturated fatty acids, induce lipid peroxidation, and alter membrane lipids, and they can potently oxidize proteins, DNA, and outer structure of cells to produce lethal effects (Laroussi, 2002; Montie et al., 2000). Leipold et al. (2011) inoculated *Listeria innocua* on glass slides and placed the slides inside a polyethylene bag, which was exposed to DBD and the plasma discharge was switched on and off. The results showed that ozone was produced immediately after plasma was generated, but that its concentration decreased over time, which indicates that switching off the discharge after a certain period can maintain a stable ozone concentration and inactivate more *L. innocua* than keeping the plasma discharge continuously switched on.

Increasing post-treatment duration also substantially reduced the pathogens on cheese slices. After the plasma treatment, the populations of *E. coli*, *S.* Typhimurium, and *L. monocytogenes* on cheese slice (approximately 5 Log CFU/g) were decreased by 1.75, 1.97, and 1.65 Log CFU/g, respectively, when the post-treatment duration was 5 min.

When a high concentration of *E. coli* suspended in a liquid within a sealed package was treated by plasma, the inactivation efficacy was influenced by the post-treatment duration, and the pH of liquid decreased during that period (Ziuzina et al., 2012). Rød et al. (2012) turned the plasma on and off for a period and repeated this process on bresaola placed inside a sealed polyethylene bag; their results showed that the treatment markedly decreased the number of *L. innocua* and potently increased lipid oxidation. Fröhling et al. (2012) turned the power on and off twice and showed that microwave plasma can also substantially reduce the total aerobic bacteria and change the colour of fresh pork inside a closed bottle.

In conclusion, the results of this study indicated that using the newly developed encapsulated DBD system successfully inactivated pathogens and potently lowered pathogen numbers on agar plates and cheese slices. Furthermore, increasing post-treatment duration enhanced inactivation efficiency, which improves the applicability of this system because foods are usually packaged and sealed.

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