

Research Paper

Inactivation of *Escherichia coli* O157:H7 and Aerobic Microorganisms in Romaine Lettuce Packaged in a Commercial Polyethylene Terephthalate Container Using Atmospheric Cold Plasma

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ABSTRACT

The effects of dielectric barrier discharge atmospheric cold plasma (DACP) treatment on the inactivation of *Escherichia coli* O157:H7 and aerobic microorganisms in romaine lettuce packaged in a conventional commercial plastic container were evaluated during storage at 4°C for 7 days. Effects investigated included the color, carbon dioxide (CO₂) generation, weight loss, and surface morphology of the lettuce during storage. Romaine lettuce pieces, with or without inoculation with a cocktail of three strains of *E. coli* O157:H7 (~6 log CFU/g of lettuce), were packaged in a polyethylene terephthalate commercial clamshell container and treated at 34.8 kV at 1.1 kHz for 5 min by using a DACP treatment system equipped with a pin-type high-voltage electrode. Romaine lettuce samples were analyzed for inactivation of *E. coli* O157:H7, total mesophilic aerobes, and yeasts and molds, color, CO₂ generation, weight loss, and surface morphology during storage at 4°C for 7 days. The DACP treatment reduced the initial counts of *E. coli* O157:H7 and total aerobic microorganisms by ~1 log CFU/g, with negligible temperature change from 24.5 ± 1.4°C to 26.6 ± 1.7°C. The reductions in the numbers of *E. coli* O157:H7, total mesophilic aerobes, and yeasts and molds during storage were 0.8 to 1.5, 0.7 to 1.9, and 0.9 to 1.7 log CFU/g, respectively. DACP treatment, however, did not significantly affect the color, CO₂ generation, weight, and surface morphology of lettuce during storage (*P* > 0.05). Some mesophilic aerobic bacteria were sublethally injured by DACP treatment. The results from this study demonstrate the potential of applying DACP as a postpackaging treatment to decontaminate lettuce contained in conventional plastic packages without altering color and leaf respiration during posttreatment cold storage.

Key words: Cold plasma; Dielectric barrier discharge; *Escherichia coli* O157:H7; Lettuce; Nonthermal processing

The health benefits associated with the consumption of fresh produce maintain a high consumer demand for a wide range of prepacked ready-to-use products (50). However, unfortunately, potential foodborne pathogens are able to grow on fresh produce surfaces (12, 25). In recent years, various produce commodities have been implicated in foodborne infections, such as lettuce, tomatoes, spouted seeds, and mixed salads (11, 33, 50). Produce can become contaminated during growing and harvesting and even storage and distribution after packaging (7). Chlorine-based washing is widely used by fresh produce processors for microbial decontamination (28). However, the possible formation of chlorinated organic compounds raises public health concerns (11). *Escherichia coli* O157:H7 has been one of the target microorganisms involved in these outbreaks and has been found frequently in lettuce. Since 2000, 29 foodborne disease outbreaks caused by *E. coli* O157:H7

were due to the consumption of contaminated lettuce (6). The number of outbreaks of foodborne illnesses has increased, accompanying the increased consumption of produce (7, 30). This indicates that the currently used intervention processes, such as conventional postharvest washing and sanitizing treatments, may not be sufficient for controlling the biological hazards in these foods, highlighting the need for new intervention technologies (7, 30, 35).

Cold plasma (CP) treatment has been investigated intensively as a nonthermal technology for the microbial decontamination of fresh fruits and vegetables (11, 16, 30). CP treatment has advantages over conventional technologies, such as its nonthermal nature, short treatment time, and nontoxic nature (49). Corona discharge, plasma jet, microwave discharges, and dielectric barrier discharge (DBD) are commonly used to generate CP in treatment systems (44). DBD reactors contain three main parts: metallic electrodes, in which the applied voltage and current can be measured, dielectric barriers that resist the flow of the conducted current through the reactor, and gas gaps in which

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electric discharges takes place (1). In DBD, plasma is generated between two electrodes at a high potential difference separated by dielectric barriers (27).

CP generated by using air at atmospheric pressure by a DBD atmospheric cold plasma (DACP) apparatus contains reactive oxygen and nitrogen species, such as atomic oxygen, ozone, singlet oxygen, metastable oxygen molecules, peroxide, superoxide, and hydroxyl radicals, nitric oxide, and nitrogen dioxide (21, 38, 47, 48), which inactivate bacteria in foods (21). DACP offers a further advantage in that it allows treatment of produce inside sealed packages (26). The plastic food package itself can serve as the dielectric barrier, which permits electric discharge inside the package (27). In-package decontamination of fresh foods is highly desirable in the food industry because it minimizes the possibility of postprocessing contamination after packaging (26, 46, 47).

CP generation by using air at atmospheric pressure is also of interest, both technically and commercially, to the food industry because CP treatment can be implemented at ambient conditions and at reduced cost without using a specific plasma-forming gas (e.g., helium and argon) (28). Nonetheless, as yet, little research has focused on in-package DACP decontamination of fresh produce and the effects of such treatments on the quality properties of the produce. Additionally, no previously published report has described the effectiveness of DACP treatment on conventional rigid plastic container-packaged produce. Thus, the objectives of this study were to (i) study the effects of DACP treatment on the growth of *E. coli* O157:H7 and mesophilic aerobic microorganisms in romaine lettuce during postprocessing storage at 4°C for 7 days, including the determination of sublethal injury and (ii) evaluate the effects of DACP treatment on the quality properties of lettuce during storage.

MATERIALS AND METHODS

Lettuce sample preparation. Romaine lettuce (*Lactuca sativa* L.) was selected as the vegetable for evaluation because it has been commonly associated with recent foodborne illness outbreaks and represents a common type of produce (6). Romaine lettuce was purchased prewashed (Harvest Originals, Fresh Express, Charlotte, NC) from a local supermarket and stored at 4°C for up to 4 days until use. The outer lettuce leaves (three to five leaves) were removed, and the intact inner mature leaves were selected for the experiment. The lettuce leaves were trimmed to pieces (~4 by 7 cm; 2.0 g), in preparation for analyses of microbial inactivation, color, and weight loss, and pieces (~2 by 7 cm; 1.0 g) for carbon dioxide (CO₂) generation analysis. The samples for microbial inactivation studies were cut aseptically by using sterile scissors.

Cut lettuce leaves were washed with running deionized water once and dried in a laminar flow biohazard hood (type A/B3, NuAire, Inc., Plymouth, MN) for 30 min and then used without further preparation to evaluate the effects of DACP treatment on the inactivation of total mesophilic aerobes and yeasts and molds and on the color, CO₂ generation, weight loss, and surface morphology of lettuce samples. For those studies, untreated (control) and treated samples were prepared in pairs from one leaf for effective comparison.

To prepare the samples inoculated with *E. coli* O157:H7, lettuce leaves were first immersed in a sodium hypochlorite

solution (300 mg/liter) for 3 min to reduce the background microbial load before surface inoculation (36). The sanitizer was prepared from a commercial stock solution (Clorox Company, Oakland, CA), purchased from a local store. The sanitizer temperature at the time of treatment was 23°C. The leaves were subsequently rinsed in sterilized deionized water to remove any remaining sanitizing agent residue and dewetted by using a salad spinner, dried in the laminar flow biohazard hood at 23 ± 2°C for 30 min, and then exposed to UV light (TUV 30W G30T8 lamp, Philips, Amsterdam, The Netherlands) for 20 min per side (40 min in total) in the hood to decontaminate the surface of the samples. Three strains of *E. coli* O157:H7, C9490, ATCC 35150, and ATCC 43894, were obtained from the Eastern Regional Research Center Culture Collection (U.S. Department of Agriculture, Wyndmoor, PA). Fresh cultures of each strain were grown overnight in tryptic soy broth (BD, Franklin Lakes, NJ) in an orbital shaker at 37°C and 150 rpm until reaching the early stationary phase, after ~18 h (4). The cells were harvested by centrifugation (2,988 × *g*, 10 min) and washed three times in sterile buffered peptone water (Remel, Lenexa, KS). The cocktail was prepared by combining equal portions of each strain to produce an inoculum of ~10⁹ CFU/ml, which was used as the working inoculum. Lettuce samples were placed in a single layer on a plastic screen in the laminar flow biohazard hood at 28 ± 2°C and 30% relative humidity. Inoculum was spotted directly onto the adaxial surface of lettuce, using approximately equal volumes, at 26 to 30 locations per lettuce sample. The total inoculum volume was 100 µl. The inoculum spots were spread evenly by using a presterilized disposable plating hockey stick (Fisher Scientific, Pittsburgh, PA). Inoculated samples were dried in the hood for ~1 h before DACP treatment.

DACP treatment system. The DACP system generates the plasma field between the base dielectric barrier and the upper pinboard electrodes (Fig. 1). Rather than having a discharge caused by the electrical breakdown potential of air, thousands of tiny discharges are generated within the electrical field. This plasma field surrounds material placed within the DACP device. Because the field permeates the objects, plasma is generated within the closed container, surrounding the product to be treated in a uniform corona discharge. The electrical components for the DACP consist of two inductor coils, direct current power supply, and a function generator. Combined, these components form the variable frequency and amplitude, bipolar-pulsed direct current high-voltage generator module. Using a function generator, the frequency range can be varied between 0 and 2,400 Hz. To generate the high voltage necessary to create a stable plasma field, two inductive coils are used to create the bipolar pulse, one positive and one negative, by connecting these coils in an inversed polarity parallel configuration. The voltage supplied is rapidly switched from the charge to discharge state by using a high-voltage-integrated gate bipolar transistor, driven by the previously mentioned function generator. This integrated gate bipolar transistor energizes the coils from the direct current power supply. The peak-to-peak voltage measured between the pinboard and the dielectric ranges from 0 to 76 kV. The mechanical components of the DACP consist of the upper pinboard electrode and the lower dielectric barrier. The upper pinboard electrode was developed internally at the USDA Eastern Regional Research Center. The electrode consists of two copper-clad glass fiber prototyping printed circuit boards (10 by 15 cm) separated by 1.8 cm. Approximately 2,000, no. 20 steel pins were inserted through the holes of the prototype board and covered with a highly conductive aluminum electromagnetic field shielding tape to interconnect all

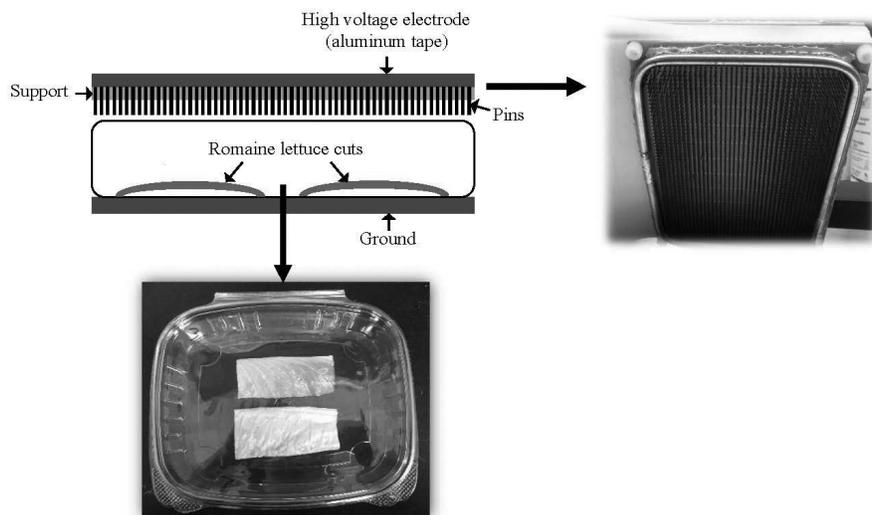


FIGURE 1. Schematic diagram of the CP reactor and the photographs of the pin-board array in the reactor (right) and packaged lettuce sample in the conventional commercial plastic container (bottom).

of the pins. The dielectric barrier was developed by using a 3.5-mm-thick sheet of borosilicate glass (approximately 40 by 70 cm) and with electromagnetic field shielding tape attached to the underside of the glass. Voltage and current measurements were accomplished by using a high-voltage probe (PN PVM-1, North Star, Marana, AZ) and a current transformer (PN 2100, Pearson Electronics, Palo Alto, CA). The output of these devices was presented on a digital scope (PN DSO-X 4034A, Agilent Technologies, Santa Clara, CA). To determine the actual power used during the plasma generation, the area under the voltage and current curves was integrated. The plasma treatment chamber, where the treatment occurs, was placed in a house connected to an ozone destruct unit (Ozone Solutions, Inc., Hull, IA). The ozone concentration of air out of the house was 0 ppm, according to the measurement using an ozone analyzer (Ozone Analyzer UV-100, Eco Sensors, Santa Fe, NM).

DACP treatment. A set of lettuce samples (two lettuce pieces), with or without inoculation with *E. coli* O157:H7, was transferred aseptically to a commercial rigid polyethylene terephthalate clamshell container (14.8 by 12.8 by 2.7 cm; AD16S, Genpak, Glens Falls, NY; Fig. 1), which was sterilized with a 70% ethanol solution, and the lid of the container was closed. Previous research in our laboratory established the upper bounds of CP treatment that this type of packaging will tolerate before breakdown. The treatment regime and operating parameters for this microbial inactivation study were chosen from the range of parameters that do not result in damage to the packaging. The samples were treated by CP using atmospheric air (relative humidity: $22\% \pm 2\%$) as a CP-forming gas at atmospheric pressure. The highest level of electrical power that did not cause any dielectric breakdown (arcing) on the lettuce samples in the DACP reactor was delivered by applying a high voltage 34.8 kV at 1.1 kHz across the electrodes (Fig. 1). The current was 1 A. The samples (Fig. 1) were subjected to each DACP treatment for 5 min, which was the longest time in which no significant increase in current occurred. This treatment time also resulted in a constant and reproducible reduction of *E. coli* O157:H7 (~ 1 log CFU/g of lettuce) in preliminary studies. The treatment conditions did not alter visual quality of the plastic package used in this study. Temperatures of lettuce samples before and after DACP treatments were measured in uninoculated samples by using an infrared camera (Fluke Ti32, Fluke Corporation, Everett, WA). Treatments were carried out at an ambient temperature of $24.5 \pm 1.4^\circ\text{C}$. After treatment, both treated and untreated samples were subsequently

stored for 7 days at $4.0 \pm 0.4^\circ\text{C}$. The relative humidity inside the sample containers during storage was 97 to 100%.

Microbiological analysis. Uninoculated untreated control samples (to determine initial background microflora), inoculated untreated control samples (to estimate the initial attached *E. coli* O157:H7 population), and inoculated or uninoculated DACP-treated samples were analyzed on days 0, 1, 2, 3, 5, and 7 of storage at 4°C . The samples were transferred aseptically into separate sterile stomacher bags (384 ml, Whirl-Pak, Nasco, Fort Atkinson, WI) with 38-ml of sterile buffered peptone water and blended for 2 min at 230 rpm by using a stomacher (Stomacher 400 Circulator, Seward, London, UK), followed by hand rubbing for 1 min for additional blending. The resulting suspension was serially diluted in buffered peptone water, and diluted samples were plated on MacConkey II with sorbitol (MCS; BD), tryptic soy agar supplemented with 0.6% (wt/vol) yeast extract (TSAYE; BD), or TSAYE supplemented with 3% (wt/vol) sodium chloride (TSAYE+NaCl; Columbus Chemical Industries, Columbus, WI), which was prepared for the enumeration of injured cells. Both TSAYE and TSAYE+NaCl were prepared according to Saldaña et al. (42). Total mesophilic microorganisms and yeasts and molds were counted by using aerobic plate count (APC) Petrifilm and yeast-mold Petrifilm (3M, St. Paul, MN), respectively. MCS and TSAYE plates were incubated for 24 h at 37°C , while TSAYE+NaCl plates were incubated for 48 h at 37°C . APC Petrifilm was incubated at 37°C for 48 h and yeast-mold Petrifilm at 25°C for 5 days prior to enumeration.

Analysis of color, CO₂ generation, and weight loss. To prepare DACP-treated samples, the samples in the containers were treated with DACP under conditions identical to those used for the microbial inactivation study. After treatment, both treated and untreated samples were stored at 4°C and analyzed on days 0, 1, 2, 3, and 7. For effective comparison of the properties of treated and untreated samples, the compared treated and untreated sample pairs were prepared from the same leaves.

For CO₂ generation analysis, both treated and untreated lettuce samples were carefully moved from the containers into a glass vial (40-ml volume) on each day of analysis, and CO₂ and O₂ in the headspace of the vials were analyzed after equilibrium for 4 h by using a portable O₂ and CO₂ analyzer (DuralTrak 902 D, Quantek Instruments, Grafton, MA). Gas in the headspace of the vials was withdrawn through a needle using a built-in pump. The

volume collected from the vial headspace for gas analysis was $\sim 20 \text{ cm}^3$.

The lettuce samples were removed carefully from the containers and weighed immediately by using a precise three-digit balance (PB 303, Mettler Toledo, Inc., Columbus, OH) for weight loss determination on each day of sampling. Weight loss was expressed as a percentage of the initial sample weight.

The color of adaxial lettuce surface was quantified via the CIELAB L^* (lightness), a^* (redness), and b^* (yellowness) values by using a colorimeter (Hunter UltraScan VIS, Hunter Associates Lab, Reston, VA). D65/10° was used as the illuminant-viewing geometry. Five readings were made on each leaf sample. The total color difference (ΔE^*) of lettuce samples was also determined during storage by using the following equation

$$(\Delta E^*) = \left[(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2 \right]^{0.5} \quad (1)$$

where L_0^* , a_0^* , and b_0^* are the L^* , a^* , and b^* values of untreated lettuce samples on day 0 of storage.

Surface morphology. Scanning electron microscopy (SEM) analysis was conducted, following the method of Keskinen et al. (20), with minor modifications. Lettuce samples (1 by 1 cm) with and without DACP treatment were fixed with 1.0 ml of 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA). The samples were rinsed twice with 3 ml of 0.1 M imidazole (Electron Microscopy Sciences) and then dehydrated in 50%, 80%, and absolute ethanol. Samples were further dried in a critical point drying apparatus (Denton Vacuum, Inc., Cherry Hill, NJ) by using liquid CO_2 (Welco Co., Allentown, PA). The samples were mounted on stubs and sputter coated with a thin layer of gold (EMS 150R ES, EM Sciences, Hatfield, PA). They were then observed by using a scanning electron microscope (Quanta 200 F, FEI Co., Hillsboro, OR), with an accelerating voltage of 5 to 10 kV in the high vacuum in the secondary electron imaging mode.

Statistical analysis. Entire experiments were performed in triplicate. Five measurements were made for each set of experiments determining the surface color and weight loss of the lettuce samples, while three measurements were made for determining CO_2 generation. Analysis of variance was used to evaluate differences among means, and in cases of statistical significance, means were evaluated by Tukey's multiple range test for significant differences ($\alpha = 0.05$) using the PASW Statistics software (version 18.0.0, IBM SPSS Inc., New York, NY).

RESULTS AND DISCUSSION

Inactivation of *E. coli* O157:H7. The initial counts of inoculated lettuce samples after treatment were 5.0 ± 0.5 , 5.1 ± 0.7 , and 5.0 ± 0.7 log CFU/g of lettuce on MCS, TSAYE, and TSAYE+NaCl plates, respectively, while the counts of untreated samples were 6.0 ± 0.4 , 6.2 ± 0.5 , and 6.0 ± 0.5 log CFU/g, respectively (data not shown). The average microbial counts of treated lettuce samples were in the range of 4 to 5 log CFU/g on all MCS, TSAYE, and TSAYE+NaCl plates during storage for 7 days at 4°C (data not shown). The difference between the viable (counts of TSAYE) and noninjured cells (counts of TSAYE+NaCl) corresponds to the sublethally injured cells (41). However, the counts in the different media were not significantly different on each day ($P > 0.05$), which suggests that sublethal injury of *E. coli* O157:H7 by DACP was not

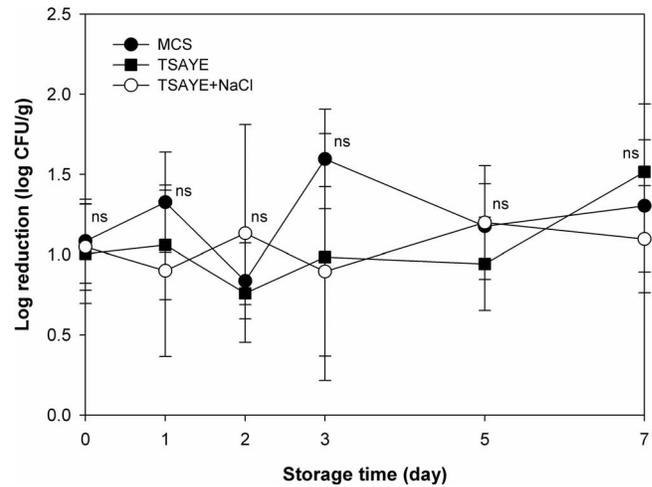


FIGURE 2. Reductions in the number of *E. coli* O157:H7 in romaine lettuce treated with dielectric barrier discharge atmospheric cold plasma (DACP) during storage at 4°C. Treatment voltage applied to the electrode, current through the CP reactor, and treatment time were 47.6 kV, 1 A, and 5 min, respectively. Error bars represent standard deviations. ns, no significant differences between the lettuce samples with and without DACP treatment within a sampling day ($P > 0.05$).

obvious. The *E. coli* O157:H7 inactivation data may suggest that the DACP treatment was more likely to have killed *E. coli* O157:H7 than to have injured the bacteria. The bactericidal action of CP is partly based on the diffusion of reactive species in plasma across the cell membrane into the cell, where they damage proteins and nucleic acids, leading to cell death (49).

The log reductions during storage were in the range of 0.8 to 1.6 log CFU/g in the counts for MCS, TSAYE, and TSAYE+NaCl (Fig. 2), which may be lower than that reported previously with DACP treatment (24, 45, 47). However, the interaction between CP and microorganisms is quite complex, depending on plasma generation factors, food factors, and microbiological factors (15), which makes it difficult to compare results obtained by different CP treatments. The complex surface of the food product is one of the food factors affecting the efficiency of the CP treatment (3, 15, 47). Baier et al. (2) reported differences in the inactivation of *E. coli* by CP on gel compared with corn salad leaves, suggesting the need to account for surface characteristics. Irregularities on the produce surface may provide many niche areas for bacteria, protecting them from CP treatment (50). The micrographs show that the lettuce surface contains stomata, cracks, and grooves (11, 15). These surface characteristics of lettuce could create physical barriers concealing microorganisms from CP and contribute to the reduced DACP effect against *E. coli* O157:H7 on lettuce (5, 50). The high standard deviations seen in the reductions (Fig. 2) may also be due to the surface characteristics of lettuce. Both even and uneven areas coexist in lettuce leaves (50). Inactivation levels could vary depending on surface evenness, resulting in high variation (17). Relatively even parts could result in a higher reduction in the cells compared with relatively uneven parts. A longer

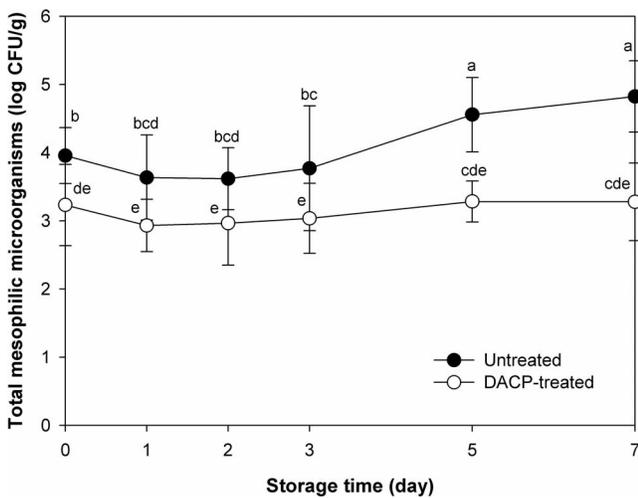


FIGURE 3. Effects of dielectric barrier discharge atmospheric cold plasma (DACP) treatment on the growth of total mesophilic aerobic microorganism in romaine lettuce during storage at 4°C for 7 days. Treatment voltage applied to the electrode, current through the CP reactor, and treatment time were 47.6 kV, 1 A, and 5 min, respectively. Error bars represent standard deviations. Different letters denote significant differences at $P < 0.05$.

plasma treatment time may increase the inactivation rate with smaller variations. However, although greater inactivation may be achieved by increasing the treatment time, in practice, cost and negative impacts on quality must be considered (22). A commercial-scale CP treatment system should be developed to significantly increase microbial inactivation levels by targeting bacteria obscured by surface convolutions.

Based on visual inspection, CP-treated lettuce did not display any signs of gross physical damage, including burns and leaf wilting, compared with the untreated control. In comparing the quality of produce treated with CP, it is important to delineate the nature of the system used to make valid comparisons. CP systems, which use externally applied CP, directed at the produce (5, 23, 39), would be expected to have a different range of impacts than systems that generate the CP inside the package, such as the prototype apparatus described herein. The temperature increase (ΔT) was $2.1 \pm 0.2^\circ\text{C}$. Temperature on the lettuce did not exceed 29°C , suggesting that the DACP was conducted nonthermally, and the microbial reduction was due solely to CP (37). The temperature was lower than that of the warm chlorinated water (47°C , 100 ppm of chlorine) often used for dipping shredded lettuce to extend the product shelf life (8).

Plasma inactivation efficiency was found to be dependent on the posttreatment storage time, which allowed for diffusion of reactive species, relatively long-lived species, in food (50). Ziuzina et al. (50) reported that 24-h posttreatment storage time facilitated atmospheric CP action on the bacterial cells by retaining generated reactive species within a closed container, promoting diffusion of the species inside the produce tissue. Ozone is a reactive species of DACP and can be retained inside the package for varying times, leading to extended reductions in microbial load (28).

However, a significant increase in the reduction of *E. coli* O157:H7 due to posttreatment storage was not observed in the current study. This is likely due to incomplete sealing of the lid of the commercial plastic container, allowing reactive species to escape from the container, preventing their continuous exposure to microorganisms on the lettuce. Although ozone is not an extremely toxic gas at low concentrations, it may be injurious to humans at a high concentration (14). Depending on exposure time, ozone at ≥ 0.2 ppm can cause varying degrees of damage to the respiratory tract (43). Thus, for future study, it may be worth investigating the ozone concentration in the package containing food during and after treatment.

The reduction of *E. coli* O157:H7 due to storage at refrigeration temperature for 7 days was less than 1.4 log CFU/g, according to the MCS plate results (data not shown). The number of *E. coli* O157:H7 in the untreated samples on day 0 (6.0 ± 0.4 log CFU/g), counted from MCS plates, was not significantly different from that on day 7 (5.2 ± 0.6 log CFU/g; $P > 0.05$), while it was significantly different from the number in the DACP-treated samples on day 7 (3.8 ± 0.7 log CFU/g; $P < 0.05$; data not shown). The total reduction achieved by the combination of CP treatment and storage for 7 days at 4°C resulted in ~ 2 -log reductions (1.9 to 2.2 log CFU/g). Storing produce at lower temperatures was beneficial for reducing pathogens on lettuce treated by DACP (28).

Inactivation of indigenous microorganisms. During storage at 4°C , APC counts of the untreated controls demonstrated a steady logarithmic growth, increasing from 4.0 ± 0.4 to 4.8 ± 0.5 log CFU/g of lettuce, while those of treated samples were constant at ~ 3.3 log CFU/g (Fig. 3). This suggests that a majority of the surviving indigenous aerobic bacteria were sublethally injured and failed to multiply during storage. Enhanced reduction in the APC counts of blueberries treated with an air plasma jet observed after storage for 7 days at 4°C was also reported by Lacombe et al. (23). The DACP treatment reduced the initial counts on APC (day 0) by 1.1 log CFU/g (Fig. 3). The log reduction in APC appeared to increase with storage time (Fig. 4). The enhanced reduction was likely due to increased APC counts in the untreated samples, with the relatively constant APC counts in the treated samples during storage (Fig. 3).

The yeast-mold counts of both treated and untreated lettuce increased steadily during storage, from 2.8 ± 0.7 to 4.6 ± 0.3 log CFU/g and from 2.2 ± 0.9 to 3.5 ± 0.3 log CFU/g, respectively (data not shown). The average log reductions exhibited in yeast-mold counts during storage were 0.9 to 1.7 log CFU/g and did not appear to depend on storage time (Fig. 4).

The treatment used resulted in reductions in the populations of native aerobic microflora on lettuce, which persisted throughout the storage period of the study (Fig. 4). Despite the potential diversity of indigenous aerobic microflora (50), the DACP treatment effectively inactivated the microorganisms ($P < 0.05$) and retarded their growth on lettuce.

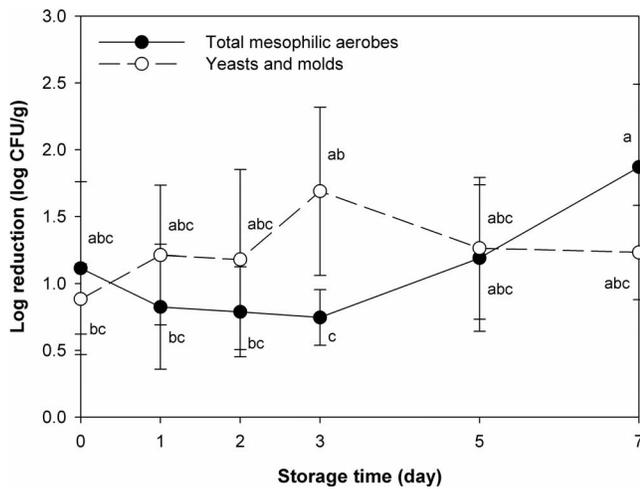


FIGURE 4. Reductions in the number of total mesophilic aerobic microorganism and yeasts and molds in romaine lettuce treated with dielectric barrier discharge atmospheric cold plasma (DACP) during storage at 4°C. Treatment voltage applied to the electrode, current through the CP reactor, and treatment time were 47.6 kV, 1 A, and 5 min, respectively. Error bars represent standard deviations.

The microorganism reductions in a commercial plastic package demonstrated in this study suggest microbial decontamination by DACP of possible postprocessing contamination of treated lettuce. Further optimization of this process is necessary to improve microbial reductions while preserving the product's physical integrity. The optimization may include packaging requirements, such as food package volume ratio.

Effects on color. The Hunter L^* , a^* , and b^* values were not significantly different between the treated and untreated lettuce samples on each day of sampling ($P > 0.05$; Table 1). The average total color difference (ΔE^*) values were 2.17 to 3.48 and were not significantly different during storage ($P > 0.05$). The color results suggest that the DACP treatment (47.6 kV, 1.0 A, and 5 min) did not affect the color of the lettuce stored at 4°C for 7 days. For lettuce,

color is probably the first quality factor judged by consumers, and browning is considered critical in perceived loss of quality (13). Some sanitizers with the most effective activity against *E. coli* O157:H7 have been reported to produce noticeable discoloration of romaine lettuce during storage at 4°C for 14 days (20). An insignificant change in color agrees with previous results of CP-treated tomatoes, strawberries, and apples (5, 28, 32).

The evaluation on the quality properties of food treated by CP is worthy of investigation because CP containing various reactive species can affect food components, and depending on the treatment conditions and food type, it can change food properties, including color (27). In a study conducted using ready-to-eat meat, a significant loss of redness was observed in the product, indicating a possible chemical reaction between the meat pigments and a plasma reactive species (41). However, our DACP treatment achieved lettuce decontamination without altering its color.

Effects on CO₂ generation. Changes in CO₂ generation in lettuce packages during storage at 4°C are presented in Table 1. Immediately after treatment, the levels of CO₂ were 1.4% for both untreated and treated lettuce samples (Table 1). During posttreatment storage, CO₂ levels tended to decrease (Table 1), suggesting decreased respiration with time (10). If the produce undergoes physiological stress, often due to processing, the respiration rate of the produce changes (39). Thus, the respiration rate is often a good index of the storage life of horticultural products (40). In this study, the respiration rate decreased with time for both treated and untreated samples. However, the levels of CO₂ generated were not significantly different between the treated and untreated samples on each day of sampling ($P > 0.05$; Table 1). The results indicate that the DACP treatment did not affect the respiration of lettuce stored at 4°C for 7 days.

Processing of fresh horticultural produce promotes a faster physiological deterioration, biochemical changes, and microbial degradation of the product (34, 39), because produce is often subjected to stress during processing (45).

TABLE 1. Effects of dielectric barrier discharge atmospheric cold plasma (DACP) treatment on the color, CO₂ generation, and weight loss of romaine lettuce during storage at 4°C for 7 days^a

Parameters	Sample	Storage time (days):				
		0	1	2	3	7
L^*	Untreated	51.52 ± 4.96 A ^b	50.49 ± 5.09 A	50.68 ± 4.75 A	49.46 ± 4.95 A	49.28 ± 4.81 A
	DACP treated	51.95 ± 5.07 A	51.88 ± 5.43 A	51.52 ± 4.57 A	50.71 ± 4.66 A	49.99 ± 4.38 A
a^*	Untreated	-8.75 ± 1.66 A	-8.69 ± 1.44 A	-9.04 ± 1.52 A	-8.28 ± 1.05 A	-8.31 ± 1.13 A
	DACP treated	-9.08 ± 1.11 A	-8.61 ± 1.12 A	-8.88 ± 1.31 A	-8.01 ± 1.23 A	-8.29 ± 0.94 A
b^*	Untreated	28.00 ± 1.86 A	27.36 ± 1.64 A	28.31 ± 1.29 A	26.27 ± 2.87 A	26.12 ± 2.20 A
	DACP-treated	28.72 ± 2.66 A	27.52 ± 2.32 A	27.6 ± 2.08 A	26.47 ± 2.26 A	26.29 ± 2.09 A
CO ₂ concn (%)	Untreated	1.4 ± 0.2 AB	1.2 ± 0.3 ABC	0.9 ± 0.1 BC	0.9 ± 0.1 BC	0.7 ± 0.1 C
	DACP treated	1.4 ± 0.3 A	1.3 ± 0.4 AB	1.1 ± 0.2 ABC	1.0 ± 0.1 ABC	0.9 ± 0.1 BC
Weight loss (%)	Untreated	0.77 ± 0.36 B	0.11 ± 0.43 B	0.10 ± 0.23 B	1.44 ± 0.69 B	4.11 ± 0.56 A
	DACP treated	0.31 ± 0.29 B	0.42 ± 0.77 B	0.53 ± 0.83 B	1.36 ± 0.95 B	4.50 ± 0.99 A

^a Treatment voltage applied to the electrode, current through the CP reactor, and treatment time were 47.6 kV, 1 A, and 5 min, respectively.

^b Different letters indicate a significant difference ($P < 0.05$) within the same parameters.

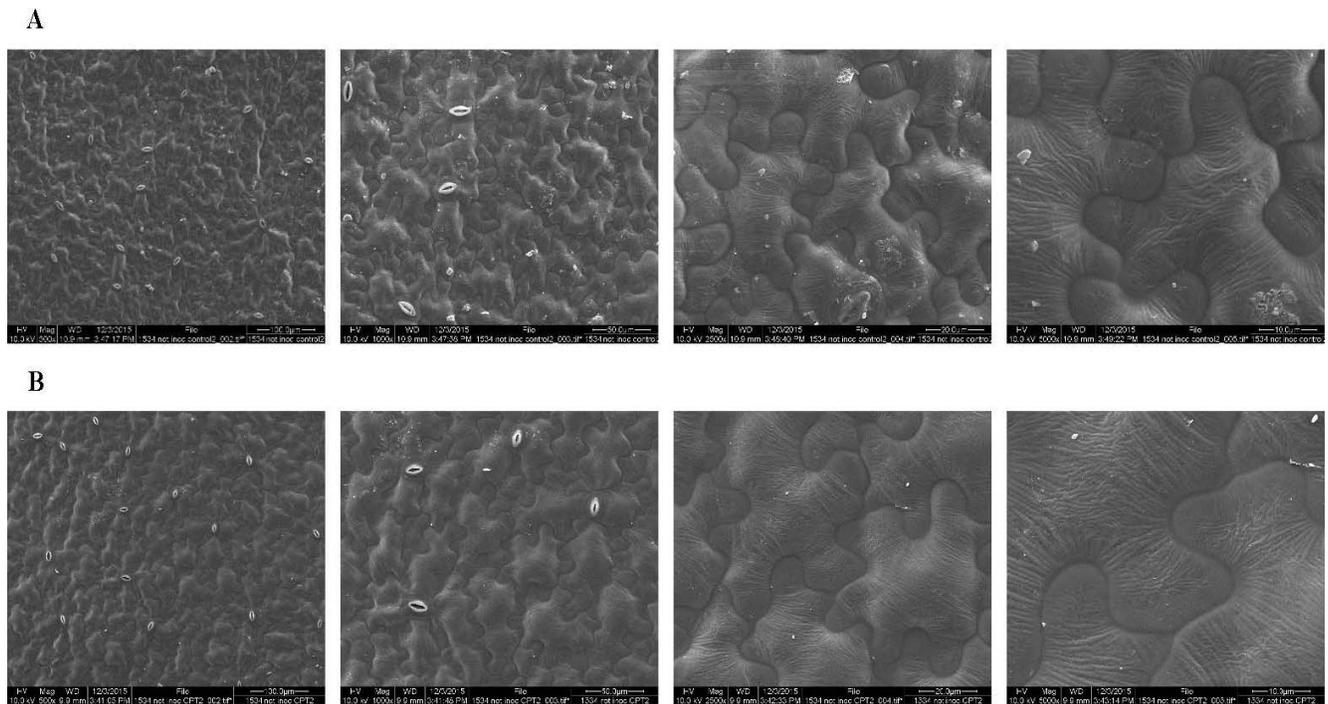


FIGURE 5. Scanning electron micrographs of the surfaces of romaine lettuce samples prepared with (B) and without (A) dielectric barrier discharge atmospheric cold plasma (DACP) treatment. Treatment voltage applied to the electrode, current through the CP reactor, and treatment time were 47.6 kV, 1 A, and 5 min, respectively. The magnifications were $\times 500$, $\times 1,000$, $\times 2,500$, and $\times 5,000$, from the left to the right rows.

When a treatment induces stress or damages the tissue of the produce, a higher respiration rate is exhibited (28, 29), which can negatively influence its postharvest life (9). Based on these observations, we conclude that the DACP did not induce significant stress in lettuce treated under the conditions evaluated.

Effects on weight loss. No significant difference in weight loss between the CP-treated and untreated samples was observed on each sampling day ($P > 0.05$; Table 1). The total loss in weight on day 7 did not exceed 6% in any sample. The weight loss can be accounted for, in part, by diffusion of gases (e.g., CO_2) across the fruit boundary and loss of water vapor from lettuce through its skin (18, 19). Scanning electron microscopy images, shown in Figure 5, demonstrate that DACP treatment did not induce any change in the surface morphology of the lettuce. This could support insignificant changes in weight loss, as well as CO_2 generation, in lettuce after DACP treatment. The results from the physical property studies indicate that the DACP treatment used in this research did not alter those properties of lettuce right after treatment and during storage at 4°C.

DACP treatments on packaged foods have been reported (27, 47). The packages in those studies were specially designed with patterned metal sheets and conductive and dielectric layers (47), as well as a rigid polypropylene package covered with a polymeric film (27), and are not directly available commercially. A flexible high barrier film (Cryovac, Sealed Air, Cambridgeshire, England) was also used for packaging cherry tomatoes for DACP treatment.

However, a rigid plastic container (clamshell container) is the most common package for fresh-cut produce and mixed salads, and this is the first study to investigate the effects of DACP treatment on produce packaged in a commercial standard conventional rigid plastic container. The results from this study suggest that the DACP treatment is compatible with food products prepared using current packaging equipment. Future research will specifically address potential interactions of the DACP with packaging materials under optimized treatment conditions. Although the results obtained in this study with single leaves are promising, part of the development of this technology to viability for the produce industry must define the efficacy of this in-package treatment for larger samples. A recent study treated multiple romaine lettuce leaves, stacked three, five, or seven high, inside commercial packaging using the same prototype DACP apparatus used herein (31). In that study, *E. coli* O157:H7 was significantly reduced on packaged leaves with very good process uniformity for leaves at all positions and for all bulk packaging configurations. Future research with in-package treatment of produce will address the technical challenges associated with the scale up from this first-generation prototype to increase the speed, efficacy, and cost efficiency to better meet the needs of industry. This research will include determination of texture, nutritional content, aroma, taste, and other quality factors for DACP-treated produce.

Overall, the results of this study demonstrated the potential of applying DACP to decontaminate lettuce contained in conventional plastic packages as a terminal processing step. Antimicrobial effects were achieved

without altering the color or leaf respiration during posttreatment cold storage.

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REFERENCES

1. Ashpis, D. E., M. C. Laun, and E. L. Griebeler. 2012. Progress toward accurate measurements of power consumptions of DBD plasma actuators. Proceedings of the 50th American Institute of Aeronautics and Astronautics Aerospace Sciences Meeting Including the New Horizons Forum and Aerospace Exposition, American Institute of Aeronautics and Astronautics, Reston, Virginia.
2. Baier, M., J. Foerster, U. Schnabel, D. Knorr, J. Ehlbeck, W. B. Herppich, and O. Schlüter. 2013. Direct non-thermal plasma treatment for the sanitation of fresh corn salad leaves: evaluation of physical and physiological effects and antimicrobial efficacy. *Postharvest Biol. Technol.* 84:81–87.
3. Baier, M., T. Janen, L. H. Wieler, J. Ehlbeck, D. Knorr, and O. Schlüter. 2015. Inactivation of Shiga toxin-producing *Escherichia coli* O104:H4 using cold atmospheric pressure plasma. *J. Biosci. Bioeng.* 120:275–279.
4. Bermúdez-Aguirre, D., and G. V. Barbosa-Cánovas. 2013. Disinfection of selected vegetables under nonthermal treatments: chlorine, acid citric, ultraviolet light and ozone. *Food Control* 29:82–90.
5. Bermúdez-Aguirre, D., E. Wemlinger, P. Pedrow, G. Barbosa-Cánovas, and M. Garcia-Perez. 2013. Effect of atmospheric pressure cold plasma (APCP) on the inactivation of *Escherichia coli* in fresh produce. *Food Control* 34:149–157.
6. Centers for Disease Control and Prevention. 2015. Foodborne outbreak online database (FOOD). Available at: <http://www.cdc.gov/foodborneoutbreaks/#>. Accessed 8 October 2015.
7. Cevallos-Cevallos, J. M., G. Gu, M. D. Danyluk, N. S. Dufault, and H. C. Ariena van Bruggen. 2012. *Salmonella* can reach tomato fruits on plants exposed to aerosols formed by rain. *Int. J. Food Microbiol.* 158:140–146.
8. Del Nobile, M. A., A. Baiano, A. Benedetto, and L. Massignan. 2006. Respiration rate of minimally processed lettuce as affected by packaging. *J. Food Sci.* 74:60–69.
9. Eskin, N. A. M. 1990. Biochemical changes in raw foods: fruits and vegetables, p. 70–78. In N. A. M. Eskin (ed.), *Biochemistry of foods*, 2nd ed. Academic Press, San Diego, CA.
10. Fan, X., and K. J. B. Sokorai. 2011. Effects of gamma irradiation, modified atmosphere packaging, and delay of irradiation on quality of fresh-cut iceberg lettuce. *HortScience* 46:273–277.
11. Fernández, A., E. Noriega, and A. Thompson. 2013. Inactivation of *Salmonella enterica* serovar Typhimurium on fresh produce by cold atmospheric gas plasma technology. *Food Microbiol.* 33:24–29.
12. Gómez-López, V. M., A. Rajkovic, P. Ragaert, N. Smigic, and F. Devlieghere. 2009. Chlorine dioxide for minimally processed produce preservation: a review. *Trends Food Sci. Technol.* 20:17–26.
13. Guan, W., L. Huang, and X. Fan. 2010. Acids in combination with sodium dodecyl sulfate caused quality deterioration of fresh-cut iceberg lettuce during storage in modified atmosphere package. *J. Food Sci.* 75:S435–S440.
14. Guzel-Seydim, Z. B., A. K. Greene, and A. C. Seydim. 2004. Use of ozone in the food industry. *LWT - Food Sci. Technol.* 37: 453–460.
15. Hertwig, C., K. Reineke, J. Ehlbeck, D. Knorr, and O. Schlüter. 2015. Decontamination of whole black pepper using different cold atmospheric pressure plasma applications. *Food Control* 55:221–229.
16. Jahid, I. K., N. Han, and S. D. Ha. 2014. Inactivation kinetics of cold oxygen plasma depend on incubation conditions of *Aeromonas hydrophila* biofilm on lettuce. *Food Res. Int.* 55:181–189.
17. Jahid, I. K., N. Han, C. Y. Zhang, and S. D. Ha. 2015. Mixed culture biofilms of *Salmonella* Typhimurium and cultivable indigenous microorganisms on lettuce show enhanced resistance of their sessile cells to cold oxygen plasma. *Food Microbiol.* 46:383–394.
18. Javanmardi, J., and C. Kubota. 2006. Variation of lycopene, antioxidant activity, total soluble solids and weight loss of tomato during postharvest storage. *Postharvest Biol. Technol.* 41:151–155.
19. Kader, A. A., and M. E. Saltveit. 2003. Atmosphere modification, p. 229–246. In J. A. Bartz and J. K. Brecht. (ed.), *Postharvest physiology and pathology of vegetables*, 2nd ed. Marcel Dekker, New York.
20. Keskinen, L. A., A. Burke, and B. A. Annous. 2009. Efficacy of chlorine, acidic electrolyzed water and aqueous chlorine dioxide solutions to decontaminate *Escherichia coli* O157:H7 from lettuce leaves. *Int. J. Food Microbiol.* 132:134–140.
21. Kim, H. J., H. I. Yong, S. Park, K. Kim, W. Choe, and C. Jo. 2015. Microbial safety and quality attributes of milk following treatment with atmospheric pressure encapsulated dielectric barrier discharge plasma. *Food Control* 47:451–456.
22. Kim, J. E., D. U. Lee, and S. C. Min. 2014. Microbial decontamination of red pepper powder by cold plasma. *Food Microbiol.* 38:128–136.
23. Lacombe, A., B. A. Niemira, J. B. Gurtler, X. Fan, J. Sites, G. Boyd, and H. Chen. 2015. Atmospheric cold plasma inactivation of aerobic microorganisms on blueberries and effects on quality attributes. *Food Microbiol.* 46:479–484.
24. Lee, T., P. Puligundla, and C. Mok. 2015. Inactivation of foodborne pathogens on the surfaces of different packaging materials using low-pressure air plasma. *Food Control* 51:149–155.
25. Lianou, A., and J. N. Sofos. 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *J. Food Prot.* 70:2172–2198.
26. Misra, N. N., K. M. Keener, P. Bourke, J. P. Mosnier, and P. J. Cullen. 2014. In-package atmospheric pressure cold plasma treatment of cherry tomatoes. *J. Biosci. Bioeng.* 118:177–182.
27. Misra, N. N., T. Moiseev, S. Patil, S. K. Pankaj, P. Bourke, J. P. Mosnier, K. M. Keener, and P. J. Cullen. 2014. Cold plasma in modified atmospheres for post-harvest treatment of strawberries. *Food Bioprocess Technol.* 7:3045–3054.
28. Misra, N. N., S. Patil, T. Moiseev, P. Bourke, J. P. Mosnier, K. M. Keener, and P. J. Cullen. 2014. In-package atmospheric pressure cold plasma treatment of strawberries. *J. Food Eng.* 125:131–138.
29. Mitcham, E. J., and R. E. McDonald. 1993. Respiration rate, internal atmosphere, and ethanol and acetaldehyde accumulation in heat-treated mango fruit. *Postharvest Biol. Technol.* 3:77–86.
30. Niemira, B. A. 2012. Cold plasma decontamination of foods. *Annu. Rev. Food Sci. Technol.* 3:125–142.
31. Niemira, B. A., S. C. Min, S. H. Roh, G. Boyd, and J. E. Sites. 2016. In-package inhibition of *E. coli* O157:H7 on bulk romaine lettuce using cold plasma, P1-33. Abstr. Annu. Meet. IAFP 2016, St. Louis, MO. International Association for Food Protection, Des Moines, IA. Available at: <https://iafp.confex.com/iafp/2016/webprogram/Paper12274.html>. Accessed 14 October 2016.
32. Niemira, B. A., and J. Sites. 2008. Cold plasma inactivates *Salmonella* Stanley and *Escherichia coli* O157:H7 inoculated on golden delicious apples. *J. Food Prot.* 71:1357–1365.
33. Niemira, B. A., C. H. Sommers, and X. Fan. 2002. Suspending lettuce type influences recoverability and radiation sensitivity of *Escherichia coli* O157:H7. *J. Food Prot.* 65:1388–1393.
34. O’Beirne, D., and G. A. Francis. 2003. Reducing pathogen risks in MAP-prepared produce, p. 231–286. In R. Ahvenainen (ed.), *Novel food packaging techniques*. CRC Press, Boca Raton, FL.
35. Olaimat, A. N., and R. A. Holley. 2012. Factors influencing the microbial safety of fresh produce: a review. *Food Microbiol.* 32:1–19.
36. Olanya, O. M., B. A. Niemira, and J. G. Philips. 2015. Effects of gamma irradiation on the survival of *Pseudomonas fluorescens* inoculated on romaine lettuce and baby spinach. *LWT - Food Sci. Technol.* 62:55–61.
37. Pankaj, S. K., N. N. Misra, and P. J. Cullen. 2013. Kinetics of tomato peroxidase inactivation by atmospheric pressure cold plasma based on

- dielectric barrier discharge. *Innov. Food Sci. Emerg. Technol.* 19:153–157.
38. Pavlovich, M. J., H.-W. Chang, Y. Sakiyama, D. S. Clark, and D. B. Graves. 2013. Ozone correlates with antibacterial effects from indirect air dielectric barrier discharge treatment of water. *J. Phys. D Appl. Phys.* 46:145202. Available at: <http://iopscience.iop.org/article/10.1088/0022-3727/46/14/145202>. Accessed 14 October 2016.
 39. Rico, D., A. B. Martín-Diana, J. M. Barat, and C. Barry-Ryan. 2007. Extending and measuring the quality of fresh-cut fruit and vegetables: a review. *Trends Food Sci. Technol.* 18:373–386.
 40. Robertson, G. L. (ed.). 2012. *Food packaging: principles and practice*, 3rd ed. CRC Press, Boca Raton, FL.
 41. Rød, S. K., F. Hansen, F. Leipold, and S. Knøchel. 2012. Cold atmospheric pressure plasma treatment of ready-to-eat meat: inactivation of *Listeria innocua* and changes in product quality. *Food Microbiol.* 30:233–238.
 42. Saldaña, G., E. Puértolas, S. Condón, I. Álvarez, and J. Raso. 2010. Modeling inactivation kinetics and occurrence of sublethal injury of a pulsed electric field-resistant strain of *Escherichia coli* and *Salmonella* Typhimurium in media of different pH. *Innov. Food Sci. Emerg. Technol.* 11:290–298.
 43. Schwartz, L. W., D. L. Dungworth, M. G. Mustafa, B. K. Tarkington, and W. S. Tyler. 1976. Pulmonary responses of rats to ambient levels of ozone: effects of 7-day intermittent or continuous exposure. *Lab. Invest.* 34:565–578.
 44. Toshifuji, J., T. Katsumata, H. Takikawa, T. Sakakibara, and I. Shimizu. 2003. Cold arc-plasma jet under atmospheric pressure for surface modification. *Surf. Coat. Technol.* 171:302–306.
 45. Watada, A. E., N. P. Ko, and D. A. Minott. 1996. Factors affecting quality of fresh-cut horticultural products. *Postharvest Biol. Technol.* 9:115–125.
 46. Yong, H. I., H. J. Kim, S. Park, A. U. Alahakoon, K. Kim, W. Choe, and C. Jo. 2015. Evaluation of pathogen inactivation on sliced cheese induced by encapsulated atmospheric pressure dielectric barrier discharge plasma. *Food Microbiol.* 46:46–50.
 47. Yong, H. I., H. J. Kim, S. Park, K. Kim, W. Choe, S. J. Yoo, and C. Jo. 2015. Pathogen inactivation and quality changes in sliced cheddar cheese treated using flexible thin-layer dielectric barrier discharge plasma. *Food Res. Int.* 69:57–63.
 48. Ziuzina, D., L. Han, P. J. Cullen, and P. Bourke. 2015. Cold plasma inactivation of internalised bacteria and biofilms for *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes* and *Escherichia coli*. *Int. J. Food Microbiol.* 210:53–61.
 49. Ziuzina, D., S. Patil, P. J. Cullen, K. M. Keener, and P. Bourke. 2012. Atmospheric cold plasma inactivation of *Escherichia coli* in liquid media inside a sealed package. *J. Appl. Microbiol.* 114:778–787.
 50. Ziuzina, D., S. Patil, P. J. Cullen, K. M. Keener, and P. Bourke. 2014. Atmospheric cold plasma inactivation of *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* inoculated on fresh produce. *Food Microbiol.* 42:109–116.