Viability of chlorine-injured Escherichia coli O157:H7 on Fresh-cut Cabbage during Cold Storage in High CO₂ Atmospheres

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Viability of chlorine-injured E. coli O157:H7 inoculated onto shredded cabbage was evaluated during storage in air or high CO₂ controlled atmospheres (CA) of 5%, 10%, and 15% at 10°C and in a modified atmosphere packaging (MAP) at 5°C and 10°C. When shredded cabbage was inoculated with chlorine-injured E. coli O157:H7 (% injury = 65%) and then stored in air or CA at 10°C, counts of E. coli O157:H7 increased during storage and injured E. coli O157:H7 (% injury = 34-66%) were detected on samples throughout the storage regardless of the CO₂ atmosphere. When shredded cabbage inoculated with chlorine-injured E. coli O157:H7 (% injury = 45-59%) were stored in a MAP using either a high or low oxygen transmission permeability (OTR) package film, the counts of E. coli O157:H7 increased during storage at 10°C and they remained constant during storage at 5°C. Injured E. coli O157:H7 were detected on shredded cabbage at a 54-56% level in a low OTR film at 10°C and a 73-74% level in a high OTR film at 5°C. These results indicated that chlorine-injured E. coli O157:H7 inoculated on fresh-cut cabbage exhibited different degrees of injury during storage in a high CO₂ CA and MAP at 5°C or 10°C.

Key words: Injured bacteria / Escherichia coli O157:H7 / Chilling temperature / High CO₂ atmospheres / Shredded cabbage.

INTRODUCTION

It is noted that an increase in produce-associated outbreaks of foodborne illness has been observed worldwide, because foodborne pathogens such as Escherichia coli O157:H7, Salmonella, and Listeria monocytogenes could be present in fresh-cut produce due to persistence from raw materials or cross-contamination during processing (Harris et al., 2003; Ramos et al., 2013). The method commonly used for decontamination of pathogens on fresh-cut produce involves washing with disinfectant such as chlorine, ozone, and other antimicrobial agents during processing (Watada et al., 2005). In addition, controlled atmosphere (CA)/modified atmosphere (MA) that means removal or addition of gasses to obtain the desired low O₂ and high CO₂ atmospheres has been shown to be beneficial in reducing the physiological and microbial decay of several fresh-cut produce (Gorny, 2003). The practice of chill temperature and CA/MA with high CO₂ levels of >10% leads to a bacteriostatic effect for native microorganisms on fresh-cut produce during storage (Izumi et al., 2016b). However, the high CO₂ conditions could result in the growth of foodborne pathogens that are facultative anaerobic bacteria by reducing the levels of competing aerobic microorganisms (Izumi, 2018). Current, it may be difficult to reduce levels of foodborne pathogens on fresh-cut produce using only high CO₂ CA and MA package (MAP), so the use of combination of antimicrobial treatments and CA/MA should be considered.

In order to ensure the microbial safety of fresh-cut produce, the presence of sublethally injured bacteria as the result of exposure to chemical and physical stresses must be taken into account (Ray, 1979; Wesche et al., 2009). Injured bacteria are not normally detected in selective media, but they resuscitate and subsequently multiply in a suitable environment such as nonselective
media. Besides their rejuvenation, many injured bacteria either retain or exhibit enhanced virulence after resuscitation, as noted for enteropathogenic E. coli exposed to seawater and sunlight using an enzyme-linked immuno-sorbent assay (ELISA) (Pommepuy et al., 1996) and heat-stressed L. monocytogenes using a bioassay (Bundukli et al., 1996) and Staphylococcus aureus using the ELISA (Hernández et al., 1993).

Chemically injured cells can occur when fresh-cut produce are washed with chemical disinfectants and the extent varies with kind and concentration of disinfectants and bacterial species and strain (Scheusner et al., 1971). Chlorine treatment induced more injured E. coli O157:H7 than quaternary ammonium treatment (Moosekian et al., 2014) and more injured Salmonella Typhimurium and L. monocytogenes than lactic acid and chlorous acid treatment (Lee et al., 2002). Lee et al. (2002) also reported that S. Typhimurium was more sensitive to chemical injury than L. monocytogenes when inoculated on mung bean sprouts. The stress from chemical disinfectants induces structural damage to the membrane and/or damage to functional components of the cells (Wesche et al., 2009). Cold-stress can also induce sublethally injured pathogens on fresh-cut produce during cold storage by transiently synthesizing a number of cold shock proteins and/or inducing lipid changes in the membrane due to decrease of the membrane fluidity and membrane-associated metabolic processes (Russell, 2002). The cold shock response has been linked with generation of injured S. Typhimurium in a broth system at 4°C and 10°C (Kinsella et al., 2006) and injured E. coli O157:H7 in apple juice stored at 7°C (Wu et al., 2008).

On the contrary, recovery of injured E. coli (Koseki and Yamamoto, 2006; Tsuchido et al., 1989) and E. coli O157:H7 (Bozoglu et al., 2004; Moosekian et al., 2014) has been indicated under favorable growth conditions after sublethal treatment. The resuscitation of sublethally injured bacteria is thought to be influenced by atmospheric conditions. An in vitro study revealed that anaerobic conditions after a heat treatment resulted in higher recovery of injured E. coli O157:H7 (Murano and Pierson, 1993) when compared with aerobic conditions. It is speculated that this may have been due to the spontaneous formation of toxic oxygen radicals in aerobic media, where heated cells are unable to deactivate due to the destruction of detoxifying enzymes like catalase and superoxide dismutase.

When fresh-cut produce are washed with chemical disinfectants, chemically injured bacteria can arise and then remain injured, inactivate, or recover on the produce during subsequent cold storage in a MA packaging (MAP) equilibrated to high CO2 levels. Little work has been done on the behavior of injured pathogens on fresh-cut produce during storage; therefore, in this study we determined the viability of chlorine-injured E. coli O157:H7 inoculated onto fresh-cut cabbage during cold storage in a high CO2, CA and MAP. The storage temperature of 10°C was mainly used for the CA and MAP, because the storage and transportation temperature of fresh-cut produce is commonly 10°C in Japan.

MATERIALS AND METHODS

Cell suspension and chlorine treatment

Nontoxicogenic E. coli O157:H7 JCM18426 obtained from the Japan Collection of Microorganisms (Riken BioResource Center, Tsukuba, Japan) was maintained at -80°C in 20% skim milk for use in this study. The culture was prepared by transferring the strain to 25 ml of tryptic soy broth (TSB; Nissui Pharmaceutical, Tokyo, Japan) and incubating at 30°C for 24 h. Cells were harvested by centrifugation (3000 x g at 20°C for 10 min), washed twice in sterile saline solution (0.85% NaCl in water), and adjusted with diluent (sterile saline solution) to approximately 106 CFU/ml for the detection of chlorine-injury by mixture with electrolyzed water and 106 CFU/ml for the inoculation onto fresh-cut cabbage using nephelometry. The number of bacteria in inoculum suspension was confirmed using the direct plating technique on MacConkey sorbitol agar (Nissui Pharmaceutical).

Since we previously reported that electrolyzed water containing chlorine caused chlorine-injury to a pure culture of E. coli (Izumi et al., 2016a), E. coli O157:H7 was injured sublethally by using electrolyzed water. Electrolyzed strongly acidic water (pH 2.7, 25 ppm available chlorine) was generated using a water electrolyzer α-Light (Amano, Yokohama, Japan), and then diluted to the concentration of 0.5, 1, 2, 3, and 4 ppm of available chlorine with sterile distilled water. The concentration of available chlorine was confirmed by the sodium thiosulfate titration method as previously described (Izumi, 1999). A 9 ml quantity of each chlorine solution was dispensed into a test tube containing 1 ml of cell suspension and mixed vigorously. After 10 min, a 1-ml sample was neutralized by dilution into TSB. A 9 ml quantity of sterile saline solution was substituted for the chlorine solution mixture as the control. The chlorine treatment was replicated three times.

Shredded cabbage and inoculation

Heads of cabbages were purchased from a local market in the Wakayama Prefecture immediately following their arrival at the store in the morning. After removal of the outer two leaves by hand, a food processor (Model MK-K78, National, Osaka, Japan) was used to prepare shredded cabbage (ca 2 mm wide, 50 mm long, and 1 mm thick). A 600 g sample of
shredded cabbage was immersed in 3 L of suspension of chlorine-injured E. coli O157:H7, using electrolyzed water containing 1 ppm of available chlorine, for 5 min at room temperature (≈ 23°C) and then dried in a laminar flow hood for 30 min with the fan running. Since our preliminary study revealed that chlorine-injured E. coli O157:H7 recovered almost completely after 30 min in the cell suspension, the inoculation was performed immediately after preparation of injured E. coli O157:H7 suspension.

High CO2 controlled atmosphere storage and modified atmosphere packaging

For CA storage, a 50 g sample was placed in a 500 ml plastic container containing 5 ml of distilled water in a beaker in order to maintain high relative humidity. Three replicated samples were stored at 10°C for 5 days under a continuous flow of air or high CO2 atmospheres (5%, 10%, and 15%) with the balance being air at a flow rate of 10 ml/min. For MAP, a 50 g sample was packaged in two types of films with an oxygen transmission permeability (OTR) of either 500 or 10000 ml/m2/d/atm [30 μm thick, 22 × 23 cm; Sumitomo Bakelite Co., Ltd, Tokyo, Japan] and stored at 10°C for 5 days or 5°C for 7 days. The storage period at 5°C was 7 days according to the estimated shelf of fresh-cut cabbage at 5°C. The O2 and CO2 concentrations in 1-ml samples taken from three replicated packages were measured daily during MAP storage using a gas chromatograph equipped with a thermal conductivity detector (GSC-8AIT-TCD, Shimadzu, Kyoto, Japan). The columns used for O2 and CO2 analyses were Molecular Sieve SA at 60°C and Porapak Q at 90°C, respectively.

Enumeration of injured E. coli O157:H7

Injured E. coli O157:H7 from cell suspension and cabbage samples treated with electrolyzed water were enumerated using the one-step TAL method (Kang and Fung, 2000), which involves a layer of selective medium on nonselective medium overlaid on the top of it. When injured target cells were inoculated directly onto the TAL medium, the injured cells resuscitated and grew on the nonselective medium and then formed typical reaction colonies when the selective agents from selective medium diffused to the nonselective medium. CHROMagar O157 medium (CHROMagar, Paris, France) was used as the selective medium, allowing E. coli O157:H7 to be detected by a characteristic mauve color colony. Standard method agar (Nissui Pharmaceutical) with 0.03% sodium pyruvate was used as the nonselective medium because pyruvate is well established as an injury repair agent to quench peroxides generated either by auto-oxidation or photo-oxidation of medium components (Tandon et al., 2007). For TAL medium, 20 ml of solidified selective agar was overlaid with two layers, 7 ml each of melted nonselective agar in a petri dish. The serial dilutions from a suspension of E. coli O157:H7 or a 10-g sample homogenate of cabbage in 90 ml of sterile saline solution using an Elmex stomacher (Promedia SH-001; ELMEX, Tokyo, Japan) were made in sterile saline solution. Each sample (0.1 ml) was spread onto both TAL medium that allowed the growth of both noninjured and injured cells and selective medium that allowed the growth of noninjured cells, and then incubated at 37°C for 24 h to enumerate colonies. The number of injured bacteria was represented by the difference between the counts on TAL and selective media. The percent of injury was expressed as follows: % injury = [(No. of CFU/ml or CFU/g from TAL plate - No. of CFU/ml or CFU/g from selective plate)/ No. of CFU/ml or CFU/g from TAL plate] x 100.

Data analysis

Three replicated microbiological plate count data sets were converted to log CFU/ml of pure cultures and log CFU/g of cabbage samples. Statistically significant differences (P ≤ 0.05) between the TAL medium and selective medium were determined for the injured bacterial population data based on t tests using the SAS system, release 6.12 (SAS Inst. Inc, Cary, NC). The mean values were separated by least significant difference.

RESULTS

Injury percentage of E. coli O157:H7 exposed to different concentrations of chlorine

When E. coli O157:H7 in pure culture was treated with electrolyzed water containing 0 to 4 ppm of available chlorine, counts of E. coli O157:H7 exposed to 1 to 2 ppm were 0.3 to 1.5 log higher on the TAL medium than on the selective medium, indicating that 46 to 97% of the viable cells were injured by a chlorine level of 1 to 2 ppm (Fig.1). Bacterial counts were below the lower limit of detection (2.0 log CFU/g) at 3 ppm, and no bacteria were detected on a plate at 4 ppm. Thus, electrolyzed water containing 1 ppm available chlorine was used to prepare a suspension of chlorine-injured E. coli O157:H7 with an injury level of approximately 50% for use as an inoculum.

Chlorine-injured E. coli O157:H7 on shredded cabbage stored in a high CO2, CA

When shredded cabbage inoculated with chlorine-injured E. coli O157:H7 (% injury = 65%) was stored in air or high CO2 atmospheres (5%, 10%, and 15%) at 10°C for 5 days, counts of E. coli O157:H7 on TAL plates
from 3.5 log CFU/g to 4.3 log CFU/g for all samples after 3 days of storage (Fig. 2). On day 5, the counts on TAL plates were 0.4 to 0.5 log higher in 10% and 15% CO₂ atmospheres than in air and a 5% CO₂ atmosphere. The injured E. coli O157:H7 were detected on samples stored in 15% CO₂ on day 1, air, 5% CO₂, 10% CO₂, and 15% CO₂ on day 3, and 5% CO₂ and 10% CO₂ on day 5. The percentage of injury ranged from 34 to 66%. A 15% CO₂ atmosphere reduced the growth of noninjured bacteria on selective plates and increased the growth of injured bacteria on TAL plates on day 1, resulting in survival of injured bacteria. On day 3 it was observed that all atmospheres accelerated the growth of injured bacteria on TAL plates, and on day 5 the detection of injured bacteria indicated that the 5% and 10% CO₂ atmospheres appeared to delay recovery of the injured cells. This result indicated that injured E.

**FIG. 1.** Counts of E. coli O157:H7 on TAL and selective medium from a pure culture mixed with electrolyzed water containing 0 to 4 ppm of available chlorine. Vertical lines represent the standard error of the mean. Means with * are significantly different (P ≤ 0.05) between the counts on paired TAL and selective media. Values of percentage (log difference in parentheses) represent the degree of injured cells, as estimated by the difference in counts between TAL and selective media. Means less than 2.0 (< 2.0) is below the level of detection. ND (not detectable) means no colonies were detected on a plate of the original culture.

**FIG. 2.** Counts of E. coli O157:H7 on TAL and selective medium from shredded cabbage inoculated with chlorine-injured E. coli O157:H7 and stored in air or high CO₂ controlled atmospheres at 10°C. Vertical lines represent the standard error of the mean. Means with * are significantly different (P ≤ 0.05) between the counts on paired TAL and selective media. Values of percentage (log difference in parentheses) represent the degree of injured cells, as estimated by the difference in counts between TAL and selective media.

**FIG. 3.** Concentrations of CO₂ (a) and O₂ (b) in two types of package films (OTR with either 500 or 10000 ml/m²/d/atm) containing shredded cabbage inoculated with chlorine-injured E. coli O157:H7 (% injury = 45%) during storage at 10°C. Vertical lines represent standard error of the mean. Symbols with * indicate significant at P ≤ 0.05 between paired low and high OTR films in the same day.
coli O157:H7 on shredded cabbage were capable of exhibiting different degrees of injury during storage at 10°C regardless of the CO₂ atmosphere.

**Chlorine-injured *E. coli* O157:H7 on shredded cabbage stored in a MAP**

Shredded cabbage inoculated with chlorine-injured *E. coli* O157:H7 was stored at 10°C for 5 days in a MAP using two types of package films with an OTR of either 500 or 10000 ml/m²/d/atm. The CO₂ concentration approached a 20% and 5% equilibrium in a low and high OTR film, respectively, after 3 days of storage (Fig.3a). The O₂ concentration decreased from 20 to 2% in a low OTR film and from 20 to 15% in a high OTR film after 3 days of storage (Fig.3b). The counts of *E. coli* O157:H7 on TAL plates increased 1 log in a low OTR film and 0.5 log in a high OTR film from the initial counts of 6.1 log CFU/g during storage of 5 days (Fig.4). Inoculated cabbage had 45% of its *E. coli* O157:H7 injured on the initial day, and injured cells ranging from 54 to 56% were present on samples stored in a low OTR film on days 3 and 5 where the CO₂ concentration was 20% and 17%, respectively.

When shredded cabbage inoculated with chlorine-injured *E. coli* O157:H7 was packaged inside a film with either an OTR of 500 or 10000 ml/m²/d/atm and was stored at 5°C for 7 days, the packages approached an equilibrium of 15% CO₂ and 6% O₂ in a low OTR film, and 4% CO₂ and 17% O₂ in a high OTR film during the storage period (Fig.5a and 5b). The counts of *E. coli* O157:H7 on TAL plates remained constant in both OTR films throughout 7 days of storage (Fig.6). For an initial injury level of 59%, injured cells at 73 to 74% level of *E. coli* O157:H7 were found on shredded cabbage stored in a high OTR film on days 3 and 7 when the CO₂ concentration ranged from 2 to 5%. These results emphasized that injured *E. coli* O157:H7 could remain viable in a MAP with low O₂ and high CO₂ that could be used commercially to extend the shelf life of fresh-cut produce.

**FIG. 4** Counts of *E. coli* O157:H7 on TAL medium and selective medium from shredded cabbage inoculated with chlorine-injured *E. coli* O157:H7 and stored in two types of package films (OTR with either 500 or 10000 ml/m²/d/atm) at 10°C. Vertical lines represent the standard error of the mean. Means with * are significantly different (*P* ≤ 0.05) between the counts on paired TAL and selective media. Values of percentage (log difference in parentheses) represent the degree of injured cells, as estimated by the difference in counts between TAL and selective media.

**FIG. 5** Concentrations of CO₂ (a) and O₂ (b) in two types of package films (OTR with either 500 or 10000 ml/m²/d/atm) containing shredded cabbage inoculated with chlorine-injured *E. coli* O157:H7 (% injury = 59%) during storage at 5°C. Vertical lines represent standard error of the mean. Symbols with * indicate significant at *P* ≤ 0.05 between paired low and high OTR films in the same day.
FIG. 6. Counts of E. coli O157:H7 on TAL medium and selective medium from shredded cabbage inoculated with chlorine-injured E. coli O157:H7 and stored in two types of package films (OTR with either 500 or 10000 ml/m²/d/atm) at 5°C. Vertical lines represent the standard error of the mean. Means with * are significantly different (P ≤ 0.05) between the counts on paired TAL and selective media. Values of percentage (log difference in parentheses) represent the degree of injured cells, as estimated by the difference in counts between TAL and selective media.

DISCUSSION

In a pure culture of E. coli O157:H7, the percentage of chlorine-injured cells was 46 to 97% following exposure to electrolyzed water containing 1 to 2 ppm of available chlorine. This finding was similar to our previous report that available chlorine of 1 to 2 ppm in electrolyzed water was effective in sublethally injuring generic E. coli in pure culture (Izumi et al., 2016a). These results followed essentially the same trends as previous reports that short-term exposure to chlorine at low levels caused sublethal injury to a large proportion of E. coli (Scheusner et al., 1971) and E. coli O157 cells (Moosekian et al., 2014). Sensitivity of E. coli to chlorine may be related to a lipopolysaccharide (Tsutcho et al., 1992) and teichoic acid-like O-polysaccharide (Perepelov et al., 2006) in its surface structure, which are responsible for the impaired permeability of cells, leading to injured cells (Ray, 1979).

We also found that chlorine-injured E. coli O157:H7 inoculated onto shredded cabbage remained injured to some extent during CA and MAP storage at 5°C or 10°C. There is no literature available documenting behavior of injured pathogens inoculated on produce during storage, although we previously reported that the injured coliform bacteria on shredded cabbage rinsed with electrolyzed water exhibited different degrees of injury during storage in air and high CO₂ CA (5%, 10%, and 15%) at 5°C and 10°C (Izumi and Inoue, 2018). Our previous report also indicated that when nonrinsed shredded cabbage was stored in air and high CO₂ atmospheres at 5°C and 10°C, injured coliform bacteria ranging from 25 to 85% were found on days 3, 5, and 7 days at 5°C and days 1 and 3 at 10°C in spite of uninjured cells on shredded cabbage on the initial day. This fact might be attributed to a cold shock response that contributed to cell injury of bacteria (Russell, 2002). In our study, the surviving injured cells on shredded cabbage did not die off during cold storage. It is conceivable that recovery of injured E. coli O157:H7 did not occur during storage because of the different degrees of injury exhibited on samples stored in CA of 5-10% CO₂ and MAP of 5-17% CO₂, but we cannot deny a new population of injured cells due to cold injury, especially after 3 days of storage. Since sublethal injury resulting from a combination of stresses may have induced a stress-sensitive state, survival of chlorine-injured E. coli O157:H7 during storage at 5°C and 10°C in this study likely involves the synergistic action of chlorine-stress and cold-stress. Survival of E. coli O157:H7 following synergistic stress was confirmed by other studies involving E. coli in apple juice that were REF-stressed followed by cold-stress (Ukuku et al., 2010) and E. coli on a corn product or whey protein product paste that were extrusion-stressed followed by cold-stress (Ukuku et al., 2013). Further research is needed to clarify the recovery rate and new proportion of injured cells during storage.

The action of CO₂ on bacteria is speculated to alter the properties of cell membranes and disrupt intercellular enzymes and organelles due to dissolved CO₂ in the aqueous phase and a decrease in intracellular pH (Farber, 1991). Thus, cell damage produced by sublethal stresses would facilitate the penetration of CO₂ into microorganisms or bring an increased sensitivity to subsequently applied CO₂, causing a greater lethal effect on microorganisms. In the presence of CO₂ of > 40%, injured bacteria were inactivated or their recovery was further delayed during storage in high CO₂ atmospheres as noted with L. innocua in fish soup (Rode et al., 2015) and S. Enteritidis and L. monocytogenes on fish products (Tassou et al., 2004). Although the application of synergistic actions of sublethal treatment and high CO₂ > 40% has been suggested for non-living products, the applied CO₂ atmospheres are too high to apply to living produce. Thus, the high CO₂ atmospheres ranging from 5 to 20% for fresh-cut cabbage would not be helpful in reducing the hazardous rise of E. coli O157:H7 populations and also the undesirable survival of injured E. coli O157:H7.

Temperature management rather than supplemental high CO₂ atmospheres could be involved in controlling
both proliferation of foodborne pathogens and the existence of injured foodborne pathogens. Although a low temperature < 5°C would prevent the growth of the pathogen, it seems to delay the repair of injury resulting from sublethal stresses according to several *in vitro* studies (Kinsella et al., 2006; Koseki and Yamamoto, 2006; Ukuku et al., 2010; Williams and Golden, 2001). With liquid foods, the repair of injured foodborne pathogens was inhibited in milk (Bozoglu et al., 2004), apple juice (Ukuku et al., 2010), and orange juice (Syed et al., 2013) during storage at 4-5°C. For fresh-cut produce, further research is needed to find the optimum temperature between 5°C and 10°C to inhibit both proliferation of bacterial pathogens and their recovery from injury while produce quality is maintained for 5-7 days.

In conclusion, we demonstrated that injured *E. coli* O157:H7 exhibited different degrees of injury on shredded cabbage during storage at 5°C or 10°C in high CO₂ atmospheres ranging from 5 to 20% in a CA or MAP. This result suggests that injured *E. coli* O157:H7 could exist in fresh-cut cabbage commercially stored in a MAP when *E. coli* O157:H7 adhered on fresh-cuts and were not inactivated lethally by disinfectants. To ensure the microbiological safety of fresh-cut produce treated with chemical disinfectants, it is necessary to adjust the concentration of disinfectants to a level lethal to bacteria and apply them multiple times to produce a synergistic effect of the two and more agents. In addition, consideration of the storage temperature used to inhibit the recovery of injured bacteria resulting from cold stress should be used as key points for further investigations on the viability of injured pathogens on fresh-cut produce during storage.

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