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ORIGINAL ARTICLE

# The effects of washing and chlorine dioxide gas on survival and attachment of *Escherichia coli* O157: H7 to green pepper surfaces

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*The effects of washing and chlorine dioxide ($ClO_2$) gas treatment on survivability and attachment of* Escherichia coli *O157: H7 C7927 to uninjured and injured green pepper surfaces were investigated using scanning electron microscopy and colony enumeration methods.* Escherichia coli *O157: H7 preferentially attached to coarse and porous intact surfaces and injured surfaces. The bacterial attachment to injured green pepper surfaces may be determined mainly by the hydrophilic properties of the injured surfaces and might not be assisted by the exocellular polymers of the bacteria. Injuries to the wax layer, the cuticle and underlying tissues increased bacterial adhesion, growth, and resistance to washing and disinfecting treatments. No significant growth of* E. coli *O157: H7 was found on uninjured surfaces after inoculation and incubation for 24 h at 37°C, whereas significant growth and multiplication were found on injured surfaces ($P < 0{\cdot}05$). $ClO_2$ gas treatment was more effective as a disinfecting method than water washing. Using a membrane-plating method for resuscitation and enumeration of $ClO_2$-treated* E. coli *O157: H7 on surface-injured green peppers, $3{\cdot}03 \pm 0{\cdot}02$ and $6{\cdot}45 \pm 0{\cdot}02$ log reductions were obtained after treatments by 0·62 and 1·24 mg $l^{-1}$ $ClO_2$, respectively, for 30 min at 22°C and 90–95% relative humidity. In contrast, water washing achieved log reductions of $1{\cdot}5 \pm 0{\cdot}05$–$1{\cdot}67 \pm 0{\cdot}10$ on injured surfaces and $2{\cdot}44 \pm 0{\cdot}04$ on uninjured surfaces.*



## Introduction

Minimally processed and refrigerated (MPR) fruits and vegetables are an important and rapidly developing class of foods (Wiley 1994). However, MPR fruit and vegetables can serve as vehicles for many different foodborne pathogenic micro-organisms (Brackett 1994, Beuchat 1996, Tauxe et al. 1997). In most cases, MPR fruit and vegetables are consumed without cooking, making the presence of pathogens a concern. The usual sources of pathogen contamination are from irrigation or wash water, fertilizers of animal waste and municipal biosolids, infected operators, and operation of

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facilities with poor sanitation (FDA et al. 1998). There have been numerous reports of foodborne outbreaks associated with these contaminated foods (Nguyen-the and Carlin 1994, Beuchat 1996, CDC 1997a, Odumeru et al. 1997, Gill 1998).

*Escherichia coli* O157: H7 is recognized as an important foodborne pathogen and can cause hemorrhagic colitis when as few as ten cells are ingested (Buchanan and Doyle 1997). It can be present on MPR fruits and vegetables (Beuchat 1999, Fisher and Golden 1998, Diaz and Hotchkiss 1996, Rosario and Beuchat 1995, Abdul-Raouf et al. 1993). It may survive in contaminated MPR fruits and vegetables due to insufficient heating, washing, or sanitation treatment. Outbreaks of *E. coli* O157: H7 infections involving fresh produce and fruit juices have been reported with increasing frequency in the USA (CDC 1994, 1996, 1997b, 1997c).

Washing with tap water or chlorinated water (50–200 ppm chlorine) is widely used for decontamination of fresh fruits and vegetables on a commercial scale; however, no more than 3 log reductions of bacteria after these treatments were reported (Beuchat 1992 and 1999, Beuchat et al. 1998, Brackett 1987 and 1992, Pao and Brown 1998, Taormina and Beuchat 1999). This may be due to attachment of bacteria to the surface and/or protection by some special sites of attachment. Seo and Frank (1999) found many live *E. coli* O157: H7 cells in the stomata and on cut edges of lettuce after treatment with 20 mg $l^{-1}$ chlorine solution. Itoh et al. (1998) also found the presence of viable *E. coli* O157: H7 in the inner tissues and stomata of cotyledons of radish sprouts grown from the seeds experimentally inoculated with the bacterium and treated by $HgCl_2$. MPR fruits and vegetables not only can have complex surface properties (smooth or coarse, intact or injured) for bacterial attachment, but also may provide nutrients for bacterial growth. Therefore, it is very important that a good sanitation technology should be used for sufficient reduction of pathogens in MPR fruits and vegetables.

One potential way to reduce micro-organisms on the surface of fruits and vegetables is to use $ClO_2$ gas, which is a strong oxidizing agent with about 2·5 times the oxidation capacity of chlorine (Bernarde et al. 1965). Because $ClO_2$ is less reactive with organic compounds than chlorine (White 1972), its application as a sanitizer in the food industry is of greater significance than chlorine. As gas has greater penetration ability than liquid, $ClO_2$ gas may be a more effective sanitizer for surface sanitation. $ClO_2$ gas effectively sanitized the surface of aseptic juice storage tanks. Han et al. (1999) found that all the spoilage micro-organisms inoculated to the surface of a model storage tank were completely killed after a treatment by 10 mg $l^{-1}$ $ClO_2$ gas for 30 min at 9–28°C and above 90% relative humidity (RH). However, information related to the use of $ClO_2$ gas as a surface disinfectant for MPR fruits and vegetables is lacking.

Scanning electron microscopy (SEM) has been used successfully to examine the attachment of spoilage or pathogenic micro-organisms to food and food contact surfaces. Using SEM, the colonization and infection of *Pseudomonas* on the interior surfaces of PVC pipes (Anderson et al. 1990), on stainless steel surface (Schwach and Zottola 1984), and on tomato surfaces (Getz et al. 1983) have been studied. SEM was also used to investigate the presence of *E. coli* O157: H7 in and on radish sprouts (Itoh et al. 1998), microbial biofilms on leaf surfaces of eight vegetables (Morris et al. 1997), attachment of *E. coli* O157: H7 to beef tissues (Fratamico et al. 1996), infection of table grape bunches by *Alternaria alternata* (Swart et al. 1995), and biofilm development of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-N rubber (Ronner and Wong 1993). Herald and Zottola (1988) also studied the attachment of *L. monocytogenes* to stainless steel surfaces at various temperatures and pH values. Mattila and Frost (1988) and Lillard (1988) used SEM to investigate the colonization of *E. coli* and *S. typhimurium* on poultry skin and muscle, respectively.

Bell-shaped green pepper (*Capsicum annuum*) is an important type of MPR vegetable. It has a smooth and easily washable surface with a thick cuticle. Waxes embedded within and over the surface (cuticle) of green pepper help resist water loss. Its surface properties are similar to those of many fruits and vegetables, such as apple, pear, eggplant and

cucumber. Because the information is limited on the attachment of pathogens to this kind of surface, green pepper was selected as a model sample for MPR fruit and vegetables for this study.

The objectives of this study were: a) to examine the attachment of *E. coli* O157: H7 to uninjured and injured surfaces of green pepper, and b) to compare the effectiveness of $ClO_2$ gas treatment and water washing on inactivation and removal of *E. coli* O157: H7 from green pepper surfaces using SEM and conventional colony enumeration techniques.

## Materials and Methods

### *Green pepper*

Bell-shaped, organic green peppers (*C. annuum*) were purchased from a local supermarket and stored at 7°C. The green peppers were rinsed with cold tap water (<3 ppm chlorine) for 1 min at 22°C. Smooth and uninjured surface sections were selected and cut into pieces (2 × 2 cm, about 5 g). These surface-uninjured green pepper pieces were placed into 100 × 15 mm sterile plastic petri dishes (Fisher Scientific, Pittsburgh, Philadelphia, USA), and treated by UV-light (30 W, about 50 cm irradiation distance) in a class II biosafety cabinet (Labconco Corporation, Kansas City, Missouri, USA) for 40 min (20 min each side) to inactivate naturally existing micro-organisms on the green pepper. Surface-injured green pepper samples were made from the surface-uninjured green peppers by using a sterile blade to gently scrape the surface so that some cuticle tissues were damaged and/or wax layer was scraped off.

### Escherichia coli *O157: H7*

*Escherichia coli* O157: H7 C7927 was provided by Dr M. P. Doyle at the University of Georgia, Athens, Georgia, USA. It was maintained at 7°C on slants of tryptic soy agar (TSA) (Difco Laboratories , Detroit, Michigan) and cultured in tryptic soy broth (TSB) (Difco Laboratories) at 37°C. The culture was transferred twice to TSB by loop inoculation at successive 24-h intervals. Cells (approximate $1 \times 10^9$ cfu $ml^{-1}$) from a 24-h static culture incubated at 37°C were used to inoculate the green pepper. The inoculum suspension was enumerated by surface plating duplicate samples on TSA after serial dilution in 0·1% peptone solution. The plates were incubated for 24 h at 37°C.

### *Inoculation of green pepper*

The outside surfaces of surface-uninjured and surface-injured green pepper pieces were evenly inoculated with 20 or 100 µl droplets of the above inoculum suspension in the class II biosafety cabinet. 20 or 100 µl inoculation achieved a total population of $2{\cdot}1 \times 10^7$ cfu or $1{\cdot}05 \times 10^8$ cfu on each sample. The inoculated samples were dried by air-blowing for 2 h at 22°C in the cabinet. The 2-h drying allowed the inoculated cells to attach to the surfaces of green peppers and minimized the growth of inoculated cells during drying.

### *Recovery and enumeration of* E. coli *O157: H7 on the surface of green pepper*

Each green pepper piece was combined with 50 ml of sterile 0·1% peptone solution in a 400 ml sterile stomacher bag (Fisher Scientific Inc.) and was blended with a Seward Stomacher 400 for 4 min at low speed. The low speed can minimize the breakage of green pepper so that few bacteria will attach to the broken tissues. The wash fluid was serially diluted and surface plated in duplicate to sorbital-MacConkey agar (SMAC) (Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with cefixime-tellurite (CT) (Dynal, Inc., Lake Success, New York, USA). The CT-SMAC plates were incubated at 37°C for 24 h. For each plate, two typical *E. coli* O157: H7 colonies were chosen and identified by an *E. coli* O157 Latex Test (Oxoid Inc., Ogdensburg, New York, USA).

To resuscitate and enumerate $ClO_2$-injured *E. coli* O157: H7 cells, a direct membrane-plating method was used, which was developed by McCarthy et al. (1998). 100 µl $ClO_2$-treated bacteria dilution was first spread over a sterile polycarbonate filter membrane (Osmonics Co., Westboro, Massachusetts, USA) previously placed on the surface of a TSA plate. The coarse side of the membrane was faced up. Plates

were incubated at 37°C for 4 h for repairing injured cells. Then the membranes were gently and aseptically transferred onto CT-SMAC plates using sterile tweezers. The membrane-CT-SMAC plates were further incubated at 37°C for 20 h. *Escherichia coli* O157: H7 colonies were counted after the same Latex confirmation procedure as the above.

### *Preparation of samples of green pepper for microscopic analysis*

Six samples for microscopic analysis were made from surface-injured green peppers (B, C, D, E, F and G), and two samples (A and H) were from surface-uninjured green peppers. The procedures to prepare the six samples are summarized in Table 1.

Samples A and B were prepared to study the effectiveness of attachment of *E. coli* O157: H7 to uninjured and injured surfaces of green pepper. Samples A and B were inoculated with 20 µl *E. coli* O157: H7 suspension ($2{\cdot}1 \times 10^7$ cfu per sample), dried for 2 h at 22°C, and incubated for 24 h at 37°C. Then, Samples A and B were prepared for scanning electron microscope (SEM) as described below.

Samples C, D, E, F, G and H were prepared to study the effectiveness of $ClO_2$ gas treatment or water washing to inactivate or remove *E. coli* O157: H7 inoculated on uninjured and injured surfaces of green pepper. These six samples were inoculated with 100 µl *E. coli* O157: H7 ($1{\cdot}05 \times 10^8$ cfu per sample), dried for 2 h, and then subjected to one or more of the following treatments: a) incubation, b) $ClO_2$ gas treatment, or c) washing with water. Sample C was incubated for 12 h at 37°C after inoculation and drying and used as a positive control for Samples D, E, F, G and H. The 12-h incubation period allows bacteria to grow and attach to the injured surfaces. Inoculated Sample D was washed with 50 ml sterile deionized water (SDW) for 1 min in an electronic shaker (Lab-Line Instrument, Inc., Melrose Park, Illinois, USA) at 200 rpm, then incubated for 12 h at 37°C. Instead of washing as for Sample D, inoculated Samples E and F were treated with $0{\cdot}62$ mg $l^{-1}$ and $1{\cdot}24$ mg $l^{-1}$ $ClO_2$ gas, respectively, for 30 min at 22°C and 95–98% RH, and then were incubated for 12 and 24 h at 37°C, respectively. The 24-h incubation period would enable injured or living cells on Sample F to grow after the treatment with high concentration of $ClO_2$. Samples G and H were incubated for 12 h at 37°C and then subjected to the same washing procedure as Sample D. After the above preparation, Samples C, D, E, F, G and H were prepared for SEM as described below.

### *Chlorine dioxide treatment of green pepper samples*

$ClO_2$ gas treatment was carried out in a 10 l Irvine Plexiglass cylinder with a stainless steel shelf, on which green pepper samples were placed. A Thermo-Hygro recorder (Control Company, Friendswood, Texas, USA) was used to monitor relative humidity and temperature inside the treatment cylinder. $ClO_2$ gas was generated from a CDG laboratory generator (CDG Technology, Inc., New York, USA).

**Table 1.** Preparation of Samples (A–H) for SEM analysis and surface-plating colony enumeration

| Samples | Green pepper surface | Incubation time after inoculation and 2-h drying (h) | Treatment | Incubation time after treatment (h) |
|---|---|---|---|---|
| A | Uninjured | 24 | No treatment | No incubation |
| B | Injured | 24 | No treatment | No incubation |
| C[a] | Injured | 12 | No treatment | No incubation |
| D | Injured | No incubation | Water washing | 12 |
| E | Injured | No incubation | $0{\cdot}62$ mg $l^{-1}$ $ClO_2$ | 12 |
| F | Injured | No incubation | $1{\cdot}24$ mg $l^{-1}$ $ClO_2$ | 24 |
| G | Injured | 12 | Water washing | No incubation |
| H | Uninjured | 12 | Water washing | No incubation |

[a]Sample C was a control for Samples D, E, F, G and H.

The generated $ClO_2$ gas was collected in a 4·7 l Teflon PEP gas sampling bag (Cole-Parmer Instrument Co., Vernon Hills, Illinois, USA). That was placed in a light-protected black outer bag to prevent light-decomposition of $ClO_2$. A 60 ml plastic gas sampling syringe was used to deliver specific volumes of $ClO_2$ gas into the cylinder containing the green pepper samples. During treatment, the $ClO_2$ gas inside the cylinder was circulated by a diaphragm vacuum pump (KNF Neuberger, Inc., Trenton, New Jersey, USA), and the cylinder was covered with aluminum foil to prevent light-decomposition of $ClO_2$.

The concentration of $ClO_2$ gas was measured by a modified amperometric method (Greenberg et al. 1992). Using a 10-ml gas sampling syringe, 5 ml freshly generated $ClO_2$ gas was immediately dissolved in 1 l deionized and distilled water. Before injecting the gas into the water, the gas was first dissolved in the syringe by drawing some water in and out repeatedly. Duplicate $ClO_2$ solutions were made in 10 min. A 200 ml $ClO_2$ solution was used for measurement of $ClO_2$ concentration following the procedures of the amperometric method. The $ClO_2$ concentration was measured in triplicate and the data were recorded as mg $l^{-1}$ available $ClO_2$.

### *Preparation and observation of specimens for SEM*

Pieces of green pepper (about $2 \times 2$ mm area and 0·5 mm thickness) were gently cut off the inoculated surface of each green pepper sample using a sterile blade. The cut pieces were fixed overnight in 4% glutaraldehyde, and rinsed twice with 0·1 M sodium phosphate buffer pH 7·0. The samples were further fixed in 2% osmium tetroxide for 1 h and rinsed twice with 0·1 M sodium phosphate buffer. Fixed samples were dehydrated in a graded ethanol series (30%, 50%, 70% and 100%). All procedures through dehydration were carried out at about 4°C. The samples were dried in a LADD Critical-Point drier (LADD Research Industries, Inc., Burlington, Vermont, USA) with $CO_2$ as the transition gas. They were then mounted on specimen stubs and coated with approximate 30 nm layer of gold-palladium using a Hummer I sputter coater (ANATECH, LTD, Springfield, Virginia, USA). The samples were examined with a JEOL JSM-840 scanning electron microscope (JEOL USA Inc., Peabody, Massachusetts, USA) at an accelerating voltage of 5 KV. Digital micrographs were collected at a resolution of $1280 \times 960$ and dwell time of 160 s. The digital images were adjusted using Adobe PhotoShop 5·0 and printed with a Codonics 1660 dye sublimation/thermal printer (Codonics, Inc., Middleburg Heights, Ohio, USA) using the thermal method.

### *Statistical analysis*

All the samples used for microscopic analysis were made in duplicate. At least five different locations in each sample were examined using SEM. All the samples used for the enumeration of *E. coli* O157: H7 by colony enumeration methods were prepared in triplicate. The mean values of duplicate plate counts of triplicate samples were calculated and reported with 95% confidence interval. Data were subjected to analysis of variance and Student Newman-Keuls' (SNK) multiple range tests (SAS Inc., Cary, North Carolina, USA) to determine if significant differences ($P < 0{\cdot}05$) in populations of *E. coli* O157: H7 existed between mean values.

## Results and Discussion

### *Attachment of* E. coli *O157: H7 to green pepper surface*

Microphotographs of Samples A and B show *E. coli* O157: H7 cells on the smoothly intact uninjured surface of Sample A (Fig. 1) and the injured surface of Sample B (Figs 2 and 3). The bacteria appeared not to penetrate the intact cuticle of green pepper, and seemed to preferentially attach to coarse, porous and injured surfaces (Fig. 2, indicated by an arrow) as compared to the uninjured surfaces. Seo and Frank (1999) and Itoh et al. (1998) also found that *E. coli* O157: H7 preferentially attached to a cut surface.

Enumerated by a surface-plating method, total populations of *E. coli* O157: H7 on Samples A and B were $7{\cdot}41 \pm 0{\cdot}06$ and $9{\cdot}62 \pm 0{\cdot}02$ log cfu,

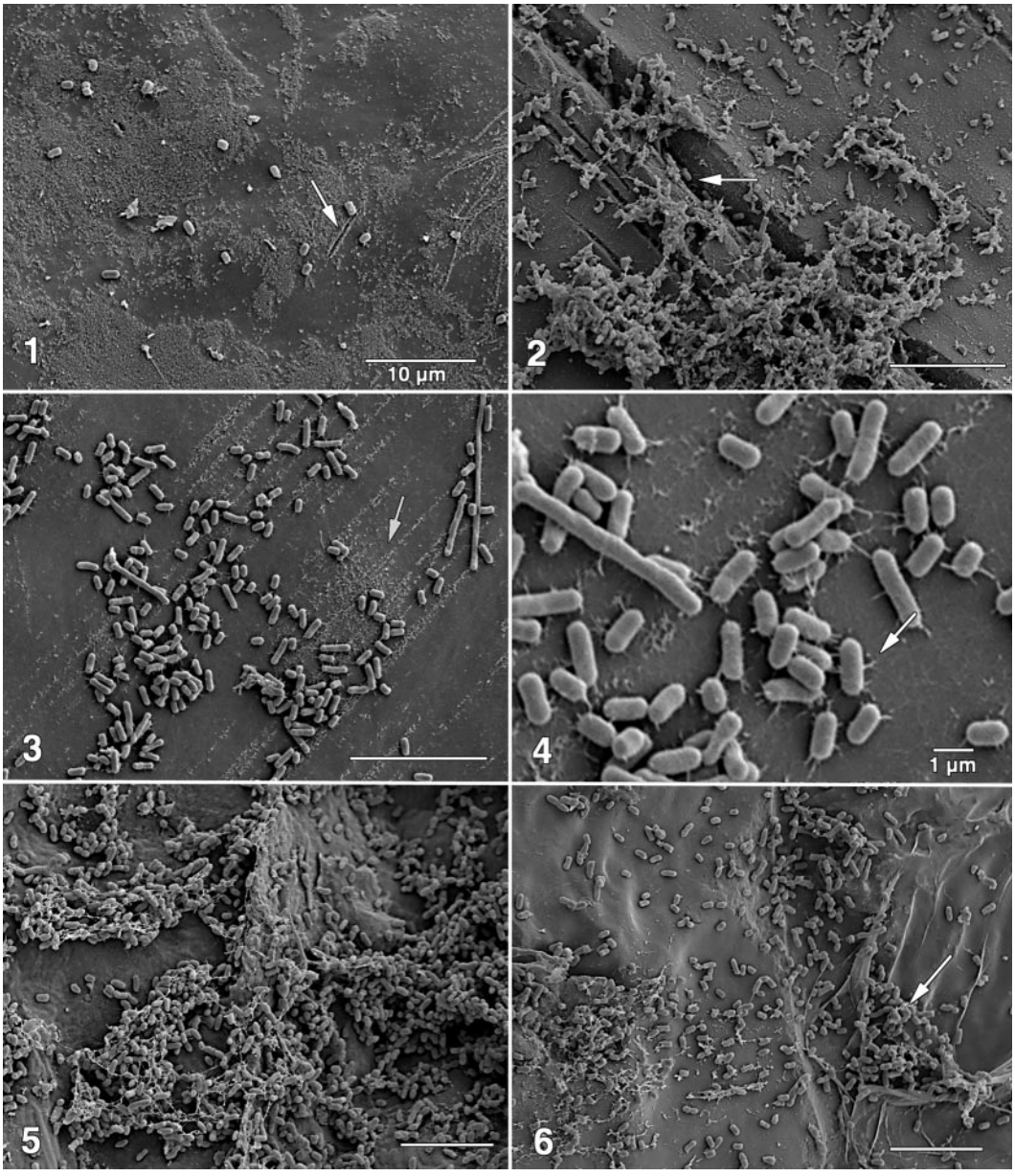


**Figure 1.** *Escherichia coli* O157: H7 cells attached to uninjured surface of Sample A after inoculation, 2-h drying, and 24-h incubation at 37°C (×3000). Bar = 10 μm, SEM.

**Figure 2.** *Escherichia coli* O157: H7 cells growing and attached to injured surface of Sample B after inoculation, 2-h drying, and 24-h incubation at 37°C (×2500). Bar = 10 μm, SEM.

**Figure 3.** *Escherichia coli* O157: H7 cells growing and attached to wax-layer-injured surface of Sample B (×3000). Bar = 10 μm, SEM.

**Figure 4.** *Escherichia coli* O157: H7 cells with exocellular polymers on wax-layer-injured surface of Sample B (×9000). Bar = 1 μm, Enlarged microphotograph from part of Fig. 3.

**Figure 5.** *Escherichia coli* O157: H7 cells growing and attached to injured surface of Sample C after inoculation, 2-h drying, and 12-h incubation at 37°C (×2000). Bar = 10 μm, SEM.

**Figure 6.** *Escherichia coli* O157: H7 cells growing and attached to the injured surface of inoculated Sample D after water washing and 12-h incubation at 37°C (×2000). Bar = 10 μm, SEM.

respectively, after incubation for 24 h at 37°C. Since 7·32 ± 0·01 log cfu of *E. coli* O157: H7 was inoculated on Samples A and B, Sample A had no significant increase of population after the incubation; whereas, Sample B showed a significant increase of population ($P < 0·05$). SEM photomicrographs also show that the bacterial population on Sample A (Fig. 1) was much less than that on Sample B (Figs 2 and 3). These results suggested that the injured tissues on Sample B provided nutrients for bacterial growth and multiplication. Although Sample A showed 7·41 ± 0·06 log cfu cells after incubation, few bacteria were observed on its surface by SEM. Most bacteria may have been washed off the surface during preparation of the specimen for SEM. However, many bacteria remained on the surface of Sample B after preparation of the specimen. All the above results suggested that the attachment of the *E. coli* O157: H7 cells to the surfaces of green peppers was mainly determined by the pepper surface properties.

Some small lesions (injuries), or lenticels, and/or microcracks were found on the uninjured surface of Sample A (Fig. 1, indicated by an arrow), which may have increased the possibility of bacterial attachment. Getz et al. (1983) observed that lesions on mature tomato fruit could develop from the trichomes in young fruit epidermis and would be infection sites for bacteria. Swart et al. (1995) found that pathogens could penetrate the host tissues of table grapes through stomata, lenticels and microcracks in the epidermis and would grow under certain conditions. Seo and Frank (1999) and Itoh et al. (1998) also found that *E. coli* O157: H7 was internalized in the stomata of lettuce leave and radish sprouts. However, in this study, the growth of *E. coli* O157: H7 at naturally existing injuries was not observed after 24 h incubation at 37°C, suggesting that these sites did not provide enough nutrients for bacterial growth.

As shown in Fig. 3, the natural wax layer of the pepper surface was slightly scraped by the action of the sterile blade (indicated by an arrow). A large number of bacteria seemingly attached and grew on the surface where the wax coating structure had been disrupted. This suggested that the waxes on the surface (cuticle) of green pepper not only resist water loss, but also prevent the attachment of *E. coli* O157: H7.

When comparing Figs 2 and 3, the degree of the surface injury artificially made on green pepper seemingly determined the extent of attachment and growth of bacteria. Bacteria sat deeply in injured tissues (Fig. 2, as indicated by an arrow). Those bacteria would be more difficult to remove by washing and more difficult to kill by disinfectant because of protection by the tissues. Therefore, it is very important to keep the surface uninjured to minimize the attachment and growth of bacteria. However, injuries on green pepper surfaces could occur at any stage of growth, harvest, transportation, packaging and processing. To attain the goal of safety, a killing step should be considered.

### *The exocellular polymers on growing* E. coli *O157: H7 cells on injured surfaces*

The exocellular polymers, which appeared as fibrils (Fig. 4, indicated by an arrow), on the growing bacteria on the injured surfaces of Sample B were observed, but they were not found on the bacteria on the intact surfaces of Sample A (Fig. 1). The fibrils observed in the SEM preparations might result from shrinkage of these polymers during the dehydration preparation steps (Herald and Zottola 1988). Most of these polymers are polysaccharides, while some contain protein and lipid components (Fletcher 1980). Costerton et al. (1985) suggested that the glycocalyx (exocellular polymers) of many bacteria act not only as a nutrient trap, removing dissolved material from the fluid-flow past the colony, but also as a means of protecting the micro-organisms from the many antibacterial elements that may occur in these natural environments. The growth and multiplication of bacteria on uninjured pepper surfaces may be limited because of unavailability of enough nutrients. In contrast, bacteria may grow and form the exocellular polymers on injured surfaces where nutrients and moisture are plentiful.

Many researchers have studied the role of exocellular polymers in bacterial attachment to food or food contact surfaces (Lillard 1986). Herald and Zottola (1989) indicated that the

attachment of *L. monocytogenes* to stainless steel surfaces might be related to the flagella and any exopolymer surrounding the cells. Smoot and Pierson (1998) found that proteins, rather than polysaccharides, played a role in the initial attachment of *L. monocytogenes* to Buna-N rubber and stainless steel surfaces. Marshall (1992) suggested that the attachment might occur in two phases, reversible and irreversible. In the first phase, bacteria may be weakly held on the contact surfaces by electrostatic and van der Waals forces. The second phase may involve the formation of exocellular polymers that cross-link to the attachment sites. The bacteria that adhere to the uninjured surfaces of green pepper (Sample A) apparently belong to the first phase (Fig. 1). The mechanisms of bacterial attachment to injured green pepper surfaces seem more complex. One main mechanism may be that the hydrophilic injured surfaces of green peppers are highly attractive to the hydrophilic surface of the cells and therefore resulted in greater attachment to the injured surfaces. However, the exocellular polymers on growing bacteria may or may not involve in assistance of bacterial attachment to injured pepper surfaces.

### *Reduction of* E. coli *O157: H7 on injured surface by water washing and $ClO_2$ gas treatment*

*Escherichia coli* O157: H7 decontamination of the surface-injured green peppers was compared using control Sample C, Sample D that was simply washed with water, and Samples E and F that were treated with $ClO_2$ gas (Figs 5–10). After a 12 h incubation at 37°C, a large number of bacteria were observed on the surface-injured control Sample C, indicating bacterial growth and multiplication (Fig. 5). Many bacteria also appeared on the injured surface of Sample D after water washing (Fig. 6), but the population was substantially less than that of the control Sample C. The bacteria on the injured surface of Sample D also showed their growth (Fig. 6, indicated by an arrow). These results suggested that water washing alone was not sufficient to remove many bacteria that were tightly attached to the injured surfaces.

Samples E (Fig. 7) and F (Fig. 9) treated with 0·62 and 1·24 g $l^{-1}$ $ClO_2$ gas, respectively, had much fewer bacteria on the injured surfaces than the control Sample C. The bacteria that were scattered in Figs 7 and 9 might be dead or injured. A large amount of bacteria were observed only in deeply injured surface location in Sample E (Fig. 8, arrow) after the 0·62 mg $l^{-1}$ $ClO_2$ treatment. These bacteria might be from the growth of survived bacteria after a 12 h incubation. These results suggested that the bacteria, which penetrated into injured tissues, were protected from inactivation by low levels of $ClO_2$ gas treatment. This was consistent with Seo and Frank's (1999) findings in which some *E. coli* O157: H7 cells that attached to stomata and cut edges survived after chlorine treatment. Little evidence of bacterial growth was found in Sample F after the 1·24 mg $l^{-1}$ $ClO_2$ treatment (Fig. 9), suggesting the inoculated bacteria could be killed or injured by high concentration of $ClO_2$ gas. Moreover, no visible changes in cell size and shape were observed when the bacteria treated with 1·24 mg $l^{-1}$ $ClO_2$ gas (Fig. 10) were compared to the bacteria without $ClO_2$ gas treatment (Fig. 4). Lindsay and von Holy (1999), Kim and Slavik (1996), and McCarthy (1992) also found minor changes in cell morphology of *B. subtilis*, *S. typhimurium* and *L. monocytogenes*, respectively, after iodine and chlorine-containing sanitizer treatment.

To evaluate the effectiveness of water washing and $ClO_2$ gas treatment, the viable bacteria on the surfaces of Samples C, D, E, and F after the treatments of water washing, $ClO_2$ gas treatment and incubation were enumerated by conventional colony enumeration method (Table 2). The *E. coli* O157: H7 cells treated by $ClO_2$ gas were enumerated by a membrane-plating method because it is very important to recover injured cells. The results in Table 2 were consistent with those from the above SEM analysis. Water washing achieved only $1{\cdot}5 \pm 0{\cdot}05$ log reduction, whereas 0·62 and 1·24 mg $l^{-1}$ $ClO_2$ gas treatments killed $3{\cdot}03 \pm 0{\cdot}02$ log and $6{\cdot}45 \pm 0{\cdot}02$ log *E. coli* O157: H7, respectively. After water washing or $ClO_2$ gas treatment, Samples D, E and F showed significantly different log reductions ($P < 0{\cdot}05$). After a further 12 or 24 h incubation, the

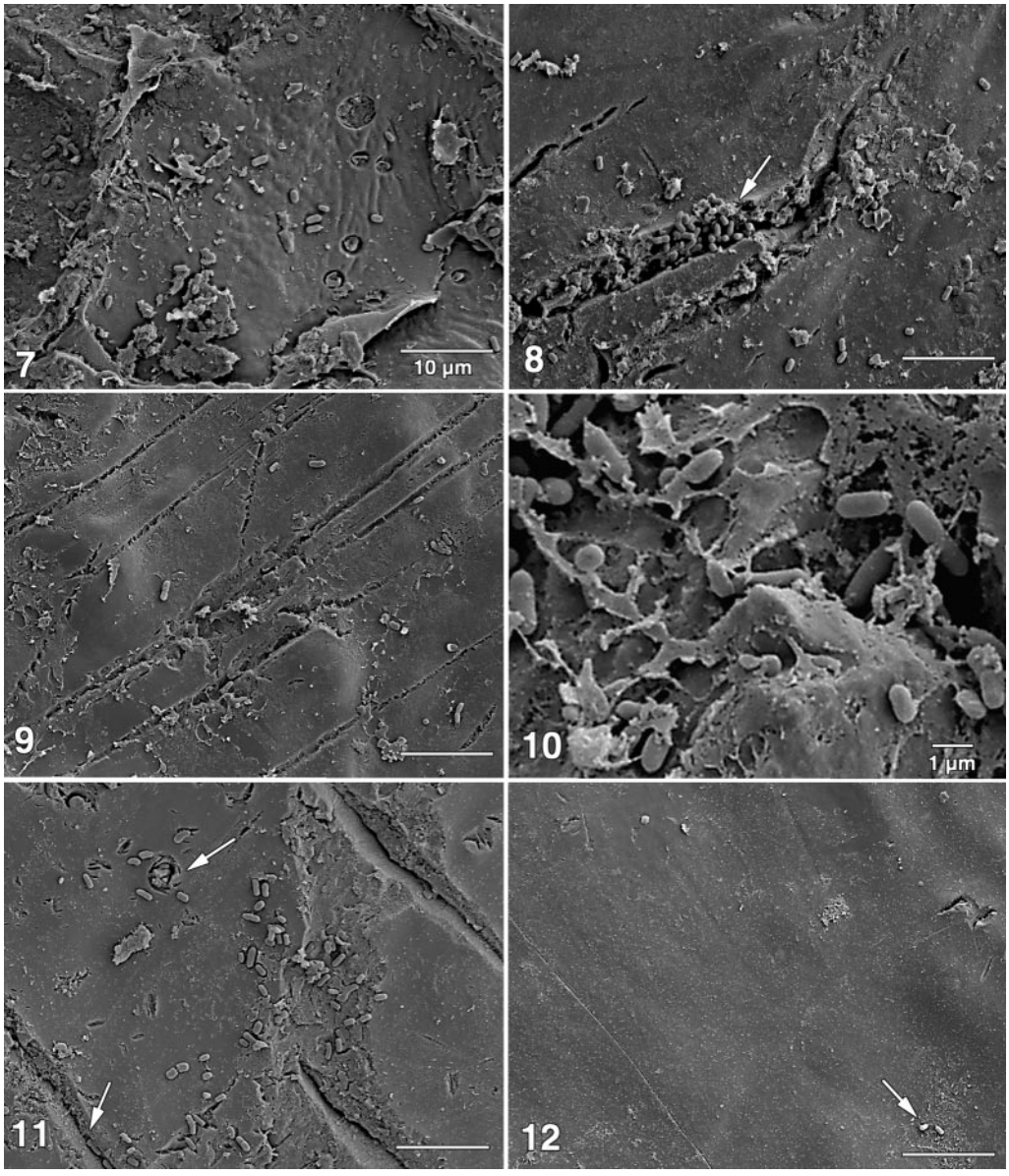


**Figure 7.** *Escherichia coli* O157: H7 cells on the injured surface of inoculated Sample E treated by 0.62 mg $l^{-1}$ $ClO_2$ for 30 min at 22°C and 90–95% RH followed by 12-h incubation at 37°C ( × 2000). Bar = 10 μm, SEM.

**Figure 8.** *Escherichia coli* O157: H7 cells growing at deeply injured section of Sample E ( × 2000). Bar = 10 μm, SEM.

**Figure 9.** *Escherichia coli* O157: H7 cells on the injured surface of inoculated Sample F treated by 1·24 mg $l^{-1}$ $ClO_2$ for 30 min at 22°C and 90–95% RH followed by 24-h incubation at 37°C ( × 2000). Bar = 10 μm, SEM.

**Figure 10.** *Escherichia coli* O157: H7 cells on the injured surface of Sample F treated by 1·24 mg $l^{-1}$ $ClO_2$ ( × 9000). Bar = 1 μm, SEM.

**Figure 11.** *Escherichia coli* O157: H7 cells attached to the injured surface of the Sample G after inoculation, incubated for 12 h at 37°C, and water washing ( × 2000). Bar = 10 μm, SEM.

**Figure 12.** *Escherichia coli* O157: H7 cells attached to the uninjured surface of the Sample H after inoculation, incubated for 12 h at 37°C, and water washing ( × 2000). Bar = 10 μm, SEM.

**Table 2.** Total populations[a] (log cfu) of *E. coli* O157: H7 on injured green pepper surface and log reduction[b] after washing and $ClO_2$ gas treatments

| Samples | Total inoculated population | Population[c] after washing or $ClO_2$ treatment | Log reduction (log cfu)[d] | Population after 12/24 h incubation |
|---|---|---|---|---|
| C | 7·91 ± 0·08 | NA[e] | NA | 9·06 ± 0·14A |
| D | 7·91 ± 0·08 | 6·41 ± 0·05 | 1·50 ± 0·05A | 8·41 ± 0·12B |
| E | 7·91 ± 0·08 | 4·88 ± 0·02 | 3·03 ± 0·02B | 6·68 ± 0·07C |
| F | 7·91 ± 0·08 | 1·46 ± 0·02 | 6·45 ± 0·02C | 3·61 ± 0·19D |

[a]Values are means ± standard deviations ($n = 3$).
[b] Log reduction (total inoculated population before treatments — population after washing or $ClO_2$ treatment).
[c] The populations of *E. coli* O157: H7 were enumerated by a surface-plating method except the populations on $ClO_2$ gas-treated samples were enumerated by a membrane-plating method.
[d] Values in the same column with different letters are significantly different ($P < 0·05$).
[e]NA: not applicable.

bacterial populations on Samples C, D, E and F also were significantly different ($P < 0·05$). Therefore, both $ClO_2$ gas treatments were more effective than water washing to reduce *E. coli* O157: H7 population on green pepper surfaces.

### *Comparison of water washing of* E. coli *O157: H7 from injured surface and uninjured surface of green pepper*

After inoculation and incubation for 12 h at 37°C, Sample G with injured surface and Sample H with uninjured surface were washed with water. Sample G showed much fewer surface bacteria (Fig. 11), with Sample H having the lowest population (Fig. 12) when compared to the surface-injured control Sample C (Fig. 5). The cells that were not removed by water washing preferentially attached to porous, deeply injured sites on Sample G (Fig. 11, indicated by an arrow). On the uninjured surfaces of Sample H (Fig. 12, indicated by an arrow), the bacteria seemed to attach to coarse surfaces.

Although not a large number of bacteria were visually observed on both Samples G and H by SEM, the surface-plating enumeration method showed 7·39 ± 0·10 log cfu and 6·61 ± 0·04 log cfu, respectively, bacteria on their surfaces after water washing. Compared to the bacterial population of 9·06 ± 0·14 log cfu on control Sample C, Samples G and H resulted in 1·67 ± 0·10 and 2·44 ± 0·04 log reduction after water washing, which differed significantly ($P < 0·05$). This result also suggested that bacterial attachment to injured surfaces was stronger than that to uninjured surfaces. However, no significant difference was seen between 1·50 ± 0·05 log reduction on Sample D, washed prior to incubation, and 1·67 ± 0·10 log reduction on Sample G, washed after incubation. This suggests that washing had a limited effectiveness in reducing bacterial population regardless of the timing of the wash.

Exocellular polymers were not observed on the bacteria that remained and attached to the injured surfaces of Sample G after water washing (Fig. 11). Costerton et al. (1985) stated that these exocellular polymers generally are water-soluble, so, they may have been removed by washing. Although these exocellular polymers were removed, many bacteria (more than 7 log) still attached to the injured surfaces (Fig. 11). This suggested that the exocellular polymers of *E. coli* O157: H7 might play no role in the formation of attachment to injured green pepper surfaces. Allison and Sutherland (1987) and Dewanti and Wong (1995) also stated that exocellular materials were not necessary for attachment and might help stabilize biofilms. The above results suggest that the mechanism of attachment is complex for these *E. coli* O157: H7 cells observed on injured surfaces after water washing.

## Conclusion

The attachment of *E. coli* O157: H7 to green pepper surfaces was affected by the properties of

the surface. No evidence of direct penetration through intact cuticle was found, but the bacteria might be entrapped in some natural lesions. *Escherichia coli* O157: H7 seemingly adhered to coarse and porous intact surfaces and preferentially attached to injured surfaces. The bacterial attachment to injured green pepper surfaces may be mainly determined by the hydrophilic properties of the injured surfaces and might not be assisted by the exocellular polymers of the bacteria. The injuries to the wax layer on the cuticle and tissues of green pepper increased the adhesion and growth of bacteria. No significant growth of *E. coli* O157: H7 was found on uninjured surfaces after inoculation and incubation for 24 h at 37°C; whereas, significant growth and multiplication was found on injured surfaces ($P < 0{\cdot}05$).

$ClO_2$ gas treatment ($1{\cdot}24$ mg $l^{-1}$ $ClO_2$) was an effective sanitation technique to achieve more than 5 log reductions of *E. coli* O157: H7 on green peppers. Using a membrane-plating method for resuscitation and enumeration of $ClO_2$ treated *E. coli* O157: H7 on surface-injured green peppers, $3{\cdot}03 \pm 0{\cdot}02$ and $6{\cdot}45 \pm 0{\cdot}02$ log reduction of inoculated *E. coli* O157: H7 were obtained after the treatment by 0·62 and 1·24 mg $l^{-1}$ $ClO_2$, respectively, for 30 min at 22°C and 90–95% RH. Water washing achieved less than 2 or 3 log reduction on injured and uninjured surfaces, respectively. Although water washing was less effective than $ClO_2$ gas treatment, it may be used to decrease the initial population of bacteria on vegetable surfaces before $ClO_2$ gas treatment or to reduce $ClO_2$ residue on vegetable surfaces after $ClO_2$ gas treatment. Therefore, $ClO_2$ gas treatment combined with water washing may be a good decontamination technique for MPR fruits and vegetables.

The bacteria entrapped in deeply injured sites may be minimally affected by low level $ClO_2$ gas treatment. This phenomenon could increase the risk for food safety because mechanical damage to surfaces of MPR fruits and vegetables can happen at any stage of harvesting, processing, packing and transportation. The safety of fresh-cut fruits and vegetables will be of concern because cut surfaces provide opportunity for bacterial attachment and growth. Therefore, an effective sanitation of the fruits and vegetables before cutting will be critical. Because any injuries on the surface of vegetables increase the resistance of bacteria to washing and killing treatments, technologies or studies applied to surface decontamination and sanitation of MPR fruits and vegetables need to be validated by using surface-injured fruits and vegetables.

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