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ORIGINAL ARTICLE

Inactivation of *Escherichia coli* O157:H7 on surface-uninjured and -injured green pepper (*Capsicum annuum* L.) by chlorine dioxide gas as demonstrated by confocal laser scanning microscopy

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Cells of Escherichia coli *O157:H7 on uninjured and injured surfaces of green pepper were inactivated by 0·15–1·2 mg l^{-1} ClO_2 gas treatments. A membrane-surface-plating method was used for resuscitation and enumeration of* E. coli *O157:H7 treated with ClO_2. The location and viability of* E. coli *O157:H7 on uninjured and injured green pepper surfaces after ClO_2 gas treatments were visualized using confocal laser scanning microscopy (CLSM). Live and dead cells of* E. coli *O157:H7 on pepper surfaces were labeled with a fluorescein isothiocyanate-labeled antibody and propidium iodide, respectively. A 7·27 log reduction of* E. coli *O157:H7 on uninjured green pepper surfaces was obtained with a 0·60 mg l^{-1} ClO_2 gas treatment for 30 min at 20°C under 90–95% relative humidity. For injured surfaces, a 6·45 log reduction was achieved with a 1·2 mg l^{-1} ClO_2 gas treatment. Each ClO_2 gas treatment (0·15–1·2 mg l^{-1} ClO_2) for inoculated bacteria on uninjured surfaces showed significantly more reductions (1·23–4·24 log) than for those on injured surfaces ($P < 0·05$). The microphotographs of CLSM showed that bacteria preferentially attached to injured surfaces and those bacteria could be protected from bacterial reduction by the injuries. This study indicates that ClO_2 gas treatment can be a potential effective method of pathogen reduction for fresh fruits and vegetables.*

Introduction

Outbreaks of *Escherichia coli* O157:H7 infections in recent years have been associated with fresh produce and fruit juices (CDC 1994, 1996, 1997a, 1997b, Ackers et al. 1996, Mermin et al. 1996). Researches have shown that *E. coli* O157:H7 can survive or grow on lettuce (Diaz and Hotchkiss 1996, Beuchat 1999, Seo and Frank 1999), apples (Fisher and Golden 1998, Buchanan et al. 1999), cantaloupe and watermelon (del Rosario and Beuchat 1995), salad vegetables (Abdul-Raouf et al. 1993), and in apple cider (Zhao et al. 1993, Miller and Kaspar 1994). Chlorinated water containing 50–200 ppm of

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chlorine is widely used to sanitize whole fruits and vegetables as well as fresh-cut produce on a commercial scale. However, its effectiveness is limited in reducing the population of micro-organisms [less than $2 \log \text{cfu}\, g^{-1}\, (cm^2)$ reductions] on fruits and vegetables (Beuchat 1992, Brackett 1992, Beuchat 1999). Therefore, highly effective sanitizers need to be developed to minimize contamination associated with pathogenic infections on fresh fruits and vegetables.

Chlorine dioxide (ClO_2) gas may be an alternative sanitizer to reduce micro-organisms on the surfaces of fruits and vegetables. Aqueous ClO_2 as a sanitizer can be used to control microbial populations in poultry processing water (FDA 1995). The Food and Drug Administration (FDA 1998) further amended the food additive regulations to allow the safe use of ClO_2 as an antimicrobial agent in water to wash fruits and vegetables in an amount not exceeding 3 ppm residual ClO_2. Costilow et al. (1984) found that 2·5 ppm ClO_2 was very effective in the destruction of micro-organisms present in water used for handling and washing fresh cucumbers, but it failed to reduce the population of micro-organisms present in fresh cucumbers, even at higher concentrations (105 ppm). Reina et al. (1995) also found that the bacterial populations in pickling cucumbers were not greatly influenced by 5·1 ppm ClO_2. These researchers concluded that many micro-organisms were bound so strongly to the cucumber fruit that they were protected from ClO_2. Because gas has a greater penetration ability than liquid, gaseous ClO_2 may be more effective than aqueous ClO_2 in reducing micro-organisms on the surfaces of fruits and vegetables. Studies have shown that ClO_2 gas had a potential application in sanitizing gas-impermeable surfaces of medical implements (Rosenblatt et al. 1987, Jeng and Woodworth 1990). Han et al. (1999) found that spoilage micro-organisms inoculated on the epoxy-coated surface of a model aseptic juice storage tank could be completely inactivated with a $10\, mg\, l^{-1}$ ClO_2 gas treatment for 30 min at 9–28°C under 90% relative humidity (RH). However, limited studies have been reported on the use of ClO_2 gas to sanitize the surfaces of fresh fruits and vegetables.

Confocal laser scanning microscopy (CLSM) has been used widely in microstructural studies of food products (Blonk and van Aalst 1993, Vodovotz et al. 1996). In recent years, CLSM has been increasingly applied to food microbiology related research (Vodovotz et al. 1996), especially in the studies of location and viability of micro-organisms in foods (Hassan et al. 1995, Kim et al. 1996, Korber et al. 1996, Morris et al. 1997, Suominen et al. 1997, Wong-Liong et al. 1997, Seo and Frank 1999). CLSM has some unique advantages compared to the scanning electron microscopy (SEM) which is traditionally used for studying microbial colonization and attachments to food or food-contact surfaces. CLSM can provide more information about microbial viability and identity, and produce 3-D images of samples. Also, samples are easily prepared for CLSM and the morphology of microbes and surfaces is fully maintained.

Some studies on attachment of micro-organisms to the surfaces of fruits and vegetables showed that the attachment could increase resistance of micro-organisms to sanitation treatments. Seo and Frank (1999) found that *E. coli* O157:H7 cells preferentially attached to cut edges of lettuce leaf, and more viable bacteria were observed in the stomata and on cut edges 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 $HgCl_2$. Buchanan et al. (1999) studied contamination of intact apples after immersion in an *E. coli* O157:H7 suspension and sanitation of contaminated apples with $2000\, mg\, l^{-1}$ sodium hypochlorite for 1 min, followed by a 1-min tap water rinse. They found that the site of greatest contamination was consistently the outer core region, and that the bacteria in the blossom-end region greatly influenced the efficacy of sanitization treatments. This treatment achieved 1–3 log reductions but did not eliminate the bacteria, particularly from the outer core region. Beuchat (1999) reported that low levels of *E. coli* O157:H7 inoculum, when applied to lettuce using bovine feces as a carrier and stored under commercial and home refrigeration conditions, could survive and were not easily reduced by washing with water or treating with 200 ppm chlorine solution.

Fruits and vegetables have complex surface properties for bacterial attachment. The bacteria not only preferentially attach to cut or mechanical injured surfaces, but also grow on these sites due to sufficient available nutrients. Therefore, it may be more significant to reduce the micro-organisms on cut or injured surfaces than on uninjured surfaces. To develop a good pathogen reduction technique for fresh fruits and vegetables, information comparing the reduction of micro-organisms on injured surfaces and on uninjured surfaces is needed.

In this study, bell-shaped green peppers were selected as a model sample for fruits and vegetables having a smooth and easily washable surface. The objectives were: a) to compare the inactivation of *E. coli* O157:H7 on uninjured and injured surfaces of green pepper by ClO_2 gas treatments, and b) to visualize the location and viability of *E. coli* O157:H7 on uninjured and injured green pepper surfaces after ClO_2 gas treatment using CLSM.

Materials and Methods

Surface-uninjured and -injured green peppers

Bell-shaped, organic green peppers (*Capsicum annuum* L.) 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^2, approximately 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 (30W, about 50 cm irradiation distance) in a class II biosafety cabinet (Labconco Corporation, Kansas City, Missouri) for 40 min (20 min for each side) to reduce naturally existing bacteria to less than 2 log cfu 5 g^{-1} green pepper. Surface-injured green pepper samples were artificially injured using uninjured green peppers. The cuticle layer (about 1–2 mm thickness) of each uninjured surface (2×2 cm^2) was gently cut into a total of 36 incisions using a sterile blade.

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, USA) 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 (approximately 1×10^9 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

To ensure that the inoculum suspension was evenly distributed, a pipette tip was used to spread 100 µl inoculum across uninjured surfaces or 36 square injured surfaces on each green pepper sample in a class II biosafety cabinet. The 100 µl inoculum achieved an average inoculum level of $7{\cdot}9 \pm 0{\cdot}29$ log cfu *E. coli* O157:H7 cells 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. The inoculated and dried samples were subjected to ClO_2 gas treatment.

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, where 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). 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). ClO_2 in solution (200 ml) was titrated with 0.00564 N phenylarsine oxide standard solution (HACH Co., Loveland, Colorado, USA) using an amperometric titrator (HACH Co.). The ClO_2 solution was prepared from the gas as following. 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 within 10 min. The ClO_2 concentration was measured in triplicate and the data were recorded as mg l^{-1} available ClO_2.

The surface-uninjured and surface-injured green peppers inoculated with *E. coli* O157:H7 were treated with four different concentrations of ClO_2 gas (0·15, 0·30, 0·60, and 1·2 mg l^{-1}) for 30 min at 20°C and 90–95% RH. After these treatments, green pepper samples were subjected to enumeration of culturable *E. coli* O157:H7 and preparation of specimens for CLSM examination as described below.

For each ClO_2 gas treatment, three controls were prepared. The positive control was inoculated green pepper without ClO_2 gas treatment. One negative control was ClO_2 gas treated green pepper without inoculation, and a second was the green pepper without inoculation and ClO_2 gas treatment. Each treatment sample and the three controls were prepared in triplicate.

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

Each green pepper sample was transferred into a 400 ml stomacher bag (Fisher Scientific Inc., Pittsburgh, Philadelphia, USA), combined with 50 ml of sterile 0·1% peptone solution, and then blended with a Seward 400 Stomacher (Seward Medical Co., London, UK) for 2 min at a normal speed. The wash fluid was serially diluted, followed by surface plating (0·1 ml) for enumeration of *E. coli* O517:H7. The sensitivity of this method is that above 250 cfu ml^{-1} of bacteria in the wash fluid could be enumerated.

To improve method sensitivity, centrifugation was used to concentrate the bacterial population in the wash fluid so that less than 250 cfu ml^{-1} of bacteria in the above washing fluid could be enumerated by the surface plating method. After making the first dilution (pipetting 1 ml wash fluid into 9 ml sterile 0·1% peptone solution), the residual of the wash fluid was poured into a 50 ml sterile plastic centrifuge tube (Fisher) and centrifuged for 15 min at 4000 rpm speed (1500 *g*) in a IEC HN-SII centrifuge (International Equipment Co., Needham, Massachusetts, USA). The pellet was resuspended in 0·5 ml sterile deionized water (SDW) so that the bacterial population in the 50 ml washing fluid was concentrated 100 times. The resuspension (0·1 ml) was further mixed with 0·9 ml SDW, giving a 10 times concentration factor for the washing fluid. This concentration method allowed the surface plating method to enumerate at least 10 cells of total bacterial population in the 50 ml washing fluid.

For those control samples that had been inoculated or uninoculated, dried, untreated with ClO_2 gas, and recovered in the same manner as ClO_2 gas treated samples, *E. coli* O157:H7 was enumerated by surface plating of 0·1 ml bacterial dilution to sorbital-MacConkey agar (SMAC) (Oxoid Inc., Ogdensburg, New York, USA) supplemented with cefix-tellurite (CT) (Dynal Inc., Lake Success, New York, USA) in duplicate. The CTSMAC plates were incubated at 37°C for 24 h and counted. For each plate, two typical *E. coli* O157:H7 colonies were chosen and confirmed by an *E. coli* O157 Latex Test (Oxoid Inc., Ogdensburg). Because no *E. coli*

O157:H7 cells were recovered on all negative controls, the initial population of *E. coil* O157:H7 on each sample was equivalent to the positive control count.

To enumerate ClO_2-treated *E. coli* O157:H7 cells including sublethally injured bacteria, a direct membrane-surface-plating method was used (McCarthy et al. 1998). Each 100-µl wash fluid, or its dilution, or resuspension of its concentrates was surface plated in duplicate over a sterile polycarbonate filter membrane (Osmonics Co., Westboro, Massachusetts, USA), which was previously placed on the surface of a TSA plate. The coarse side of the membrane was faced upward. Plates were incubated at 37°C for 4 h to repair injured cells. Then the membranes were gently and aseptically transferred onto CTSMAC plates using sterile tweezers. The membrane-CTSMAC plates were further incubated at 37°C for 20 h. *Escherichia coli* O157:H7 colonies were counted, after which the Latex confirmation test was conducted.

An end-point method, which was similar to the method used for recovery of ClO_2 gas treated spoilage micro-organisms (Han et al. 1999), was used to determine if all inoculated *E. coli* O157:H7 on uninjured surface of green pepper were inactivated by $1{\cdot}2\,\mathrm{mg\,l^{-1}}$ ClO_2 gas treatment. After the treatment, each green pepper sample was transferred into a sterilized bottle containing 100 ml of sterile tryptic soy broth and incubated for 48 h at 37°C. After the incubation, samples were further plated on CTSMAC plates followed by a 24 h incubation at 37°C. Typical *E. coli* O157:H7 colonies also were identifed by *E. coli* O157 Latex tests. Presence or absence of *E. coli* O157:H7 were recorded as positive or negative results. A negative result indicated that all of the inoculated *E. coli* O157:H7 were killed after the treatment.

Live/dead bacteria labeling methods

A fluorescein isothiocyanate-labeled affinity purified antibody (FITC-Ab) to *E. coli* O157:H7 (Kirkegaard & Perry Laboratories. Inc., Gaithersburg, Maryland, USA) was used for labeling live *E. coli* O157:H7 on the surfaces of green pepper. Propidium iodide (PI) from the Live/Dead BacLight Bacterial Viability Kit (L-7012) (Molecular Probes, Inc., Eugene, Oregon, USA) was used for labeling dead *E. coli* O157:H7. This method was developed by Seo and Frank (1999) and used to label live and dead bacteria on lettuce leaf surfaces. After staining, live bacteria could be seen in green color and dead cells in red by CLSM.

Staining and preparation of slides for microscopic analysis

Each green pepper sample was first cut into two $0{\cdot}5 \times 0{\cdot}5$ cm cubes using a sterile blade, being careful not to touch and disturb the inoculated surfaces. A $0{\cdot}5 \times 0{\cdot}5$ cm piece of cuticle layer with 0·2–0·3 mm thickness was removed from the top of the green pepper cube. The cut cuticle pieces were submerged in 1 ml 40 nM PI solution in a microtube for 15 min at room temperature, then transferred into another microtube with 1 ml SDW and gently shaken three times. The samples were submerged in FITC-Ab solution (1:200 dilution in 1% bovine serum albumin in 0·01 M PBS) and incubated for 30 min at 37°C, followed by two successive gentle washings in microtubes as described above. Because the attachment of bacteria to surfaces of green peppers may not be strong, staining and washing procedures should be carefully operated to minimize removal of bacteria from the surface of green peppers. Rinsing was not recommended for replacement of washing. After staining, each sample was put on a microscope slides (Fisher), dried for 10 min in the biosafety cabinet, and then mounted with a drop of immersion oil (DF) (Fisher) and a No. 1 coverglass (Fisher). The edges of the coverglass on each specimen were sealed with tape to hold the sample and to protect leaking of the mounting oil. Specimens were refrigerated until examined by CSLM.

Confocal Scanning Laser Microscopy

A Bio-Rad MRC-1024 confocal scanning laser microscope (Bio-Rad, Inc., Hemel Hempstead, UK) with a Krypton-Argon laser was used to view green pepper slides using $60\times$ oil immersion objective with 1·4 numerical aperture.

FITC-Ab-labeled and PI-stained *E. coli* O157:H7 on green pepper surfaces were detected using 488 nm excitation wavelength. Emission light from FITC was collected with a 522/35 filter, which was seen as green fluorescence in a mixer A. Emission from PI was collected with a 605/32 filter, observed by red fluorescence in a mixer B. The surfaces of green pepper were observed by transmission light at 488 nm and appeared in gray in a mixer C. Each final image was a combination of the individual images of those three mixers, in which the surfaces of green peppers were shown in blue color instead of gray. The size of each collected image was 512×512 pixels (each pixel is 0·55µm). At least 10 different locations in each sample were examined using CLSM. Three-dimensional images of surface of green peppers were reconstructed from multiple optical sections using Lasersharp Processing 3·2 Software (Bio-Rad, Inc., Hemel Hempstead, UK). A 15-µm-thick stack of optical sections was collected at 0·5 µm interval. All the images were adjusted and edited using Adobe PhotoShop 5·0.

Statistical analysis

All the samples used for colony enumeration, including controls and the samples for ClO_2 gas treatments, 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 ($P<0·05$) existed between mean values.

Results

Log reduction of E. coli *O157:H7 on surface-uninjured and surface-injured green peppers after* ClO_2 *gas treatments*

Using a colony enumeration method, log reductions of the *E. coli* O157:H7 inoculated on surface-uninjured and surface-injured green peppers after ClO_2 gas treatments were measured (Table 1). For both surface-uninjured and -injured samples, the log reduction of *E. coli* O157:H7 significantly increased ($P<0·05$) as the concentration of available ClO_2 gas increased. The 0·60 mg l^{-1} ClO_2 gas treatment achieved 4·37 and 1·36 more log reductions on uninjured and injured surfaces, respectively, than the 0·15 mg l^{-1} ClO_2 gas treatment. These results suggested that the concentration of ClO_2 gas was a very important factor to inactivate *E. coli* O157:H7 on green pepper surfaces. Using the end-point method, all the inoculated *E. coli* O157:H7 cells on uninjured green pepper surfaces were inactivated after 1·2 mg l^{-1} ClO_2 gas treatments, providing a more than 8·0 log reduction. However, a positive result in the end-point analysis was obtained for injured surfaces after 1·2 mg l^{-1} ClO_2 gas treatments.

Log reductions of *E. coli* O157:H7 on uninjured surfaces were found to be 1·23, 2·12,

Table 1. Log reduction[1] of *E. coli* O157:H7 inoculated on surface-uninjured and surface-injured green peppers after ClO_2 gas treatments

Samples[2]	Log reduction after ClO_2 treatments[3]			
	0·15 mg l^{-1}	0·30 mg l^{-1}	0·60 mg l^{-1}	1·2 mg l^{-1}
Uninjured suface	$2·90 \pm 0·09_{Ad}$[4]	$3·99 \pm 0·07_{Ac}$	$7·27 \pm 0·68_{Ab}$	$8·04 \pm 0_{Aa}$[5]
Injured surface	$1·67 \pm 0·08_{Bd}$	$1·87 \pm 0·03_{Bc}$	$3·03 \pm 0·02_{Bb}$	$6·45 \pm 0·02_{Ba}$

[1]Values are means ± s. d. ($n = 3$).
[2]The initial populations of *E. coli* O157:H7 on surface-uninjured and -injured green peppers were $7·9 \pm 0·29$ log cfu 5 g^{-1}.
[3]ClO_2 gas treatments included 0·15, 0·30, 0·60 and 1·2 mg l^{-1} ClO_2 gas, respectively, for 30 min at 20°C under 90–95% RH.
[4]Values in the same column with different uppercase subscript letters are significantly different ($P<0·05$). Values in the same row with different lower subscript letters are significantly different ($P<0·05$).
[5]No viable *E. coli* O157:H7 was detected by the end-point method after 1·2 mg l^{-1} ClO_2 gas treatments.

4·24 and 1·59, which were lower than the log reductions on injured surfaces after 0·15, 0·30, 0·60 and 1·2 mg l^{-1} ClO_2 gas treatments, respectively. These differences were determined to be significant ($P < 0·05$). Moreover, the differences increased as the concentration of available ClO_2 gas increased from 0·15 to 0·6 mg l^{-1}. These results suggested that injured surfaces increased protection of *E. coli* O157:H7 from ClO_2 gas treatments and the protection became more obvious under a high level of ClO_2 gas treatment than a low level of treatment. Possible explanations for protection against ClO_2 gas may be due to reduced exposure due to bacterial attachment to pepper surfaces and penetration into injured surfaces.

Location of E. coli *O157:H7 on green pepper surfaces observed by CLSM*

After FITC-Ab/PI staining, the location of *E. coli* O157:H7 on the surface-uninjured and -injured green peppers treated without or with ClO_2 gas was visualized by CLSM. The surfaces of green peppers without inoculation (negative control) and with inoculation (positive control) are shown in Fig. 1(a) and (b), respectively. Although the autofluorescence of green pepper cell walls (in green) and nuclei (in red) could be seen (Fig. 1a), they did not interfere with two-color differentiation for bacteria viability. Because the autofluorescence was much weaker than the fluorescence of bacteria stained by FITC and PI, it could be minimized using a low level of laser light. As seen in Fig. 1(b), the living bacteria on the positive control sample were stained green in color and the red green pepper cell wall gave a confirmation for visualization of the stained bacteria by CLSM.

The distribution of bacteria on uninjured surfaces was quite different from injured surfaces. On a flat uninjured surface, most bacteria were evenly distributed as seen in Fig. 1(e), (g), and (k). However, stacks of bacterial cells also were observed on some surfaces as shown in Fig. 1(c) and (i). On an injured surface, most bacteria were found in injured locations, as shown in Fig. 1(b), (d), (f), (h), and (j). This might be because the inoculated bacteria were transferred to the injured areas of green peppers as the water in the inoculum suspension flowed to those locations during drying.

Viability of E. coli *O157:H7 on green pepper surfaces after ClO_2 gas treatment observed by CLSM*

After FITC-Ab/PI staining, the viability of *E. coli* O157:H7 on the surface-uninjured and -injured green peppers treated with 0·15, 0·30, 0·60 or 1·2 mg l^{-1} ClO_2 gas was visualized by CLSM. After 0·15 mg l^{-1} ClO_2 gas treatment, surface-uninjured peppers (Fig. 1(c)) showed fewer living bacteria (green or yellow) than surface-injured peppers (Fig. 1(d)). This was consistent with the above results from colony enumeration, in which surface-uninjured and -injured peppers showed 2·9 and 1·67 log reductions, respectively. Besides green and red cells, many yellow cells were visible in Fig. 1(c) and (d). Seo and Frank (1999) suggested that the yellow bacteria might either be injured and culturable, or injured and non-culturable. In Fig. 1(d), most bacteria were in yellow, but the log reduction was quite low. Therefore, those yellow bacteria might be sub-lethally injured. The scattered bacteria seemed to be more easily inactivated than those in the center of a bacterial stack on uninjured surfaces (Fig. 1(c)).

After a 0·30 mg l^{-1} ClO_2 gas treatment, surface-uninjured peppers (Fig. 1(e)) also showed fewer viable bacteria than surface-injured peppers (Fig. 1(f)). Moreover, these samples had fewer living bacteria than those samples treated with 0·15 mg l^{-1} ClO_2 gas. These results also were consistent with the above log reduction results. Although bacteria on uninjured surfaces were scattered (Fig. 1(e)), some bacteria (in green) were still viable after the ClO_2 gas treatment, indicating their strong resistance to the ClO_2 gas treatment. In Fig. 1(f) more viable bacteria were found at injured locations (indicated by an arrow) than at intact locations, suggesting these bacteria might also be protected from ClO_2 gas treatment by injuries on pepper surface.

After a 0·60 mg l^{-1} ClO_2 gas treatment, some injured bacteria (in yellow) were found on surface-uninjured peppers (Fig. 1(g)); whereas, more living bacteria (in green) were observed on surface-injured peppers (Fig. 1(h)). Most of

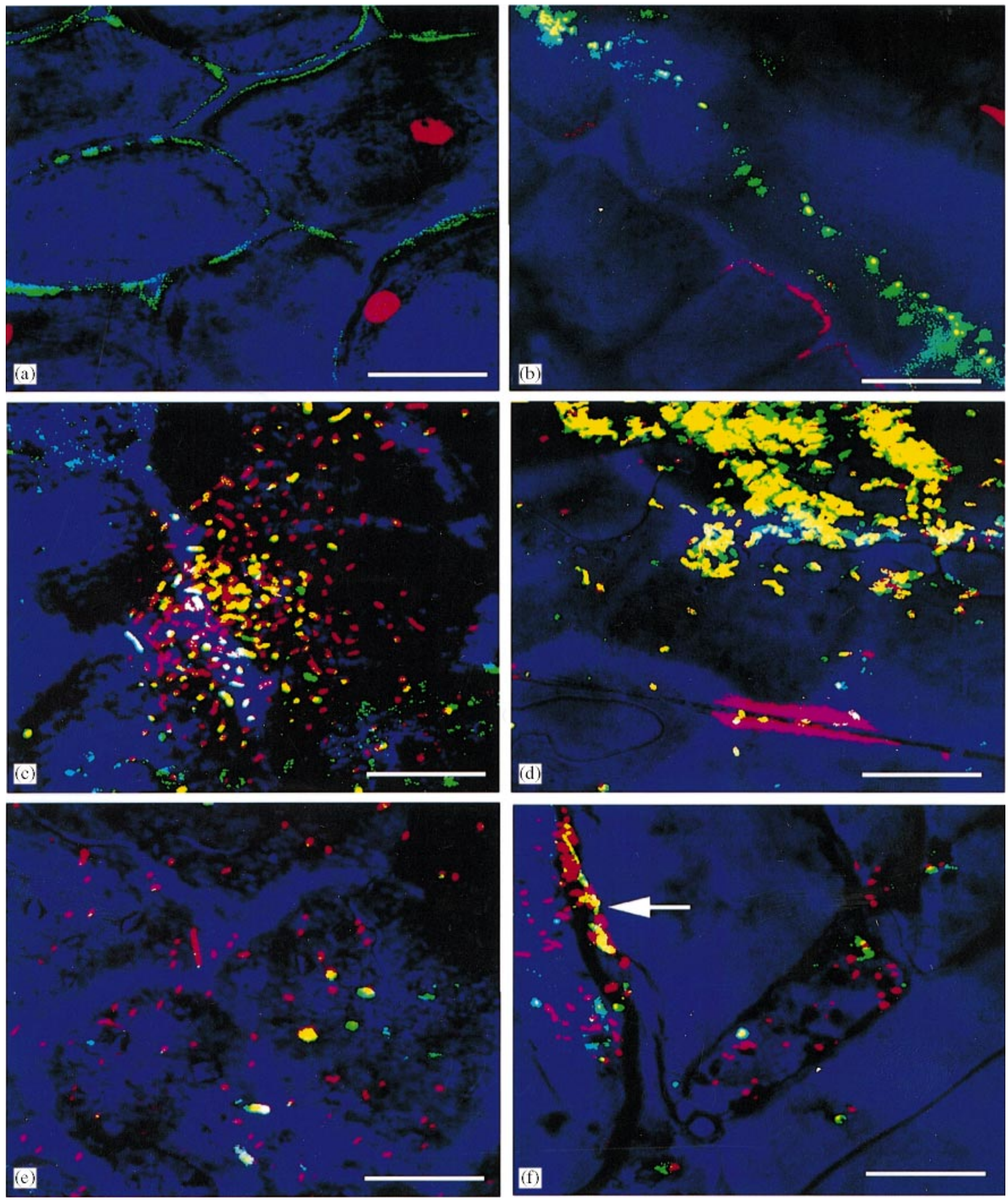

Figure 1. Microphotographs of CLSM. Bar = 50 µm. (a) Autofluoresence of green pepper cell wall and nuclei. (b) Live *E. coli* O157:H7 cells attached to injured pepper surface. (c) Live and dead *E. coli* O157:H7 cells on uninjured pepper surface after $0{\cdot}15\ \mathrm{mg\,l^{-1}}$ ClO_2 gas treatment. (d) Live and dead *E. coli* O157:H7 cells on injured pepper surface after $0{\cdot}15\ \mathrm{mg\,l^{-1}}$ ClO_2 gas treatment. (e) Live and dead *E. coli* O157:H7 cells on uninjured pepper surface after $0{\cdot}3\ \mathrm{mg\,l^{-1}}$ ClO_2 gas treatment. (f) Live and dead *E. coli* O157:H7 cells on injured pepper surface after $0{\cdot}3\ \mathrm{mg\,l^{-1}}$ ClO_2 gas treatment. (g) Live and dead *E. coli* O157:H7 cells on uninjured pepper surface after $0{\cdot}6\ \mathrm{mg\,l^{-1}}$ ClO_2 gas treatment. (h) Live and dead *E. coli* O157:H7 cells on injured pepper surface after $0{\cdot}6\ \mathrm{mg\,l^{-1}}$ ClO_2 gas treatment. (i) Live and dead *E. coli* O157:H7 cells on uninjured

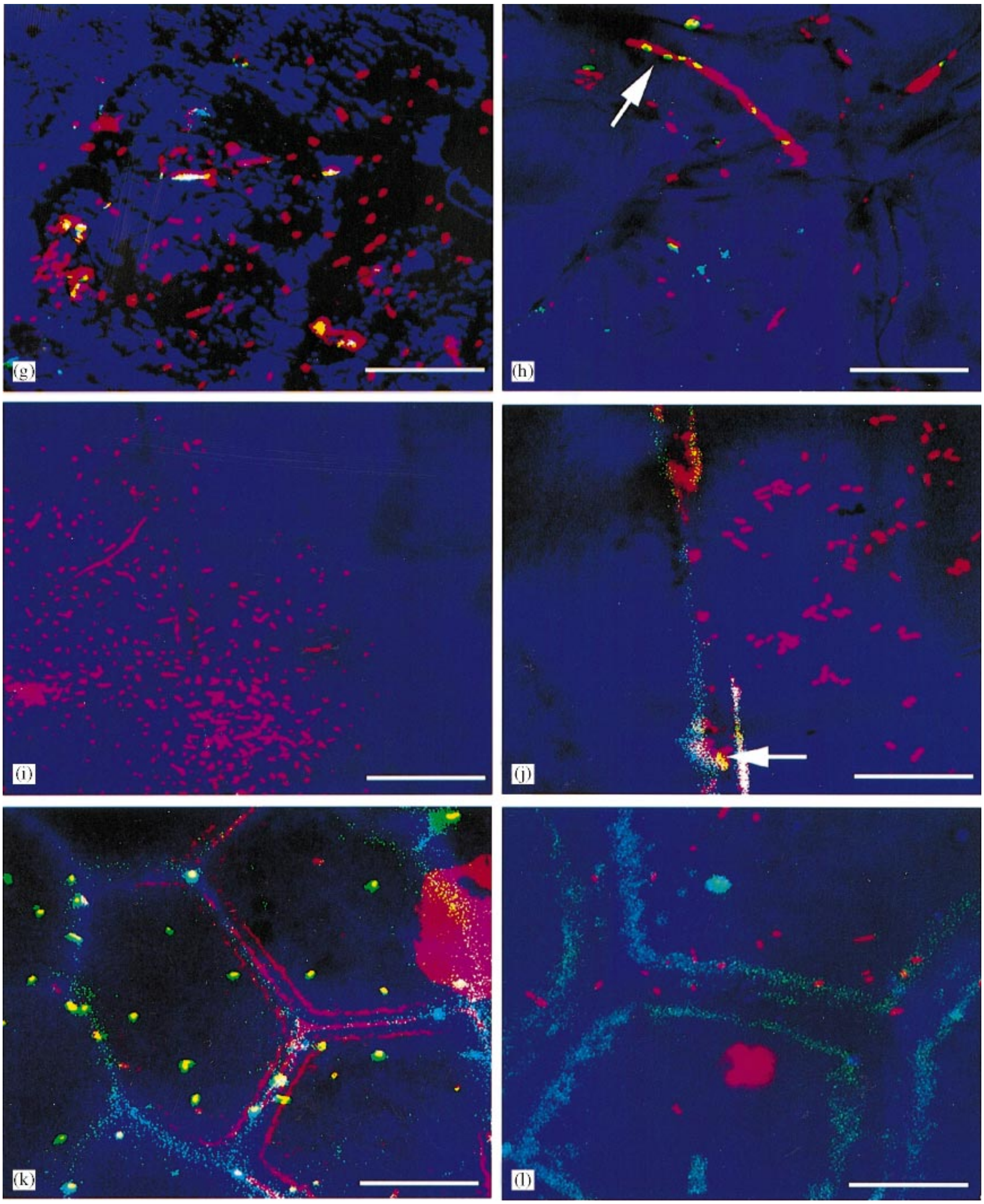

Figure 1. (Continued)

pepper surface after $1{\cdot}2\,mg\,l^{-1}$ ClO_2 gas treatment. (j) Live and dead *E. coli* O157:H7 cells on injured pepper surface after $1{\cdot}2\,mg\,l^{-1}$ ClO_2 gas treatment. (k) Three dimensional microphotograph of live and dead *E. coli* O157:H7 cells attached to uninjured pepper surface (control). (l) Three-dimensional microphotograph of live and dead *E. coli* O157:H7 cells attached to uninjured pepper surface after $0{\cdot}6\,mg\,l^{-1}$ ClO_2 gas treatment.

these living bacteria were located along injuries on green pepper surfaces (Fig. 1(h), as indicated by an arrow). These results suggested that the injuries to green pepper surfaces protected the bacterial cells from lethal effect of ClO_2.

After a $1{\cdot}2\,mg\,l^{-1}$ ClO_2 gas treatment, no living bacteria (green or yellow) were found on uninjured surfaces (Fig. 1(i)). This demonstrated complete inactivation, which also was validated by the end-point analysis. Living bacteria (Fig. 1(j)), indicated by an arrow) were still found on injured surfaces although their population was much less than that in Fig. 1(h).

Three-dimensional visualization of E. coli *O157:H7 on green pepper surfaces by CLSM*

Living and dead *E. coli* O157:H7 on uninjured green pepper surfaces were visualized in reconstructed three-dimensional images by CLSM. A 15-µm-thick stack of optical sections were collected, which allowed for visualizing all the bacteria attached to the uninjured surfaces and the cell nuclei of green pepper. Living and dead *E. coli* O157:H7 cells on a positive control of green pepper and a sample treated with $0{\cdot}60\,mg\,l^{-1}$ ClO_2 gas were showed in Fig. 1(k) and (l), respectively. Because of interference of colors, the side views of the reconstructed images were not shown.

Analysis of the optical sections used for reconstruction of the three-dimensional image in Fig. 1(k) suggested that most living bacteria were located 0–8 µm above the recognized surface of green pepper. The level of the recognized surface was defined as the level at which the surface on an image just became clear and the last cell disappeared from the image. Some living bacteria also were moving on a hydrated surface. After $0{\cdot}60\,mg\,l^{-1}$ ClO_2 gas treatment, most dead bacteria were found 0–5 µm above the recognized surface of green pepper (Fig. 1(l)). It seemed that viability of bacteria might not be an important factor for their attachment to uninjured surfaces. Visualization of an injured surface using reconstructed three-dimensional images (not shown in this paper) suggested that bacteria could be trapped at the locations as deep as injuries could occur.

Discussion

Results of this study suggest that ClO_2 gas is a potential effective sanitizer for fresh fruits and vegetables. Foschino et al. (1998) reported that a 5 log reduction of *E. coli* ATCC 11229 was achieved when the bacteria were suspended in water with $1{\cdot}4$ ppm aqueous ClO_2 for 30 s and when the bacteria were attached to a steel surface with 7 ppm for 6 min. However, Costilow et al. (1984) and Reina et al. (1995) found that the bacterial populations in cucumbers were not greatly influenced by $2{\cdot}5$ ppm or $5{\cdot}1$ ppm ClO_2, respectively. In this study, an approximate 7 log reduction of *E. coli* O157:H7 on uninjured green pepper surfaces was obtained with a $0{\cdot}60\,mg\,l^{-1}$ ClO_2 gas treatment for 30 min at 20°C under 90–95% RH. For injured surfaces, a $6{\cdot}45$ log reduction was achieved with a $1{\cdot}2\,mg\,l^{-1}$ ClO_2 gas treatment. All the inoculated bacteria ($8{\cdot}04$ log cfu 5 g^{-1}) on uninjured surfaces were completely inactivated by the $1{\cdot}2\,mg\,l^{-1}$ ClO_2 gas treatment as confirmed by an end-point method. Therefore, gaseous ClO_2 might be a better sanitizer than aqueous ClO_2 for sanitation of fruits and vegetables.

Based on the log reduction data, ClO_2 gas treatments for inoculated bacteria on uninjured surfaces showed significantly more inactivation than for those on injured surfaces ($P < 0{\cdot}05$). The differences in inactivation increased as the concentration of available ClO_2 gas increased from $0{\cdot}15$ to $0{\cdot}6\,mg\,l^{-1}$. The biggest difference of $4{\cdot}24$ log reductions was found after a $0{\cdot}6\,mg\,l^{-1}$ ClO_2 gas treatment. The microphotographs of CLSM were consistent with the log reduction data. More living bacteria were found at injured regions of surfaces, which agreed with the findings of Seo and Frank's (1999) and the results of our study on attachment of *E. coli* O157:H7 to uninjured and injured green pepper surface using scanning electronic microscopy (Han et al. 2000). Therefore, injuries to fruit and vegetable surfaces could protect attached bacteria from sanitation treatments. This is significant for minimally processed and refrigerated fruits and vegetables, especially for fresh-cut fruits and vegetables, because their cut surfaces will largely protect bacteria from sanitation treatments. For these foods, it is critical to sanitize their uninjured surfaces before cutting. Once the cut or injured surfaces are contaminated by pathogens, it will be very difficult to inactivate these attached or growing bacteria. This may explain why many researchers could not achieve a 5 log reduction of micro-organisms on cut fruits and vegetables using chemical sanitizers at concentrations that did not compromise sensory quality.

Besides protection of bacteria from sanitation by injuries to surfaces or fruits and vegetables, the stage of growth or amount of cell injury may be other important factors to affect the inactivation of bacteria on the surfaces by sanitizers. The intact surfaces of fruits and vegetables will not provide nutrients for adhered bacterial growth, therefore, the bacteria are in the worst living conditions and can be easily killed by sanitation treatments. Therefore, it is also very important to minimize mechanical damages to the surfaces of fresh fruits and vegetables before applying sanitation treatment and to keep clean processing environment after sanitation.

Numerous researchers have studied the attachment of micro-organisms to food contact surfaces and their responses to various sanitizer treatments (Zottola 1994, Foschino et al. 1998, Smoot and Pierson 1998, Lindsay and von Holy 1999). They concluded that the attachment of micro-organisms to the food contact surfaces enhanced their resistance to sanitation, which was similar to the results of studies in bacterial attachment to surfaces of fruits and vegetables. Therefore, attachment of micro-organisms to food surfaces and food contact surfaces has become a protrusive problem for sanitation. Zottola (1994) suggested that correct and sufficient cleaning and sanitizing procedures should be used for food contact surfaces. Beuchat (1999) indicated the need for the development of sanitizers more efficacious than chlorine for the removal of pathogens from raw fruits and vegetables. To solve this problem, pathogen reduction using ClO_2 gas may be an encouraging alternative approach. However, more studies on ClO_2 gas sanitation should be done, such as application technologies on a commercial processing scale, safety of residual ClO_2 in products, and safe control of the sanitation treatment.

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