Irradiation Compared with Chlorination for Elimination of *Escherichia coli* O157:H7 Internalized in Lettuce Leaves: Influence of Lettuce Variety

B.A. NIEMIRA

ABSTRACT: Pathogenic bacteria internalized in leaf tissues are not effectively removed by surface treatments. Irradiation has been shown to inactivate leaf-internalized bacteria, but many aspects of targeting these protected pathogens remain unknown. Bacterial cells of a cocktail mixture of 3 isolates of *Escherichia coli* O157:H7 were drawn into the leaves of iceberg, Boston, green leaf, and red leaf lettuce using vacuum perfusion. The inoculated leaves were treated with a 3-min wash with sodium hypochlorite solution (0, 300, or 600 ppm) or various doses of ionizing radiation (0.25 to 1.5 kGy). Leaves were stomached to recover the internalized cells and survivors enumerated. Washes with 0 ppm (water), 300 ppm, and 600 ppm chlorine solutions each gave reductions of less than 1 log. These reductions were statistically significant only in the case of green leaf lettuce. In contrast, irradiation effectively reduced *E. coli* O157:H7 on all varieties examined, with all doses tested being significantly reduced from the untreated control. The specific variety influenced the efficacy of irradiation. The greatest reduction obtained was 5 logs on iceberg lettuce treated with 1.5 kGy. The D_{10} values (the dose necessary to achieve a 1 log reduction) were significantly ($P < 0.05$) different among the varieties of lettuce tested, and ranged from 0.30 kGy (iceberg) to 0.45 kGy (Boston). These values were observed to be notably higher than previous irradiation D_{10} values for *E. coli* O157:H7 surface inoculated onto these 4 lettuce varieties. This study has shown that irradiation is able to effectively reduce viable *E. coli* O157:H7 cells internalized in lettuce, and that the variety of lettuce influences the specific response.

Keywords: foodborne, iceberg, internalization, ionizing radiation, nonthermal

Introduction

Fresh and fresh-cut fruits and vegetables are increasingly implicated in outbreaks of foodborne illness (Tauxe and others 1997; Gombas and others 2003; Horby and others 2003). In the United States, produce-associated outbreaks have risen from fewer than 20 throughout the 1970s to more than 100 in the 1990s (Sivapalasingam and others 2004). Leafy green vegetables were associated with 22 outbreaks and recalls from 1995 through 2007 (USFDA 2005). In 2006, prebagged baby spinach contaminated with *Escherichia coli* O157:H7 caused 199 illnesses and 3 deaths across 26 U.S. states and 1 Canadian province (USFDA 2006). The uncertainties that persist with respect to the mechanisms of contamination, and potential for continuing problems, have reduced consumer confidence in bagged spinach, lettuce, and other leafy vegetables (USFDA 2005). Good agricultural practices (GAP) and other proper controls can effectively serve to reduce the risk of contamination of growing leafy green vegetables with human pathogens. It is clear that identifying an effective lethal antimicrobial intervention (a "kill step") that can be incorporated into the GAP/GMP infrastructure is an industry priority. Chlorine-based sanitizers, commonly in concentrations of 50 to 200 ppm, are widely used to sanitize produce surfaces and processing equipment (USFDA 2001). However, the potential for internalization of *E. coli* O157:H7 or other pathogens into leaf tissue is a topic of ongoing discussion (Tauxe and others 1997; USFDA 1999; Solomon and others 2002a, 2002b; Jablasone and others 2004, 2005; Kutter and others 2005).

In 1999, the U.S. FDA (USFDA 1999) reviewed the potential for human pathogens and other bacteria to infiltrate into fresh fruits and vegetables, and also examined the ability of conventional surface decontamination measures to reduce contamination in this event. The FDA's conclusion was that a variety of opportunities exist for internalization events to occur, and that surface treatments are generally ineffective in eliminating pathogens that have been internalized.

Irradiation is a penetrating nonthermal kill step that effectively eliminates *E. coli* O157:H7 from lettuce (Foley and others 2002; Niemira and others 2002; Smith and Pillai 2004; Niemira 2007). It is known that differences in the supporting food matrix can significantly influence the radiation sensitivity of inoculated *E. coli* O157:H7. To date, a consistently applicable explanation for this has not been developed. The specific microenvironment chemistry is believed to play a key role, but the mechanisms are not yet understood. There were small but statistically significant differences among the D_{10} values of surface-inoculated *E. coli* O157:H7 on 4 varieties of lettuce (Niemira and others 2002). More recently, it has been shown that irradiation effectively kills *E. coli* O157:H7 that has

been internalized in romaine lettuce and baby spinach, but that the response depends on the vegetable (Niemira 2007). The variability of irradiation sensitivity for pathogens internalized in more closely related leafy vegetables is not known. Although a number of irradiation studies have been conducted on surface-contaminated leaves, the literature on the use of irradiation to kill leaf-internalized *E. coli* O157:H7 is scant.

The objectives of this study were to (1) compare the ability of sodium hypochlorite washes and irradiation to eliminate leaf-internalized *E. coli* O157:H7 cells, and (2) determine the influence of lettuce variety on the efficacy of the processes.

Materials and Methods

Microorganisms

All isolates utilized in this study were obtained from the USDA-ARS-ERRC culture collection. Three strains of *E. coli* O157:H7 previously used in this laboratory for studies of irradiated leafy vegetables were chosen for use in this study. The isolates were maintained in tryptic soy broth (TSB, Difco, Detroit, Mich., U.S.A.): C9490 (Centers for Disease Control, Atlanta, Ga., U.S.A.) and, ATCC-35150 and ATCC-43894 (American Type Culture Collection, Manasass, Va., U.S.A.). Fresh cultures (150 mL) of each isolate were grown overnight in TSB at 37 °C. The cell concentration of these individual cultures was approximately 10^9 CFU/mL, as determined by serial dilution and plate count on TSA incubated overnight at 37 °C.

Produce

Whole heads of iceberg, Boston, green leaf, and red leaf lettuce were purchased from local markets and stored at 4 °C for no more than 2 d prior to the experiments. Each variety of lettuce was evaluated separately. Produce was prepared for the studies according to the method of Niemira and others (2002). The outer leaves and any obviously damaged leaves of each head were removed and discarded, and cut leaf pieces were prepared. The basal portion of the head was removed, approximately 5 cm from the end. The leaves were sliced as a group into pieces weighing approximately 0.5 g. Before use in the experiments, the cut leaf material was washed and sanitized using a solution of 300 ppm sodium hypochlorite at room temperature. The leaf material was submerged and gently agitated for 3 min. The leaves were thoroughly rinsed by agitation under successive changes of distilled water, and spun in a sterile salad spinner-type centrifuge (Oxo Intl., New York, N.Y., U.S.A.) to remove excess surface water. This design of salad spinner incorporates a container base that captures all of the water removed from the leaf surface, and prevents the formation of aerosolized droplets. The leaf pieces were held at room temperature until inoculation, typically less than 15 min.

Inoculation

The leaves were inoculated according to the method of Niemira (2007) using an adaptation of the vacuum perfusion method, a method that irrigates the intercellular spaces without damaging the cell walls. Sterile Butterfield's phosphate buffer (BPB, 4050 mL) was placed into a sterilized glass vacuum desiccator (approximately 10 L total capacity) inside a biological safety cabinet. The 3 *E. coli* O157:H7 cultures (150 mL each) were combined with the BPB to make a 1:10 diluted cocktail inoculum, final volume 4500 mL. Leaf pieces sufficient for 1 replication were added to the desiccator and gently submerged with a stainless steel utensil, and the desiccator sealed. A 0.2 μm filter was placed in the vacuum line between the desiccator and the house vacuum to prevent contamination

of the house vacuum system, operating at approximately 27" Hg (91.4 kPa). A vacuum was drawn on the system for 4 min to pull gas from the intercellular spaces of the leaf pieces. As the gas bubbles formed on the leaf pieces, the desiccator was gently swirled to dislodge them. After 4 min, the vacuum was broken by quickly opening the vacuum desiccator to the air, thereby drawing the inoculum into the leaf pieces. The vacuum perfusion process was repeated a total of 3 times to fully perfuse the leaf pieces. In some cases, the desiccator was opened between cycles to submerge the leaf pieces with the utensil. After the final perfusion, the fully perfused leaves were removed from the inoculum bath and spun to visible dryness in a salad spinner inside the biological safety cabinet, typically with 2 or 3 spin cycles. The spun-dried fully perfused leaves were weighed into subsamples for treatments (described subsequently) within 30 min.

Samples (40 g) of leaf pieces for each variety of lettuce were weighed before and after perfusion to measure the uptake of fluid for each variety of lettuce. These values were used in the subsequent dilution calculations to determine CFU/g leaf.

Chemical wash

Perfused spin-dried leaf pieces were divided into 20-g samples. One 20-g sample of leaf pieces was placed in a nr 400 stomacher bag (Tekmar Inc., Cincinnati, Ohio, U.S.A.) and used as an untreated control. For the wash treatments, separated beakers and flasks were used to subject the separate 20-g samples with 0 ppm (a water wash), 300 ppm (pH = 9.77), or 600 ppm (pH = 9.99) sodium hypochlorite solutions prepared fresh immediately prior to treatment from concentrated stock solution. Each test solution was handled individually, and solutions were kept at room temperature during treatment. The leaf pieces were agitated in 250 mL of the appropriate test solution for 3 min, then removed to a clean beaker and agitated in sterile deionized water for 3 min. Chlorine concentrations and washing times were chosen to approximate a treatment comparable to current industry practice (USFDA 2001) and an aggressive treatment that uses a concentration much higher than current practice. The rinsed leaf pieces were then spun dry as described, and similarly placed in a stomacher bag. Each control and treatment was performed 3 times in independent replications.

Irradiation

The inoculated leaf pieces were bagged in 20-g samples as described and treated with 0.25, 0.50, 0.75, 1.0, or 1.5 kGy. In all cases, the irradiation was conducted at 4 °C. As with the controls and wash treatments, each irradiation treatment was performed 3 times in separate replications. Temperature control was maintained during irradiation by injection of gas coming from liquid nitrogen into the sample chamber. The samples were irradiated using a Lockheed-Georgia (Marietta, Ga., U.S.A.) cesium-137 self-contained gamma radiation source, with a dose rate of 5.64 kGy/h. The dose rate was established using alanine transfer dosimeters from the Natl. Inst. of Standards and Technology (Gaithersburg, Md., U.S.A.). Alanine pellets (Bruker Inc., Billerica, Mass., U.S.A.) were used for dosimetry. The pellets were read on a Bruker EMS 104 EPR analyzer and compared with a previously determined standard curve. Actual dose was typically within 5% of the nominal dose.

Sampling

BPB (80 mL) was added to each of the bags with treated leaf pieces, making a ratio of 5:1 ratio of total solution:leaf by weight. The bags were folded and stomached on high (260 rpm) for 4 min to pulp the leaf pieces. This duration resulted in sampling liquid that was as dark green as could be obtained from the pulped leaves,

indicating effective recovery of the internal fluid of the leaf pieces. An aliquot (0.1 mL) of the fluid was drawn and serially diluted using BPB. Dilutions appropriate for each treatment were spread-plated on TSA plates, inverted, and incubated overnight at 37 °C. For each of the 3 replications, 3 plates per dilution were counted by hand. Plate counts were adjusted for dilutions and for the fluid absorption ratios to determine CFU/g leaf.

Statistical analysis

The surviving population for each treatment for each leaf type was compared with that of the untreated control using 2-way analysis of variance (ANOVA, Tukey test, $P < 0.05$) (SigmaStat v. 4.0, SPSS Inc., Chicago, Ill., U.S.A.), using data pooled from each of the 3 replications ($n = 9$ per treatment). The radiation D_{10} for the *E. coli* O157:H7 cocktail was calculated based on the negative reciprocal of the slope for the linear regression line. The significance of differences among the D_{10} values (iceberg, Boston, green leaf, red leaf) was determined with analysis of covariance (ANCOVA) (Excel, Microsoft Corp., Redmond, Wash., U.S.A.).

Results and Discussion

The vacuum perfusion process effectively introduced the inoculum into the leaf pieces. As has been previously reported for romaine lettuce and baby spinach (Niemira 2007), infiltration proceeded initially along the major vascular elements, creating a distinct water-soaked appearance. Subsequent cycles rendered the leaf pieces fully perfused after 3 cycles.

The wash treatments were ineffective for iceberg (Figure 1), Boston (Figure 2), and red leaf lettuce (Figure 4). On green leaf lettuce (Figure 3), the water wash, 300 ppm, and 600 ppm washes all significantly reduced populations of *E. coli* O157:H7 compared with the untreated control, but in absolute terms resulted in less than a 1 log reduction. These results are consistent with the conclusion of the U.S. FDA that surface treatments using conventional levels of chlorine do not reduce internalized microbial populations (USFDA

1999, 2001). The method used herein can be modified in future research to incorporate a surface sterilization step to eliminate all remaining surface-adherent bacteria prior to enumeration of the internalized population, and by direct measurement of free chlorine in the chemical treatments.

Ionizing radiation effectively reduced *E. coli* O157:H7 on all varieties examined, with all doses tested being significantly reduced from the untreated control. The specific variety influenced the efficacy of irradiation. The D_{10} values formed 2 clusters, with those of iceberg (0.30 kGy) (Figure 1) and red leaf (0.35 kGy) (Figure 4) being significantly lower than those obtained for green leaf (0.37 kGy) (Figure 3) and Boston (0.45 kGy) (Figure 2). The regression R^2 value for red leaf (0.79) was lower than that of iceberg (0.95), Boston (0.88), or green leaf (0.95), a result of a tailing effect observed in the response at the highest doses. Radiation sensitivity is known to be influenced by the particular nature of the suspending food substrate (Niemira and others 2002); this was evident in the present study, with a variety-specific influence on the efficacy of irradiation.

These results are also consistent with a study that compared sodium hypochlorite washes to irradiation treatment of *E. coli* O157:H7 internalized in romaine lettuce and baby spinach (Niemira 2007). That study also found a lack of efficacy in the chemical treatments and an effective reduction of the pathogen by irradiation. Nthenge and others (2007) showed that lettuce plants grown in hydroponic solutions inoculated with *E. coli* O157:H7 contained the pathogen in the leaf tissue, and that gamma irradiation effectively killed the pathogen but 200 ppm aqueous chlorine did not.

The risk posed by pathogen internalization in the field is a subject of ongoing debate. Laboratory and greenhouse studies with lettuce (Solomon and others 2002a, 2002b), barley (Kutter and others 2005), and maize (Bernstein and others 2007) have shown that pathogen internalization can occur when introduced via irrigation water, contaminated soil, or other means. These studies generally use inoculation levels of 10^5 to 10^6 CFU/mL, and demonstrate bacterial levels in tissue of 10^2 to 10^3 CFU/g. The recoverability of
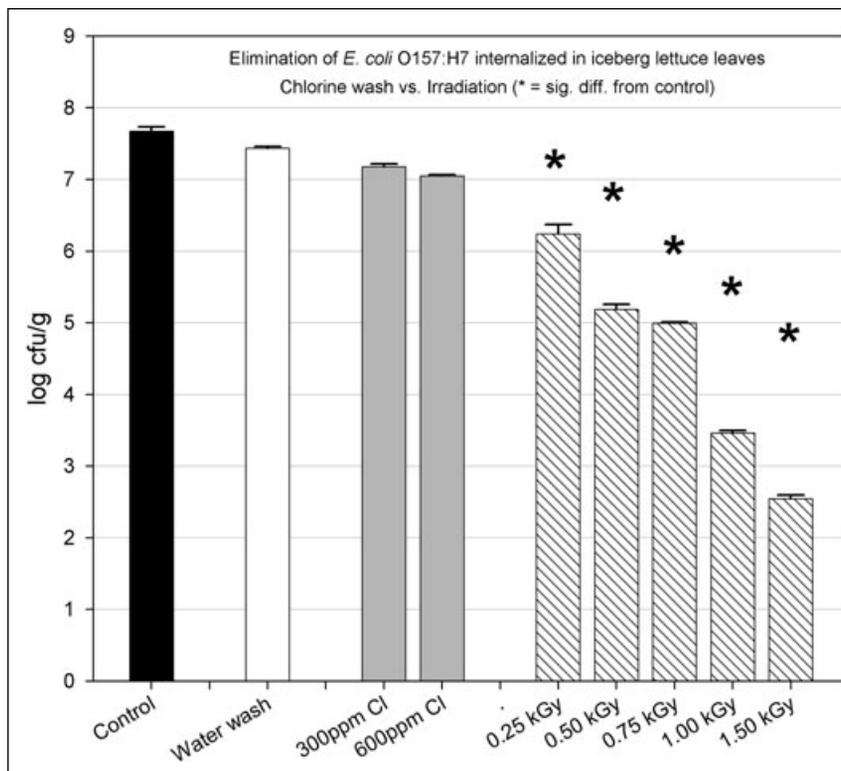

Figure 1 — Surviving populations of *E. coli* O157:H7 in iceberg lettuce leaf pieces: untreated control (black), following water wash (white), sodium hypochlorite treatments (gray), or irradiation (hashed). Bars equal standard error, *n* = 9. Asterisk (*) indicates significantly different from the control (ANOVA, *P* < 0.05).

approximately 0.1% of the inoculated bacteria may indicate a low efficiency of uptake or low survivorship within the leaf. Other studies using tomatoes have found that internalization via the root system does not occur or is extremely inefficient (Jablasone and others 2004, 2005). Additional research is needed to fully understand the events that lead to internalization via root uptake in healthy crop plants, and the actual potential for risk arising from this eventu-ality in the commercial growing environment. It should be noted that despite the uncertainties related to vascular internalization via root uptake, more conventional internalizations via wounds into stomatal cavities, hydathodes, or other natural openings are clear means by which pathogens may avoid contact with antimicrobial chemical treatments. More information is needed on how the microenvironment on and within leaves can influence the efficacy of
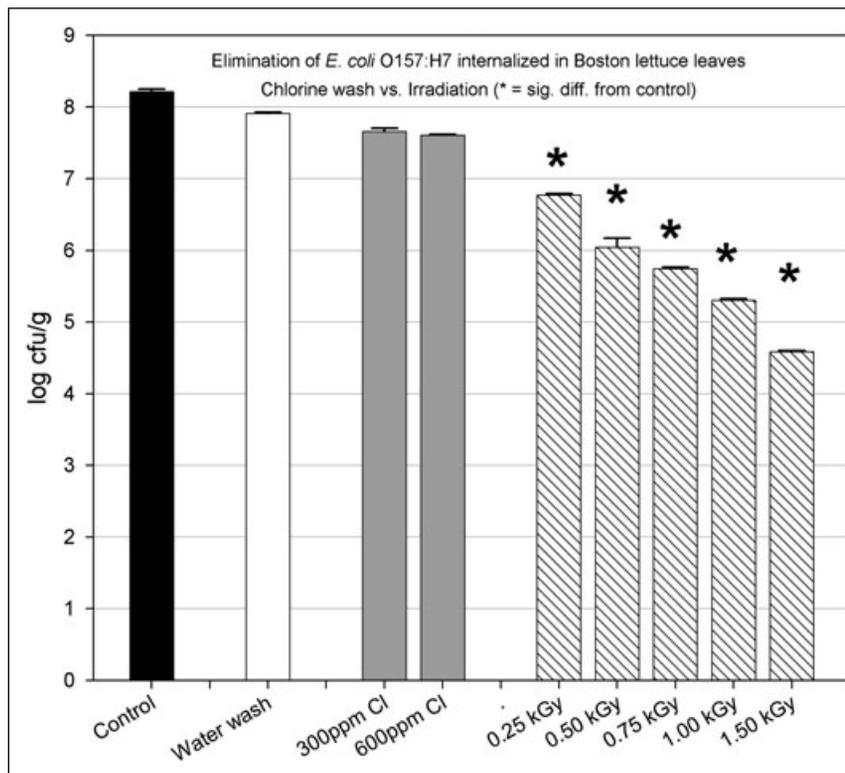

Figure 2 – Surviving populations of *E. coli* O157:H7 in Boston leaf pieces: untreated control (black), following water wash (white), sodium hypochlorite treatments (gray), or irradiation (hashed). Bars equal standard error, *n* = 9. Asterisk (·) indicates significantly different from the control (ANOVA, *P* < 0.05).
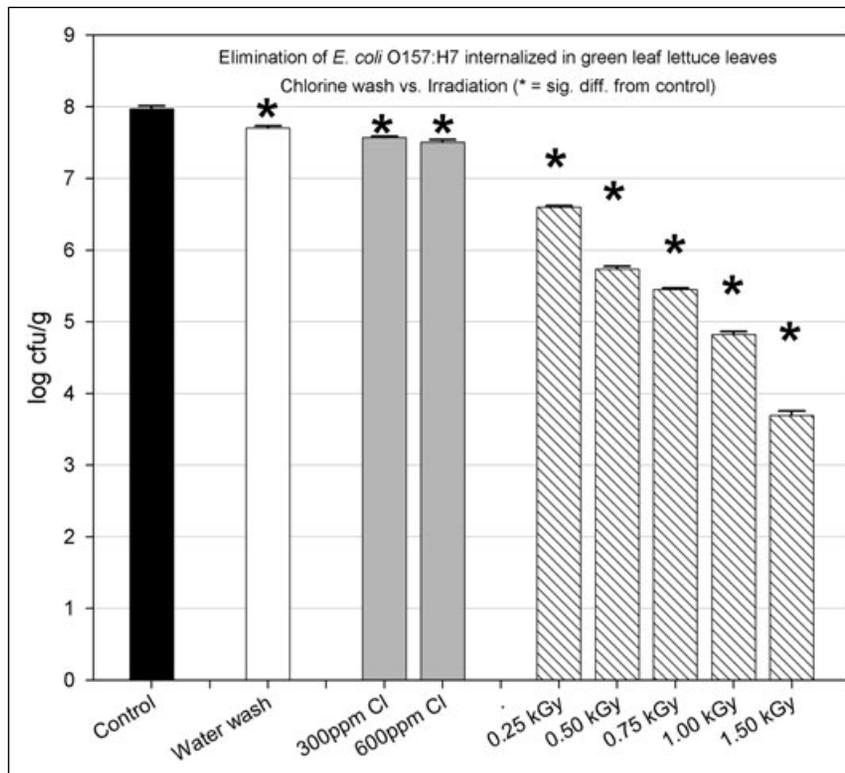

Figure 3 – Surviving populations of *E. coli* O157:H7 in green leaf pieces: untreated control (black), following water wash (white), sodium hypochlorite treatments (gray), or irradiation (hashed). Bars equal standard error, *n* = 9. Asterisk (·) indicates significantly different from the control (ANOVA, *P* < 0.05).
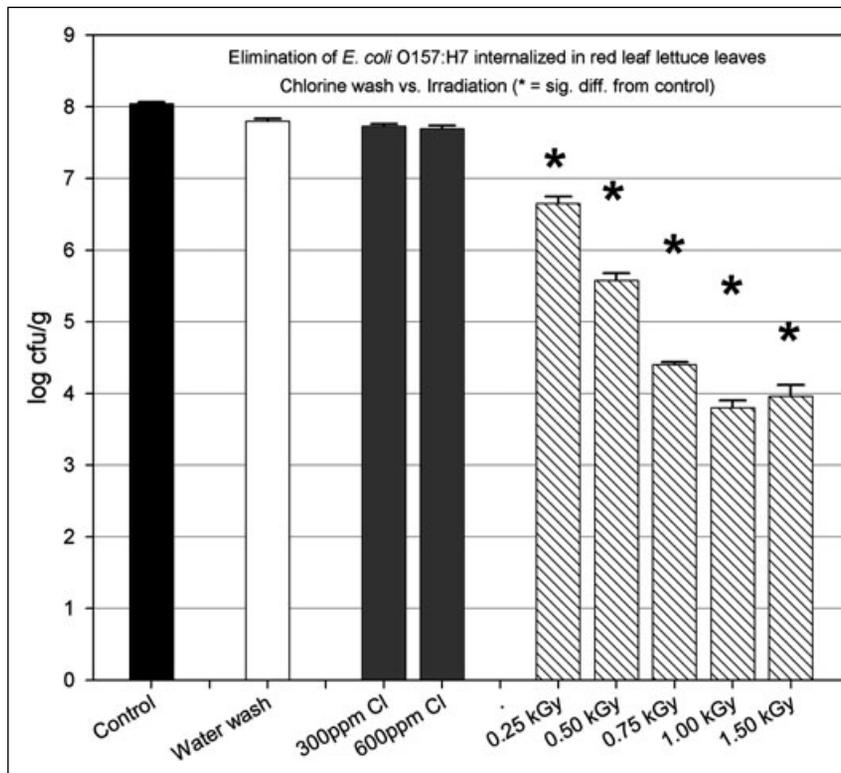
Figure 4 — Surviving populations of *E. coli* O157:H7 in red leaf pieces: untreated control (black), following water wash (white), sodium hypochlorite treatments (gray), or irradiation (hashed). Bars equal standard error, *n* = 9. Asterisk (*) indicates significantly different from the control (ANOVA, *P* < 0.05).

antimicrobial processes such as irradiation. Further research may determine how this response may be a result of the specific nature of the internal microenvironment of red leaf lettuce.

A study using the same 4 lettuce varieties examined herein (Niemira and others 2002) obtained D_{10} for surface-inoculated *E. coli* O157:H7 of 0.12 to 0.14 kGy, indicating that leaf-internalized *E. coli* O157:H7 is markedly more tolerant of irradiation than surface-associated. That study also presented D_{10} values obtained in homogenized leaf tissue from each type of lettuce. This was offered as a model system intended to predict the effect of the internal microenvironment within a leaf on internalized bacteria. In homogenized leaf tissue of Boston, green leaf, and red leaf lettuce, the D_{10} value of *E. coli* O157:H7 was 0.33 to 0.34 kGy, while in homogenized iceberg lettuce, the D_{10} value obtained was 0.09 kGy. These values are comparable to those obtained in the present study for the 2 looseleaf-type lettuces (green leaf and red leaf). For the 2 head lettuces, iceberg and Boston, D_{10} values obtained in leaf homogenate are 70% and 27% lower than those obtained by the direct vacuum perfusion internalization used herein. It should be noted that the tailing effect observed for red leaf lettuce treated with doses above 0.75 kGy is not yet understood. Red leaf lettuce is the most highly colored of the lettuces examined. This tailing effect was not observed in studies of surface-inoculated red leaf lettuce (Niemira and others 2002), but was observed with *E. coli* O157:H7 internalized in spinach, the more intensely colored vegetable examined in an earlier study (Niemira 2007). Further investigation is required to determine the extent to which homogenized leaf tissue may be an adequate model for evaluating the antimicrobial efficacy of irradiation against internalized bacteria.

Further research is necessary to understand varietal influence on the efficacy of irradiation. It has been previously demonstrated that leafy vegetables will tolerate irradiation doses comparable to those used in this study (Niemira and others 2002; Smith and Pillai 2004). A key aspect of future research will be evaluation of antimicrobial efficacy and sensory response at doses calibrated to reduce risks of

internalized pathogens. These doses must be determined based on a clearer understanding of the extent to which *E. coli* O157:H7 cells internalize in the field and at what population levels.

Conclusions

Sodium hypochlorite solutions did not result in meaningful reductions of *E. coli* O157:H7 internalized in iceberg, Boston, green leaf, or red leaf lettuce. However, irradiation effectively reduced the viable pathogen population in a dose-dependent manner. The response was variety specific, with D_{10} values ranging from 0.30 to 0.45 kGy. Irradiation is a potentially effective kill step for leaf-internalized human pathogens. Specific doses and irradiation protocols should be established for each type of lettuce in a commodity-specific process validation.

Acknowledgments

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