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# Relative Efficacy of Sodium Hypochlorite Wash Versus Irradiation To Inactivate *Escherichia coli* O157:H7 Internalized in Leaves of Romaine Lettuce and Baby Spinach†

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## ABSTRACT

Pathogenic bacteria that become internalized in leaf tissues are protected from the antimicrobial effects of surface treatments. Ionizing radiation is known to penetrate food tissues, but the efficacy of the process against internalized bacteria is unknown. Leaves of Romaine lettuce and baby spinach were cut into pieces, submerged in a cocktail mixture of three isolates of *Escherichia coli* O157:H7, and subjected to a vacuum perfusion process to force the bacterial cells into the intercellular spaces in the leaves. Scanning electron microscopy was used to evaluate the efficacy of the perfusion process. The inoculated leaves were then treated with a 3-min water wash, a 3-min wash with a sodium hypochlorite sanitizing solution (300 or 600 ppm), or various doses of ionizing radiation (0.25 to 1.5 kGy). Leaves were stomached to recover the internalized pathogen cells, which were enumerated. The vacuum perfusion effectively forced bacteria into the leaf vasculature and apoplast, as confirmed by scanning electron microscopy. For spinach leaf pieces, neither the water nor the sodium hypochlorite washes resulted in significant reductions of *E. coli* O157:H7 cells relative to the untreated control. For Romaine lettuce leaf pieces, 300 and 600 ppm sodium hypochlorite each resulted in less than 1-log reduction; water wash was ineffective. Ionizing radiation, in contrast, significantly reduced the pathogen population, with 4-log (Romaine lettuce) or 3-log (spinach) reductions at the highest dose tested. In Romaine leaves, the reduction was dose dependent across the range of doses tested, with a $D_{10}$-value (the amount of irradiation necessary to reduce the population by 1 log unit) of 0.39 kGy. In spinach leaves, the pathogen had a biphasic response, with a $D_{10}$-value of 0.27 kGy in the range of 0 to 0.75 kGy but only slight additional reductions from 0.75 to 1.5 kGy. In this study, ionizing radiation but not chemical sanitizers effectively reduced viable *E. coli* O157:H7 cells internalized in leafy green vegetables, but the response of the pathogen to irradiation was more complex in spinach leaves than in Romaine lettuce leaves.

Fresh and fresh-cut fruits and vegetables have been increasingly implicated in outbreaks of foodborne illness *(4, 6, 17).* Produce-associated outbreaks in the United States have risen from less than 20 throughout the 1970s to more than 100 in the 1990s *(13).* From 1995 through 2006, 20 outbreaks were associated with leafy green vegetables *(20).* In 2006, prebagged baby spinach contaminated with *Escherichia coli* O157:H7 caused 199 illnesses and three deaths across 26 U.S. states and one Canadian province *(21).* With these recent illnesses, deaths, and product recalls of prebagged leafy vegetables such as lettuce and spinach for contamination with *E. coli* O157:H7, the ability of growers and processors of leafy green vegetables to produce a safe product has come under scrutiny *(20).*

Good agricultural practices and other proper controls can effectively serve to reduce the risk of contaminating growing leafy green vegetables with human pathogens. Chlorine-based sanitizers are commonly used on produce surfaces and processing equipment, with concentrations of 50 to 200 ppm *(19).* However, the potential for internalization of *E. coli* O157:H7 or other pathogens into leaf tissue is a topic of ongoing discussion *(7–9, 15–18).* Throughout the 2006 outbreaks, previous concerns were renewed regarding the possibility that human pathogens may become internalized in leafy vegetables, calling into question the efficacy of surface decontamination measures. In 1999, the U.S. Food and Drug Administration (FDA) *(18)* reviewed the potential for infiltration of bacteria, including human pathogens, into fresh fruits and vegetables and the ability of surface decontamination measures to address this problem. The conclusion of that review was that a variety of opportunities exist for internalization to occur and that surface treatments are ineffective in reducing microbial populations that have been internalized into produce.

Irradiation effectively eliminates *E. coli* O157:H7 from lettuce *(2, 12).* Each of the major irradiation technologies (electron beam, X ray, and gamma ray) are penetrating processes and are used commercially to eliminate pathogens from meat products *(14).* Differences in the supporting food matrix as subtle as varietal differences among different type of lettuce can significantly influence the radiation sensitivity of inoculated *E. coli* O157:H7 *(10).* The apoplast of spinach contains ascorbate, and younger leaves (''baby spinach'') contain more apoplastic ascorbate than do older

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leaves *(10).* When in solution, ascorbate (a potent antioxidant) protects suspended bacteria from the antimicrobial effects of irradiation by preemptive quenching of the oxygen and hydroxyl radicals produced during the irradiation process *(2, 9, 12).* One hypothesis is that the chemistry of the inside of a leaf apoplast exerts a particular influence on the efficacy of the irradiation process, rendering this treatment less effective against internalized bacteria than against surface-associated bacteria. However, experimental data to support or refute this hypothesis are lacking; there have been no published studies on the efficacy of irradiation in eliminating leaf-internalized bacteria. Spinach as a commodity has been poorly studied with respect to irradiation, and little information exists on how suitable this intervention is for spinach for elimination of human pathogens such as *E. coli* O157:H7.

The objectives of this study were (i) to compare the antimicrobial efficacy of sodium hypochlorite washes and ionizing radiation for the elimination of leaf-internalized *E. coli* O157:H7 cells and (ii) to determine the influence of leaf type (lettuce versus spinach) on the efficacy of these processes.

## MATERIALS AND METHODS

**Microorganisms.** All isolates utilized in this study were from the U.S. Department of Agriculture–Agricultural Research Service–Eastern Regional Research Center culture collection. Three strains of *E. coli* O157:H7 previously used in this laboratory for studies of irradiated leafy vegetables were chosen for this study. The isolates were maintained in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.): C9490 (Centers for Disease Control, Atlanta, Ga.) and ATCC-35150 and ATCC-43894 (American Type Culture Collection, Manasass, Va.). 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 tryptic soy agar (TSA; Difco, Becton Dickinson) incubated overnight at 37°C.

**Produce.** Prebagged baby spinach leaves and whole heads of Romaine lettuce were purchased from local markets on the day of the experiments. Studies examining spinach and lettuce were conducted separately. Produce was prepared for the studies according to the method of Niemira et al. *(12).* The outer leaves of the Romaine heads 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. Whole spinach leaves were removed from the commercial packaging. To provide a spinach sample comparable to that of the Romaine lettuce, the spinach leaves were cut in half laterally into pieces weighing approximately 0.5 g.

Before use in the experiments, the cut leaf material was washed and sanitized by submerging it in a solution of 300 ppm sodium hypochlorite at room temperature and gently agitating for 3 min. The leaves were then thoroughly rinsed by agitation under successive changes of distilled water and spun in a sterile salad spinner–type centrifuge (Oxo International, New York, N.Y.) to remove excess surface water. This salad spinner has 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. The microflora of leaf material was measured for each leaf type by stomaching with Butterfield's phosphate buffer (BPB), serial dilution, pour plating with TSA, and incubation at 37°C for 24 h. The background population on Romaine lettuce was approximately $3.9 \pm 0.2 \times 10^5$ CFU/g presanitization and $7.1 \pm 0.4 \times 10^3$ CFU/g postsanitization. For spinach, the background population was $3.7 \pm 0.7 \times 10^5$ CFU/g presanitization and $4.1 \pm 0.2 \times 10^4$ CFU/g postsanitization.

**Inoculation.** The leaves were inoculated using an adaptation of the vacuum perfusion method, which irrigates the intercellular spaces without damaging the cell walls *(2).* Sterile BPB (4,050 ml) was placed into a sterilized glass vacuum desiccator inside a biological safety cabinet. The three *E. coli* O157:H7 cultures (150 ml each) were combined with the BPB to make a 1:10 diluted cocktail inoculum with a final volume of 4,500 ml. Leaf pieces sufficient for one replication of the experiment were added to the desiccator and gently submerged with a stainless steel utensil, and the desiccator was sealed. A 0.2-μm-pore-size filter was placed in the vacuum line between the desiccator and the house vacuum to prevent contamination of the house vacuum system, which operated at approximately 27 in. of 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 three 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 the salad spinner inside the biological safety cabinet, typically with two or three spin cycles. The spun-dried, fully perfused leaves were weighed into subsamples within 30 min.

Samples (40 g) of leaf pieces for lettuce and for spinach were weighed before and after perfusion to measure the uptake of fluid. Romaine lettuce leaf pieces took up 53% of their normal weight during the perfusion process, and spinach leaf pieces took up 35.5% of their normal weight. These values were used in the subsequent dilution calculations to determine the CFU per gram of leaf.

**Scanning electron microscopy (SEM).** To validate the perfusion process, perfusion-inoculated leaf pieces of both Romaine lettuce and baby spinach were prepared as described above. The perfused material was immersed in a 2.5% glutaraldehyde–0.1 M imidazole solution (pH 7.2) for more than 2 h and then washed in the buffered solution to remove the glutaraldehyde. Samples were then dehydrated by exchange in a graded series of ethanol solutions (50, 80, and 100%), frozen in liquid nitrogen, and fractured manually with cold fine-tipped tweezers, similar to the method described by Haggis and Phipps-Todd *(5).* Frozen fragments were thawed into ethanol and critical point dried with liquid carbon dioxide. Dry fragments were mounted with Duco cement (ITW Performance Polymers, Riviera Beach, Fla.) onto specimen stubs with fracture faces oriented upward and sputter coated with a thin layer of gold. Intercellular spaces near the fracture faces were examined with a Quanta 200 FEG scanning electron microscope (FEI Co., Inc., Hillsboro, Oreg.) operated at an accelerating voltage of 10 kV in the high-vacuum–secondary electron imaging mode of operation.

To forestall the possibility that the observations might be of natively occurring internalized bacteria, a companion microscopy

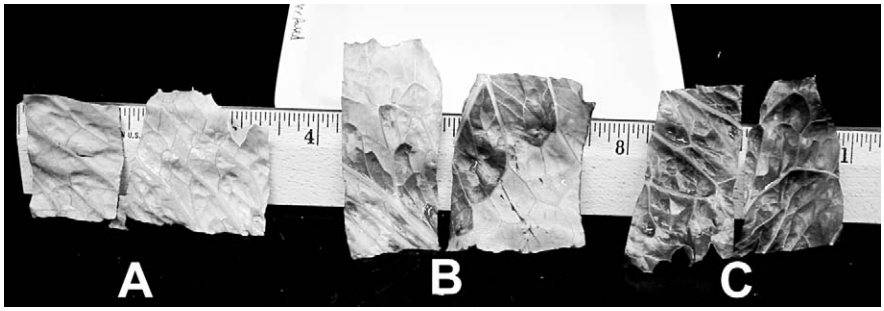


FIGURE 1. *Romaine lettuce leaf pieces before perfusion (A), after partial perfusion (B), and fully perfused (C) with* E. coli *O157:H7. Note the increasingly darkened and water-soaked area associated with the inoculum perfusion.*

study was performed using the same perfusion process with a mixed suspension of Fluoresbrite calibration-grade plastic beads of two sizes, 0.88 and 1.98 μm (Polysciences, Warrington, Pa.), which roughly approximate the dimensions of *E. coli* cells. Lettuce pieces were perfused and examined using SEM as described.

**Chemical wash.** Perfused spin-dried leaf pieces were divided into 20-g subsamples. One 20-g sample was placed in a no. 400 stomacher bag (Tekmar, Inc., Cincinnati, Ohio) 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 before treatment from a concentrated stock solution. Each test solution was handled individually, and solutions were at room temperature during treatment. The leaf pieces were agitated in the appropriate test solution for 3 min and then removed to a clean beaker and agitated in sterile deionized water for 3 min. Concentrations and washing times were chosen to approximate a treatment comparable to current industry practice *(19)* and an aggressive treatment with a much higher concentration. The rinsed leaf pieces were then spun-dried as described and placed in a stomacher bag. Each control and treatment was performed three times in independent replications.

**Irradiation.** Inoculated leaf pieces bagged in 20-g samples were treated with 0.25, 0.50, 0.75, 1.0, or 1.5 kGy of radiation. In all cases, irradiation was conducted at 4°C. As with the controls and wash treatments, each irradiation treatment was performed three 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 with a cesium-137 self-contained gamma radiation source (Lockheed-Georgia, Marietta, Ga.) at 5.64 kGy/h. This rate was established using alanine transfer dosimeters (National Institutes of Standards and Technology, Gaithersburg, Md.). Alanine pellets (Bruker, Inc. Billerica, Mass.) were used for dosimetry. The pellets were read on an EMS 104 EPR analyzer (Bruker), and results were 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 5:1 ratio of solution:leaf by weight. The bags were folded and stomached at high speed (260 rpm) for 4 min to pulp the leaf pieces. The resulting sample liquid was fully green, indicating effective recovery of the internal fluid of the leaf pieces. An aliquot (0.1 ml) of the fluid was removed and serially diluted in BPB. Dilutions appropriate for each treatment were spread plated on TSA plates, which were inverted and incubated overnight at 37°C. Three plates per dilution were counted by hand. Plate counts were adjusted for dilutions and for the fluid absorption ratios for Romaine lettuce and baby spinach to determine the CFU per gram.

**Statistical analysis.** The surviving population for each treatment for each leaf type was compared with that of the untreated control using a two-way analysis of variance (ANOVA; SigmaStat v. 4.0, SPSS, Inc., Chicago, Ill.) (Tukey test, $P < 0.05$) with data pooled from the three plates for each of the three replications. The radiation $D_{10}$ for the *E. coli* O157:H7 cocktail was calculated for Romaine lettuce using the entire data set based on the negative reciprocal of the slope for the linear regression line. Because of the apparent biphasic nature of the response of the pathogen to irradiation in baby spinach leaves, separate $D_{10}$-values were calculated for the lower dose range (0.0 to 0.75 kGy) and the upper dose range (0.75 to 1.5 kGy). The pivot value for 0.75 kGy was included in each calculation. As a comparison, this same procedure was applied to the apparently liner response data for Romaine lettuce. The significance of the differences among the $D_{10}$-values (Romaine, lower range spinach, and higher range spinach) was determined using an analysis of covariance (ANCOVA; Excel, Microsoft Corp. Redmond, Wash.).

## RESULTS

The vacuum perfusion process effectively introduced the inoculum into the leaf pieces of Romaine lettuce (Fig. 1) and baby spinach (not shown). The infiltration proceeded along the major vascular elements of the leaf in the first cycle of perfusion, with the inoculum visibly drawn into the leaf pieces and creating a distinctive water-soaked appearance. Subsequent cycles drew the inoculum into the leaf pieces more extensively, and the leaf pieces were fully perfused after three cycles.

SEM revealed bacterial cells throughout the vasculature and intercellular spaces in both Romaine lettuce (Fig. 2) and baby spinach (Fig. 3) after the vacuum perfusion. The inoculated bacterial cells in spinach leaves tended to be more clumped together into aggregates, in many cases extensively so, in comparison with those in lettuce leaves. SEM of leaves perfused with microspheres (Fig. 4) revealed extensive uptake, particularly along the major vascular elements, in agreement with the visual observation of the process of infiltration of the suspending liquid.

The concentration of bacterial cells per gram of leaf material was determined directly by sampling the untreated control and by calculation from the known CFU per gram concentration of the diluted inoculum and the known weight of the inoculum taken up during the perfusion process for Romaine lettuce and spinach. Because these results were in agreement, the sampling of the buffer from the stomached leaf pieces was considered an accurate representation of the distribution of the internalized bacteria, approximately $10^7$ CFU/g.

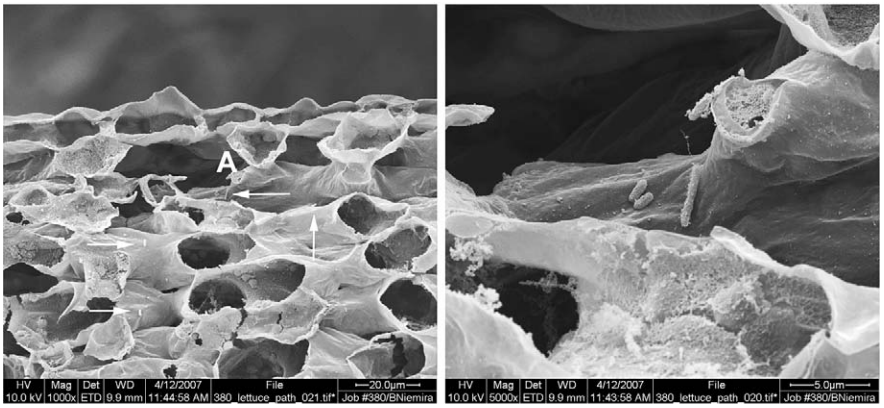


FIGURE 2. *Scanning electron micrographs of Romaine lettuce leaf pieces after perfusion with* E. coli *O157:H7 inoculum. In the left image, arrows indicate bacterial cells in intercellular spaces, and the letter A indicates area of magnification (shown on the right).*

Relative to the untreated control, the water wash treatment had no significant effect on the bacterial concentration in Romaine lettuce leaves (Fig. 5). Washes with 300 and 600 ppm sodium hypochlorite resulted in populations that were each significantly different ($P < 0.05$) from those in the control groups. Each treatment resulted in an approximately 0.5-log reduction, and the results of the two treatments were not significantly different from each other. The surviving bacterial concentrations for all of irradiation treatment groups were significantly different from those of the controls and the water wash samples, and results for all doses except 0.25 kGy were significantly different from those obtained with the sodium hypochlorite washes. The calculated $D_{10}$-value for *E. coli* O157:H7 cells internalized in Romaine lettuce leaf pieces was 0.39 kGy, based on the entire data set. The separate $D_{10}$-values for the lower and upper dose ranges were 0.33 and 0.35 kGy, respectively, confirming the linearity of the response.

Relative to the untreated control, neither the water wash nor the 300 and 600 ppm sodium hypochlorite washes had any significant effect on surviving bacterial concentrations in baby spinach leaf pieces (Fig. 6). The concentrations of surviving bacteria in all of the irradiation samples tested were significantly different ($P < 0.05$) from those in the control, water wash, and sodium hypochlorite wash samples. Unlike the response of Romaine lettuce, the irradiation dose-response for spinach appeared to be biphasic, with a relatively steep response from 0.25 to 0.75 kGy and a relatively flat response at higher doses. For the lower dose range (0 to 0.75 kGy), the calculated $D_{10}$-value for *E. coli* O157:H7 cells internalized in baby spinach was 0.27 kGy, a value not significantly different (ANCOVA, $P < 0.05$) from the value for Romaine lettuce. However, for the upper dose range (0.75 to 1.5 kGy), the calculated $D_{10}$-value was significantly higher, at 2.67 kGy, a very high value that reflects the nearly flat tailing in the upper range.

## DISCUSSION

This study is the first to demonstrate the efficacy of irradiation for the elimination of internalized *E. coli* O157:H7 in spinach and Romaine lettuce. The results presented herein with the water and sodium hypochlorite washes confirm the finding of the FDA that surface treatment is ineffective in reducing internalized microbial populations and that the concentrations of chlorine commonly used in the processing of leafy vegetables may be inadequate to address contamination *(18, 19).* The inoculation method used in this study involves the submersion of the leaf pieces in the inoculum cocktail, and some surface presence of the pathogen is therefore expected. To completely separate counts of surface-associated and internalized bacteria, a future modification of this method could incorporate complete sterilization of the leaf surface (e.g., with a 2% [wt/vol] calcium hypochlorite wash) prior to bacterial enumeration.

Surface treatments effective against surface contamination were not effective against the majority of the bacterial cells present within the leaf tissues. The vacuum perfusion process forces fluid into the intercellular spaces occupied by apoplastic fluid and has an acknowledged, though slight, potential for damage to internal leaf structures *(2).*

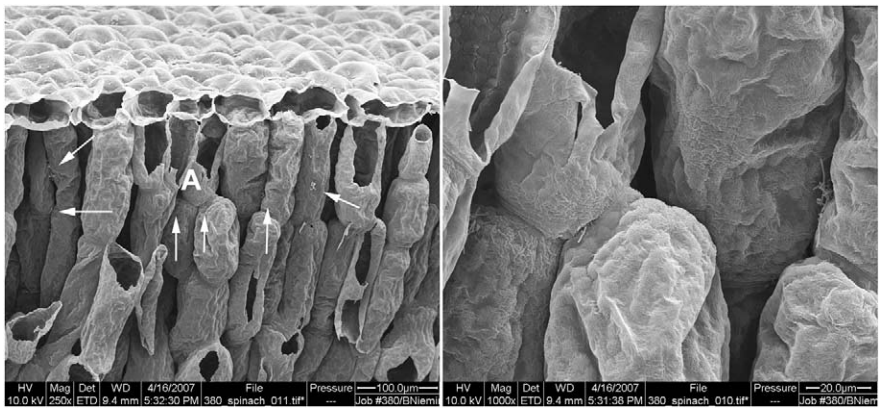


FIGURE 3. *Scanning electron micrographs of baby spinach leaf pieces after perfusion with* E. coli *O157:H7 inoculum. In the left image, arrows indicate bacterial cells in intercellular spaces, and the letter A indicates area of magnification (shown on the right)*

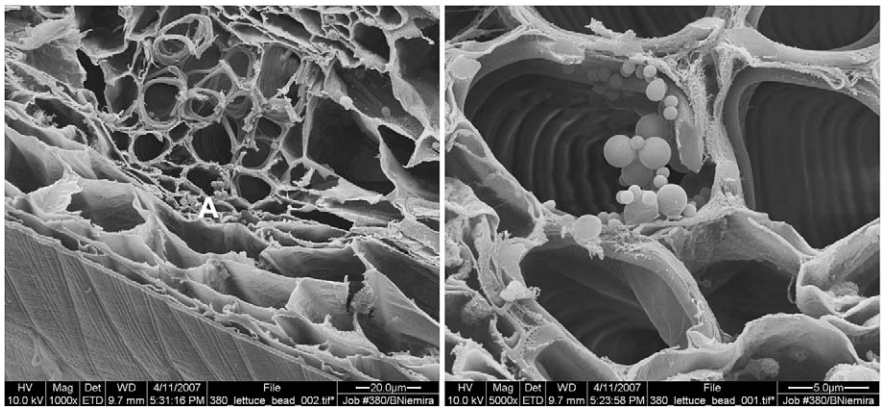


FIGURE 4. *Scanning electron micrographs of Romaine lettuce leaf pieces after perfusion with microbeads. The letter A indicates area of magnification (shown on the right), showing microbeads in vascular elements.*

The small amount of fractured solid cellular contents that may be present in the perfused cell suspensions may therefore exert a slight influence on the radiation sensitivity of the internalized bacteria. The expectation is that naturally internalized human pathogenic bacteria would not invade the cell wall but would be confined to the apoplast and/or the vasculature, but research data to confirm this assumption are lacking.

Recent research on the potential for internalization of human pathogens into crop plants has shown the complexity of the issue. Solomon et al. *(16)* found that *E. coli* O157:H7 effectively migrated from the rhizosphere into internal locations in the edible portions of the plant. The internalized *E. coli* O157:H7 cells remained viable for at least 20 days after exposure to the pathogens and were not removed by a chlorine wash *(15).* Barley plants were effectively colonized by *Salmonella enterica* and *Listeria* spp. introduced to the roots *(9).* In that study, *Listeria* tended to be restricted to the root tissues, while *Salmonella* spread throughout the plant, including into the edible tissues. In contrast, Jablasone et al. *(8)* found that *E. coli* O157:H7 internalized in seedlings but did not persist in mature plants. In a study of *Salmonella* introduced to tomato plants via irrigation water, the pathogen did not become incorporated into the edible portions of the plant *(7).* Given that in these studies various methods for inoculation and plant cultivation were used, additional research is needed to fully understand the events that lead to internalization of bacteria in healthy crop plants and the true potential for risk from this internalization in the commercial growing environment. The most probable growth phase of bacteria internalized under natural conditions is not known. Bacteria in stationary phase within plant tissues may pose an even greater challenge for sanitization processes.

Because surface treatments have limited efficacy against internalized pathogens, irradiation, which is a penetrating process, has been suggested as a possible postharvest processing step that may be useful in targeting these

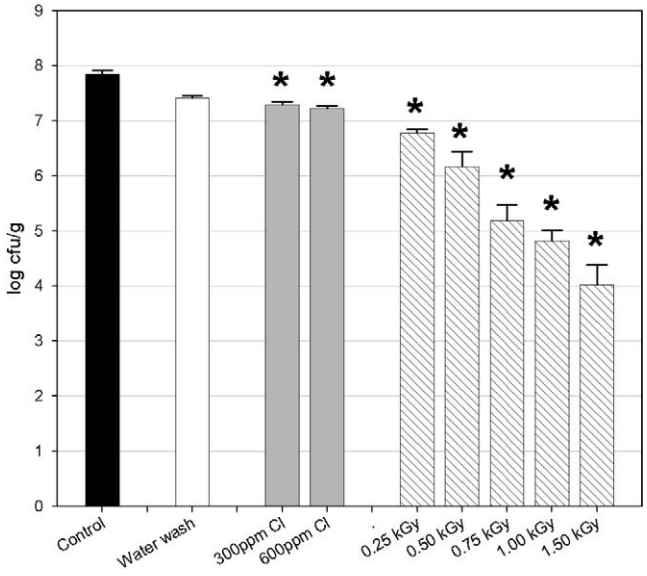


FIGURE 5. *Surviving populations of* E. coli *O157:H7 on Romaine lettuce leaf pieces: untreated control (black) and after treatment with water wash (white), sodium hypochlorite wash (gray), or irradiation (hashed). Bars equal standard error,* n = *9. Asterisk indicates significant differences from the control (ANOVA,* $P < 0.05$*).*

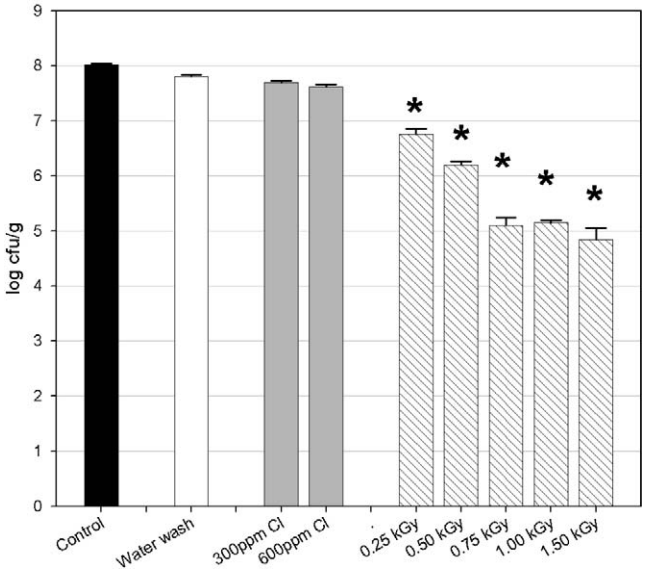


FIGURE 6. *Surviving populations of* E. coli *O157:H7 on baby spinach leaf pieces: untreated control (black) and after treatment with water wash (white), sodium hypochlorite wash (gray), or irradiation (hashed). Bars equal standard error,* n = *9. Asterisk indicates significant differences from the control (ANOVA,* $P < 0.05$*).*

inaccessible pathogens. The FDA *(21)* is currently reviewing irradiation as a sanitizing treatment for leafy vegetables. In the present study, irradiation effectively eliminated the pathogen from the internal spaces of lettuce and spinach leaf pieces. A treatment of 1.0 kGy (the highest dose currently allowed for vegetable products in the United States) resulted in an approximately 3-log reduction. The bacterial concentration used in the inoculum cocktail was approximately $10^8$ CFU/ml, a level much higher than would expected to be found in nature even under extreme contamination conditions. The degree of perfusion of the liquid also was greater than that expected to occur during natural internalization events, and the process was further facilitated by the use of cut spinach and Romaine lettuce leaf pieces. These levels of contamination, which were more extreme than the natural condition by at least an order of magnitude, were dictated by experimental necessity with regard to testing and recovery. Nevertheless, because the minimum infective dose for *E. coli* O157:H7 may be less than 1,000 cells for healthy individuals or as low as 10 cells for at-risk individuals *(1),* even a low level of contamination poses an unacceptable risk. Agricultural processes, such as vacuum cooling or hydrocooling, that can cause perfusion or uptake of potentially contaminated wash water can be a source of increased risk.

The $D_{10}$-value obtained for *E. coli* O157:H7 on Romaine lettuce in this study was 0.39 kGy. This value is higher (approximately a three- to fourfold increase) than that obtained for surface inoculated *E. coli* O157:H7 on red leaf (0.12 kGy), green leaf (0.12 kGy), Iceberg (0.14 kGy), or Boston (0.14 kGy) lettuces *(12).* However, in that study $D_{10}$-values were also obtained in homogenized leaf tissue, a model system intended to predict the effect of leaf chemistry on internalized bacteria. In homogenized leaf tissue, the $D_{10}$-value of *E. coli* O157:H7 was 0.33 to 0.34 kGy for the darker green lettuces (red leaf, green leaf, and Boston), values comparable to that obtained in the present study. This finding suggests that homogenized leaf tissue may be an adequate model for investigating the response of internalized bacteria to irradiation. However, that study *(12)* did not include samples of spinach or Romaine lettuce.

Radiation sensitivity is influenced by the particular nature of the food substrate *(12),* and this effect was evident in the present study. In spinach leaves, the reduction of internalized *E. coli* O157:H7 cells was biphasic, with a $D_{10}$-value for the lower dose range (0.27 kGy) not different than that observed in Romaine lettuce, but a remarkably high calculated $D_{10}$-value was obtained for the upper dose range (2.67 kGy). This value, approximately an order of magnitude higher than that typically observed for bacterial pathogens *(3, 12, 14),* results from the relatively flat response above 0.75 kGy. Because this biphasic response was not obtained for Romaine lettuce, although the two types of material were inoculated identically, it seems unlikely that the biphasic pattern is solely due to the mortality curves of a surface population compounded with those of the internal population. Although a clear explanation for bacterial persistence at higher radiation doses has not been formulated, SEM revealed that the bacterial cells within the spinach leaves were more aggregated than those within the Romaine lettuce leaves. The cause for this aggregation is not yet known. There was no evidence of extracellular material connecting the aggregated bacterial cells, as would be the case with a developing biofilm, which suggests that the clumping is the result of a physical process originating with the spinach leaf apoplastic environment rather than a biological process originating with the bacterial cells. The response to irradiation of biofilm-associated or otherwise aggregated cells, relative to planktonic or individual cells, has not been widely studied. In a recent study, *E. coli* O157:H7 cells grown in vitro in biofilms were variously sensitive to irradiation relative to planktonic cells, depending on the isolate and biofilm culture conditions *(11).* More information is needed on how the microenvironment on and within leaves can influence the efficacy of antimicrobial processes such as irradiation.

Although lettuce and spinach leaves are superficially similar, the data obtained in the present study suggests a significantly different response to the presence of *E. coli* O157:H7 internalized through vacuum perfusion and a different response to irradiation. These differences highlight the problems associated with extrapolating from one commodity to another in predicting how antimicrobial processes will perform. A contamination problem that persists, even as treatment levels increase, can pose a notable risk. Further research is necessary to understand how the nature of the contaminated leaf might influence the efficacy of irradiation at commercially practical doses, with specific attention paid to the differential response of spinach versus other types of leafy greens. Although previous research indicates that leafy vegetables will tolerate radiation doses comparable to those used in this study *(2, 12),* an important aspect of future research will be evaluation of the potential for sensory damage to a specific product.

Sodium hypochlorite solutions were not effective in eliminating internalized *E. coli* O157:H7 cells from Romaine lettuce or baby spinach leaf pieces. In contrast, ionizing radiation effectively reduced the viable population of the pathogen in a dose-dependent manner. The internalized pathogen had a more complex response to irradiation in spinach leaves than in Romaine lettuce leaves, with a marked tailing effect in spinach at higher doses as compared with a linear response in the lettuce. Although irradiation is a potentially effective means of eliminating internalized human pathogens from leafy vegetables, recommendations for the specific doses to be used should be determined for each product based on observed patterns of antimicrobial efficacy and specific product sensory responses.

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