

## Reduction of *Escherichia coli* O157:H7 and *Salmonella* on Baby Spinach, Using Electron Beam Radiation

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### ABSTRACT

The effect of low-dose electron beam (e-beam) radiation on the reduction of *Escherichia coli* O157:H7 and *Salmonella* in spinach was studied. Fresh baby spinach (*Spinacia oleracea*) was inoculated with a bacterial cocktail containing multiple strains of rifampin-resistant *E. coli* O157:H7 and rifampin-resistant *Salmonella*. Inoculated samples were exposed to e-beam radiation from a linear accelerator and tested for counts of both *E. coli* O157:H7 and *Salmonella*. Irradiated spinach was also stored for 8 days at 4°C, and counts were made at 2-day intervals to determine if there was any effect of radiation on the survival trend of both pathogens. When no pathogens were detected on plates, additional enrichment plating was conducted to verify total destruction. Respiration rates were measured on spinach samples exposed to e-beam radiation. Each dose of e-beam radiation significantly reduced the numbers of *E. coli* O157:H7 and *Salmonella* from initial levels of 7 log CFU/g. Treatment by e-beam radiation at a dose of 0.40 kGy resulted in a reduction in populations of *E. coli* O157:H7 and *Salmonella* of 3.7 and 3.4 log cycles, respectively. At 0.70 kGy, both pathogens were reduced by 4 log. All doses above 1.07 kGy showed reductions greater than 6 log and decreased to undetectable levels when stored for 8 days. The respiration rate of spinach showed no changes after irradiation up to 2.1 kGy. These results suggest that low-dose e-beam radiation may be a viable tool for reducing microbial populations or eliminating *E. coli* O157:H7 and *Salmonella* from spinach without product damage.

There has been an increase in the number of foodborne illnesses associated with fresh produce in the past 30 years (1, 13, 23). Between 1995 and 2005 there have been approximately 26 outbreaks of *Escherichia coli* O157:H7 associated with lettuce or leafy greens (6–8, 16, 21). The recent increase in outbreaks associated with raw produce can be attributed to changing dietary habits, new production and processing technologies, sources of produce, as well as the manifestation of pathogens previously not associated with raw produce (1–3, 23). Spinach has recently become a concern because of its involvement as the vehicle of *E. coli* O157:H7 in the 2006 multistate outbreak originating in California. This outbreak resulted in 205 confirmed cases and three deaths. Thirty-one of the 103 hospitalized case patients developed hemolytic uremic syndrome (4). For the 2006 spinach outbreak, environmental samples containing *E. coli* O157:H7 were found in cattle feces, wild pig feces, river water, and soil samples (4). *E. coli* O157:H7 can remain viable in bovine feces for up to 70 days (25). Further contamination may occur during postharvest operations by wash water spreading contamination over product units (23). Researchers are continuing to study ways to reduce the food safety risks associated with fresh produce. Despite the use of proper hygiene and good agricultural practices, under specific conditions, contamination of fresh produce

may occur at any point along the farm to table continuum (24).

The irradiation of foods is not a novel concept; however, due to the numerous outbreaks associated with produce and other commodities, application of this technology may now be utilized more frequently. Ionizing radiation kills microorganisms by causing irreparable damage to cell biomolecules. Electronic beam (e-beam) technology uses high-energy electrons to destroy microorganisms. E-beams are produced using linear accelerators, which use electricity and can accelerate electrons up to 99% of the speed of light. These accelerated electrons then collide with chemical bonds, causing breaks. In certain products, the breaks in chemical bonds (6 bonds for every 10 million present in a system) cause minimal effect on the physical appearance. Therefore, the product damage by irradiation is dependent on the individual food commodity (18). E-beam uses higher energy than gamma and x-rays use, but has low penetration potential. The depth of penetration is determined by the density of the product. For example, using a dual beam will result in a penetration depth of 8.9 cm in ground beef (18). In bagged leafy greens, the penetration and dose distribution depends on the air spaces between the leaves, and the tightness of the packaged product needs to be taken into account in developing irradiation treatments for these commodities (11). Because of the numerous possible fruits and vegetables that can be irradiated, each commodity must be studied separately (17). In an effort to reduce the number of foodborne illnesses associated with fresh produce, con-

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tinued food safety research must be conducted on each type of fruit or vegetable concerning specific pathogens, the manner in which the pathogen attaches to the produce item, growth characteristics of the microorganism on the commodity, as well as investigate multiple types of interventions or treatment methods.

The objectives of this study included examining the effectiveness of low-dose e-beam radiation on *E. coli* O157:H7 and *Salmonella* and to determine if there is any effect of e-beam radiation on the survival trend of both pathogens.

## MATERIALS AND METHODS

**Bacterial cultures.** Rifampin-resistant mutants were derived from five parent strains of *E. coli* O157:H7, according to the method published by Kaspar and Tamplin (14). The parent strains were obtained from the Texas A&M Food Microbiology Laboratory (College Station) culture collection. In addition, rifampin-resistant *Salmonella* serotypes Agona, Gaminara, Michigan, Montevideo, Poona, and Typhimurium, obtained from the Texas A&M Food Microbiology Laboratory culture collection, were used to inoculate fresh baby spinach to be treated in this study. Growth curves and radiation sensitivity of the mutant strains were determined to be virtually indistinguishable from the parent strains. Five strains each of mutant *E. coli* O157:H7 and mutant *Salmonella* Agona, Gaminara, Michigan, Montevideo, Poona, and Typhimurium were cultured onto tryptic soy agar slants (TSA; Difco, Becton Dickinson, Sparks, Md.) and incubated at 37°C for 24 h. Three days prior to each experiment the microorganisms were resuscitated by two consecutive transfers to tryptic soy broth (TSB; Difco, Becton Dickinson) and incubated at 37°C for 12 h. Rifampin resistance was confirmed by streaking TSB cultures onto plates of TSA plus 100 mg/liter rifampin (Sigma, St. Louis, Mo.) and incubated at 35°C for 24 h.

**Inoculum preparation.** Nine milliliters of a 12-h culture of each microorganism was dispensed in sterile centrifuge tubes (50 ml) and harvested by centrifugation at  $1,623 \times g$  in a Jouan B4i centrifuge (Thermo Scientific, Waltham, Mass.) for 15 min at 21°C. The pellet for each microorganism was resuspended in 5 ml of 0.1% peptone water (Difco, Becton Dickinson), and then 1-ml aliquots of each were combined to make a cocktail in a sterile bottle containing 89 ml of 0.1% peptone water. The prepared inoculum (containing each pathogen at a concentration of ca. 8 log CFU/ml) was used within 2 h after preparation and was kept at room temperature (23 to 24°C) during the experiment.

**Sample preparation and inoculation of spinach.** Fresh baby spinach typical of leafy greens entering the U.S. food supply was selected for use in this study and purchased from a major supplier. After transporting to the Texas A&M Food Microbiology Laboratory, spinach leaves were sorted to remove leaves that were bruised, cut, or had decay. Spinach leaves were randomly separated in 10-g portions in individual stomacher bags, and 1 ml of the bacterial cocktail was added to each bag. The bag then was closed and shaken for 1 min to assist in distributing uniformly. Inoculated sample bags were placed on a flat surface and pressed manually to remove as much air as possible from the bag. The bags then were folded lengthwise, placed into a secondary stomacher bag, and sealed using a heat sealer to follow an established protocol for preventing leaks when handling biohazardous materials. This procedure resulted in a sample that was sufficiently thin to permit irradiation with a maximum/minimum dose ratio of 1 for all doses tested. Inoculated spinach samples were irradiated within 2 h post-preparation. The initial concentrations of *E. coli*

O157:H7 and *Salmonella* on the baby spinach were 7.1 and 7.3 CFU/g respectively.

**Irradiation.** All e-beam radiation treatments were conducted at the Food Technology Facility for Electron Beam and Space Food Research at Texas A&M University (College Station). A pit and tower system with two 10-MeV and 15-kW linear accelerators was used for this experiment (LINAC, Varian, Palo Alto, Calif.), using a dual beam. Prior to treatments, high-precision dosing was conducted to determine the appropriate attenuation scheme and conveyor speed to achieve the target doses, using alanine dosimeter film strips (BioMax, Eastman Kodak Co., Rochester, N.Y.) placed above and below triplicate preliminary spinach samples. The high precision in dose was achieved due to the thin nature of the sample packets. High-density polyethylene sheets (King Plastic Corporation, North Port, Fla.) were used as attenuators to reduce the energy of incident electrons in order to achieve the target doses. Inoculated samples were exposed to 0.4-, 0.79-, 1.07-, 1.16-, 2.04-, or 2.48-kGy e-beam radiation from a linear accelerator. Dose absorption was calculated from the dosimeter strips, using an electron paramagnetic resonance instrument (EMS 104 EPR analyzer, Bruker Instruments, Karlsruhe, Germany). Nonirradiated spinach served as control for this experiment. All experiments, accounting for each condition and organism, were replicated three times.

**Microbiological analysis.** After e-beam radiation, 90 ml of sterile 0.1% peptone water was added to each of the spinach samples in stomacher bags and pummeled in a laboratory blender (Stomacher 400, Seward, London, UK) for 1 min. Serial dilutions were made and spread plated onto lactose-sulfite-phenol red-rifampin agar, a selective and differential medium designed for simultaneous enumeration of rifampin-resistant *E. coli* and *Salmonella* (5). Plates were incubated for 24 to 28 h at 35°C. Rifampin-resistant *E. coli* O157:H7 produced yellow colonies on the medium, whereas rifampin-resistant *Salmonella* developed colonies with a black center surrounded by a pink halo. Counts of *E. coli* O157:H7 and *Salmonella* were made independently. Confirmation tests were conducted to verify the identities of the colonies, using standard biochemical tests.

Additional enrichment plating was conducted to verify total destruction in case no colonies were detected on the count plates. Twenty-five-gram samples of irradiated, inoculated spinach were enriched in 225 ml of TSB plus 22.5 mg of rifampin, incubated at 37°C, and streaked for growth onto lactose-sulfite-phenol red-rifampin agar after days 0, 2, 4, 6, and 8.

**Respiration rate.** An additional experiment was conducted to determine the effect of radiation on respiration rates of baby spinach. Bagged spinach for wholesale distribution (1.1-kg bags) was obtained from commercial sources and subjected in their original package to e-beam radiation at doses of 1.2, 2.1 and 3.2 kGy. After treatment, triplicate 225-g samples were separated from individual bags for each dose including nonirradiated controls and placed in separate 1-liter gas-tight glass containers (Kerr, Jarden Home Brands, Daleville, Ind.) equipped with a rubber septum port for sampling and an airtight lid with an o-ring for sealing. The jars were stored at 4°C. At 1-day intervals over 3 days, the gaseous atmosphere in the triplicate jars corresponding to each dose was sampled to measure changes in the concentrations of O<sub>2</sub> and CO<sub>2</sub>. Gas samples were withdrawn from the jars, using an airtight syringe and analyzed for percentage of O<sub>2</sub> using an O<sub>2</sub> analyzer (S-3A/I AEI Technologies, Inc., Pittsburgh, Pa.) and CO<sub>2</sub>, using an infrared gas analyzer (model PIR-2000, Horiba, Irvine, Calif.).

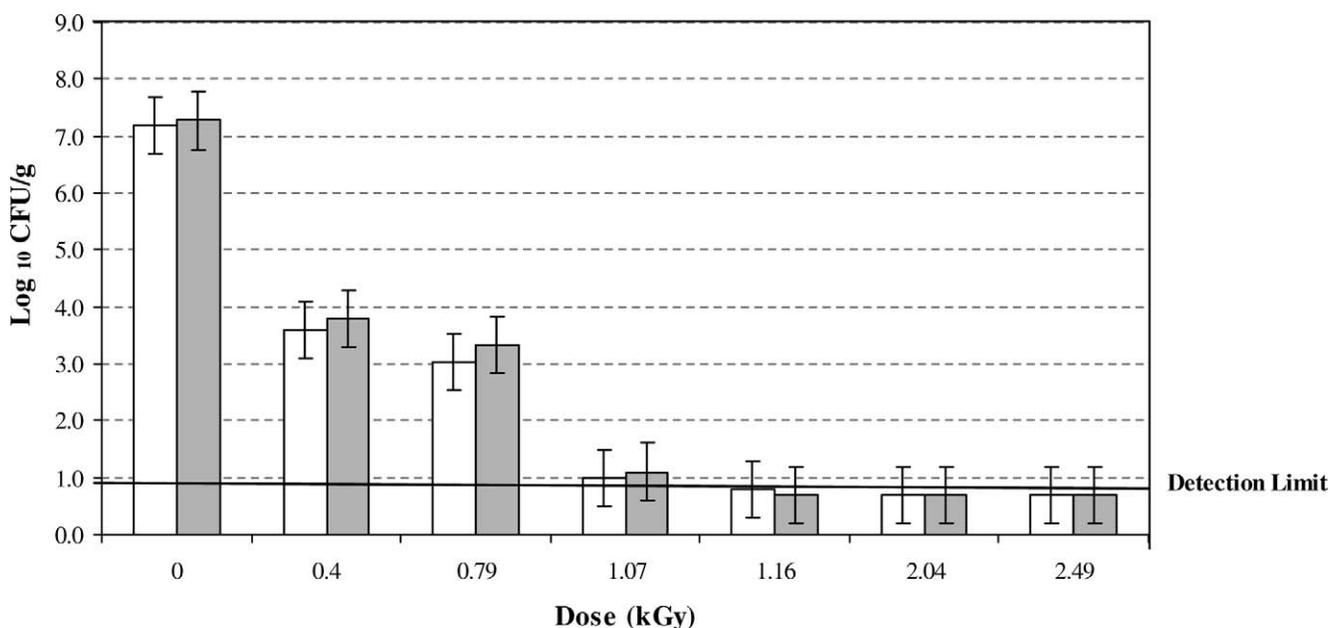


FIGURE 1. Effects of radiation dose on the populations of *E. coli* O157:H7 (white) and *Salmonella* (gray) after e-beam radiation.

Respiration rates were estimated from the O<sub>2</sub> and CO<sub>2</sub> concentrations.

**D<sub>10</sub>-value.** In a separate experiment, rifampin-resistant *E. coli* O157:H7 was prepared and inoculated onto 10-g spinach samples as described above. During preliminary studies, the resistances of all *E. coli* O157:H7 strains to radiation were determined. Since there was no significant difference in the resistances of all strains to radiation, to prevent any variation in the results, a single strain was selected for determining the D<sub>10</sub>-value. The initial concentration of *E. coli* O157:H7 on the baby spinach was 6.4 CFU/g. Alanine pellet dosimeters (Harwell Dosimeters, Oxfordshire, UK) were used for this experiment, and the spinach samples were prepared as described above to achieve a package thickness of 4 mm, which is equal to the thickness of the dosimeter pellets. This approach permitted high-precision dosing aimed at establishing treatments at target doses between 0 to 1 kGy, with increments of 0.15 kGy. Dosimeters were placed in plastic carriers to mimic

TABLE 1. Estimated log reduction for *E. coli* O157:H7 and *Salmonella* inoculated onto fresh baby spinach leaves as affected by dose of e-beam radiation<sup>a</sup>

Radiation dose (kGy)	<i>E. coli</i> O157:H7	<i>Salmonella</i>
0.4	3.7 A <sup>b</sup>	3.4 A
0.79	4.1 B	4.0 B
1.07	6.3 C	6.1 C
1.16	6.3 C	>6.6 C <sup>c</sup>
2.04	>6.6 C	>6.5 C
2.49	>6.4 C	>6.6 C

<sup>a</sup> Estimated log reduction: log CFU per gram on control spinach minus log CFU per gram on spinach after treatment. Average log CFU per gram on control spinach was 7.1 for *E. coli* O157:H7 and 7.3 for *Salmonella*.

<sup>b</sup> Means within columns with the same letter are not significantly different ( $P > 0.05$ ).

<sup>c</sup> Values preceded by the ">" sign represent reductions to levels lower than the detectable limit of the counting method (0.8 log CFU/g).

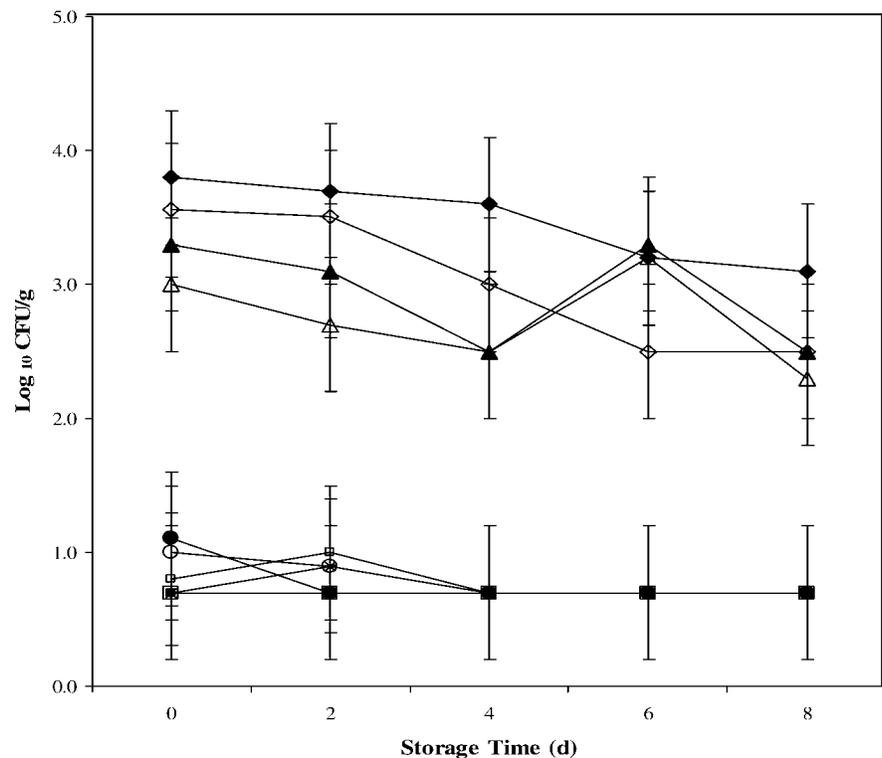
the spinach packets and placed alongside the spinach samples. Dosimeters were not placed inside the sealed samples due to the presence of pathogens. After irradiation, each sample was mixed with 90 ml of sterile 0.1% peptone water in stomacher bags and pummeled for 1 min. Aliquots of the homogenate were serially diluted 10-fold, and then spread plated onto TSA plus 100 mg/liter of rifampin. Counts of *E. coli* O157:H7 were calculated as CFU per gram. Rifampin resistance was confirmed by streaking TSB cultures onto plates of TSA plus 100 mg/liter of rifampin and incubated at 35°C for 24 h.

**Data analysis.** Colony counts were calculated as CFU per gram and converted to log values for data analysis. Estimated log reductions (ELR) were determined by subtracting the log count for the corresponding treatment from the log count on control spinach samples. The effect of radiation dose on the reduction of pathogens and their survival in the product during storage, and on the respiration rates of spinach samples were determined by the ANOVA procedures of SPSS (SPSS, Inc., Chicago, IL). For the determination of D-value, the log counts of surviving *E. coli* O157:H7 were plotted against increasing doses of radiation and analyzed by linear regression. The D-value was determined from the reciprocal of the slope of the regression line as the dose in kiloGrays required to reduce the population of *E. coli* O157:H7 by 1 log. The confidence interval was calculated for the death curve, using Excel 2007 (Microsoft Corp., Redmond, Wash.).

## RESULTS AND DISCUSSION

The populations of *E. coli* O157:H7 and *Salmonella* inoculated on baby spinach decreased significantly after e-beam radiation. The counts of both pathogens were inversely proportional to the dose of energy applied. Doses above 1.16 kGy reduced *E. coli* O157:H7 and *Salmonella* numbers near or below the detection limit of 0.8 log CFU/g (Fig. 1). The ELR for *E. coli* O157:H7 and *Salmonella* are shown in Table 1. When the pathogens were inoculated on the spinach at levels of ca. 7.0 log CFU/ml, doses of 0.4, 0.79, 1.07, and 1.16 kGy resulted in ELR of 3.7, 4.1, 6.3, and 6.3 log CFU/g, respectively, for *E. coli* O157:H7,

FIGURE 2. Survival of *E. coli* O157:H7 and *Salmonella* stored at 4°C for 8 days exposed to 0.4- ( $\diamond$ ), 0.79- ( $\Delta$ ), 1.07- ( $\circ$ ), 1.16- ( $\square$ ), 2.04- (\*), or 2.48- ( $\square$ ) kGy e-beam radiation. Open data points denote *Salmonella*, and solid data points denote *E. coli* O157:H7. Error bars reflect standard deviations of the means obtained from triplicate samples.



whereas a reduction of this pathogen to undetectable levels was observed at doses of 2.04 and 2.49 kGy. For *Salmonella*, e-beam doses of 0.4, 0.79, and 1.07 kGy produced ELR of 3.4, 4.0, and 6.1 log CFU/g, respectively, whereas doses of 1.16, 2.04, and 2.49 kGy reduced the microbial number to an undetectable level, with an ELR greater than 6.5 log (Table 1).

During refrigerated storage (Fig. 2), counts of *E. coli* O157:H7 and *Salmonella* irradiated at 0.4 kGy both remained constant for the first 2 days of storage, and then declined to 2.5 and 3.1 CFU/g, respectively, by day 8. *E. coli* O157:H7 and *Salmonella* irradiated at 0.79 kGy were reduced to 3.0 and 3.3 CFU/g on day 0 and decreased to 2.3 and 2.5 CFU/g by day 8. Both *E. coli* O157:H7 and *Salmonella* irradiated at 1.07 kGy had counts of 1.0 CFU/g on day 0 but fell below the detection limit by day 2. Similarly, both pathogens showed countable colonies through day 4 at 1.16 kGy (1 and 0.9 CFU/g, respectively); however, counts decreased to undetectable levels by day 4. *E. coli* O157:H7 irradiated at 2.04 kGy had 1 CFU on day 2, and then fell below the detection limit. *Salmonella* was not detectable at 2.04 kGy. Neither *E. coli* O157:H7 nor *Salmonella* yielded detectable counts after irradiation at 2.48 kGy. When no pathogens were detected on plates, additional enrichment plating was conducted to verify total destruction. *E. coli* O157:H7 was not recoverable after enrichment at doses above 1 kGy; however, when the spinach was irradiated at doses of 1.07 and 1.16 kGy, *Salmonella* was consistently recovered after enrichment over the 8 days of storage. No *Salmonella* was recoverable after enrichment when the spinach was treated at doses of 2.04 kGy.

In this study, we found that e-beam radiation at 1.16 kGy was successful in reducing *E. coli* O157:H7 and *Salmonella* on baby spinach from counts of 7.1 to 7.3 log

CFU/g to levels at or below the detection limit of the counting method (0.8 log CFU/g). This finding is consistent with that of Lee et al. (15), who reported that low-dose radiation was effective in eliminating pathogens inoculated in ready-to-eat vegetables. According to these authors, gamma radiation at doses of 1 kGy resulted in a 4-log reduction of *E. coli* inoculated onto seasoned spinach. Foley et al. (10) showed that chlorination and irradiation at 0.55 kGy could achieve a 5.4-log reduction in *E. coli* O157:H7 in inoculated shredded lettuce. Goularte et al. (12) showed that irradiation at 0.7 kGy could achieve a 4-log reduction in *Salmonella* and a 6.8-log reduction in *E. coli* O157:H7 in inoculated shredded lettuce. By enriching samples of which no colonies were detected on the count plates, we were not able to detect any *E. coli* O157:H7, while *Salmonella* was detected in samples with undetectable counts after enrichment when the dose was 1.16 kGy, indicating that few surviving salmonellae were still present. This is consistent with previous information indicating that *Salmonella* may be more resistant to radiation than is *E. coli* O157:H7 (18). Foley et al. (10) also reported that *E. coli* O157:H7 decreased during storage and were undetectable after 7 days.

When using irradiation, the appropriate dose must be determined to reduce the risk of foodborne illness and destroy the entire population of pathogens on a food commodity. In our study, a *D*-value of 0.2 kGy ( $\pm 0.01$ ) was obtained for *E. coli* O157:H7 in baby spinach (Fig. 3). While *D*-values differ based on moisture content and the matrix of a particular food item, Clavero et al. (9) reported a *D*-value in the range of 0.241 to 0.307 kGy for multiple strains of *E. coli* O157:H7 tested in combination on ground beef. Goularte et al. (12) reported *D*-values ranging from 0.11 to 0.12 kGy for *E. coli* O157:H7. Niemira et al. (19) reported similar *D*-values for *E. coli* O157:H7 on different

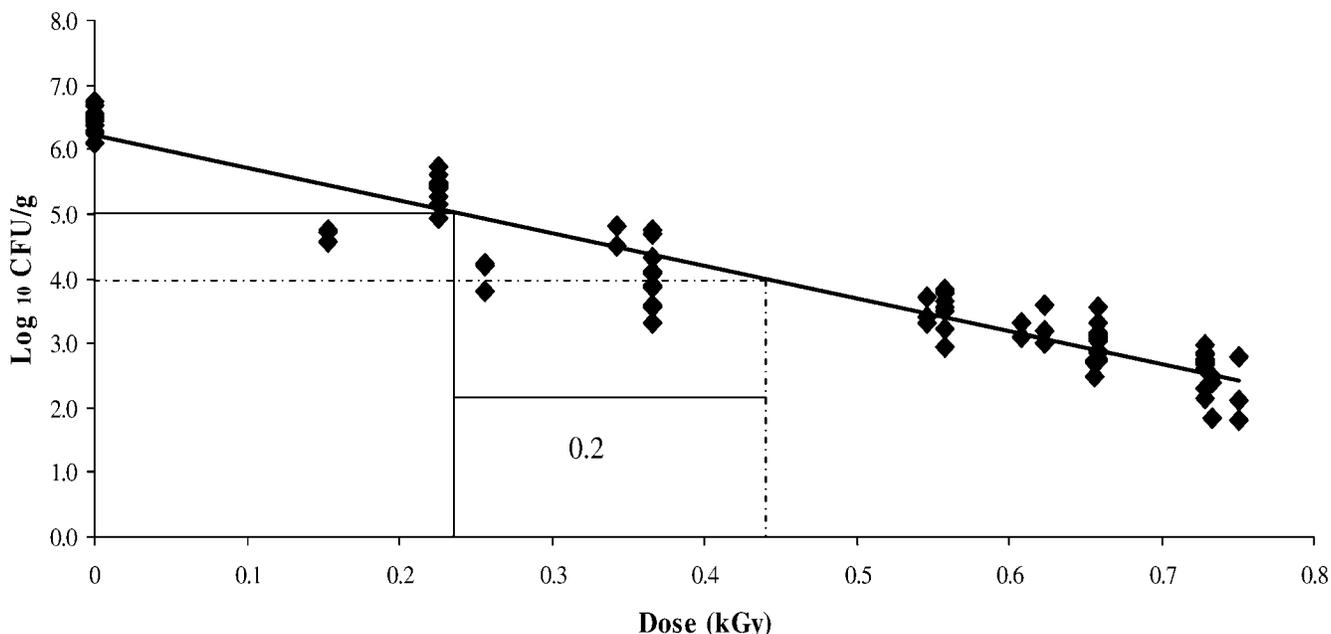


FIGURE 3. Regression line for *E. coli* O157:H7 counts on spinach leaves treated with increasing doses of radiation. Data points represent the average of triplicate samples. Lines drawn serve the purpose of illustrating the *D*-value but not for the calculation.

types of lettuce. *Salmonella* was not included in this phase of the study, since each strain used in this research corresponded to a different serotype, which may account for variations in the results. However, leafy greens are more frequently associated with *E. coli* O157:H7 disease than with salmonellosis (6–8). Microorganisms’ sensitivity to radiation differs, and certain *Salmonella* may have a higher *D*-value range than *E. coli* O157:H7 (18). Prakash et al. (22) reported a *D*-value range of 0.26 to 0.39 kGy for *Salmonella* spp. inoculated onto irradiated diced tomatoes. Niemira et al. (19) found *D*-values ranging from 0.35 to 0.71 kGy for different *Salmonella* tested in orange juice, indicating that a 5-log CFU/g reduction in *Salmonella* would require a dose of 1.3 to 1.95 kGy. E-beam radiation doses of 1.2 and 2.1 kGy had a limited impact on the respiration rates to radiation levels (Fig. 4). These results are in agreement with Foley et al. (10), who found that irradiation at 0.55 kGy did not cause adverse effects on sensory attributes. There were no obvious visual quality differences between irradiated samples and controls.

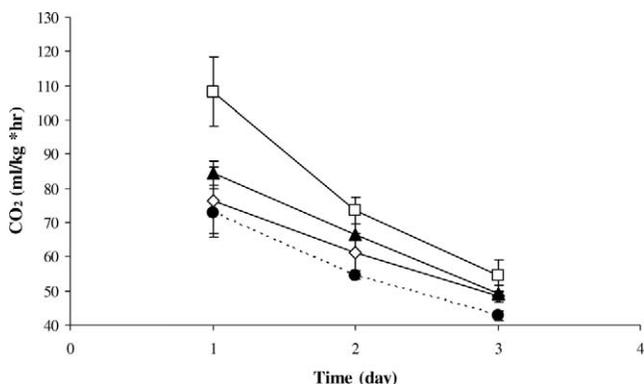


FIGURE 4. Respiration rates of spinach exposed to 0- (●), 1.2- (◇), 2.1- (▲) and 3.2- (□) kGy e-beam radiation.

Our results indicate that the use of e-beam radiation can reduce the risk of pathogenic bacteria in fresh baby spinach. Low doses of radiation (1.16 kGy) will effectively reduce *E. coli* O157:H7 and *Salmonella* by at least 6 log. Further research will be conducted to investigate in more detail the effects of e-beam radiation on sensory attributes.

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