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THE EFFECTS OF ELECTRON BEAM IRRADIATION AND SANITIZERS IN THE REDUCTION OF PATHOGENS AND ATTACHMENT PREVENTION ON SPINACH

A Dissertation

by

JACK ADAIR NEAL, JR.

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Major Subject: Food Science and Technology

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Approved by:

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May 2009

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ABSTRACT

The Effects of Electron Beam Irradiation and Sanitizers in the Reduction of Pathogens and Attachment Prevention on Spinach. (May 2009)

Jack Adair Neal, Jr., B.S., University of Houston;

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The effects of electron beam (e-beam) irradiation and sanitizers in the reduction of *Escherichia coli* O157:H7 and *Salmonella* counts and attachment prevention on spinach was studied. Survival of these pathogens in spinach was observed at multiple times and temperatures. Inoculated spinach was examined by confocal microscopy to determine attachment sites and internalization of these pathogens. To determine the effectiveness of sanitizers in reducing pathogen numbers, inoculated spinach was treated with L-lactic acid, peroxyacetic acid, calcium hypochlorite, ozone, and chlorine dioxide. Inoculated spinach was exposed to e-beam irradiation and tested for counts of both pathogens immediately after irradiation treatment to determine bacterial reduction, and at 2 day intervals over 8 days to determine effects of ionizing irradiation on pathogen survival. Respiration rates were measured on spinach exposed to e-beam. The effectiveness of e-beam irradiation on the microbiological and sensory characteristics of spinach was studied. For spinach samples stored at 4°C and 10°C for 8 days, *E. coli* O157:H7 survived and grew significantly in samples stored at 21°C for 24 h. Confocal

microscopy images provided valuable information on the attachment sites and internalization of the pathogens on spinach. The greatest reduction by a chemical sanitizer was 55°C L-lactic acid with a 2.7 log CFU/g reduction for *E. coli* O157:H7 and 2.3 log CFU/g reduction for *Salmonella*. Each dose of e-beam irradiation significantly reduced populations of both pathogens. Respiration rates of spinach increased as irradiation treatment doses increased. Total aerobic plate counts were reduced by 2.6 and 3.2 log CFU/g at 0.7 and 1.4 kGy, respectively. Lactic acid bacteria were reduced at both doses but grew slowly over the 35 day period. Yeasts and molds were not reduced in samples exposed to 0.7 kGy whereas 1.4 kGy had significantly reduced counts. Gas compositions for samples receiving 0.7 and 1.4 kGy were significantly different than controls. Irradiation did not affect the objective color or basic taste, aromatic or mouthfeel attributes of spinach. These results suggest that low dose e-beam irradiation may be a viable tool for reducing microbial populations or eliminating *E. coli* O157:H7 and *Salmonella* from spinach with minimal product damage.

DEDICATION

To my wife, Shaelyn, for all of her encouragement, prayers, support and love, and especially for giving me the freedom to pursue my dream, and to our wonderful children Micheala, Jackson, John Riley and Selah for all of their love and understanding. To the memory of John and Myr Roche for their love and support.

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TABLE OF CONTENTS

Page

ABSTRACT iii

DEDICATION v

ACKNOWLEDGEMENTS vi

TABLE OF CONTENTS vii

LIST OF FIGURES xi

LIST OF TABLES xiii

INTRODUCTION 1

REVIEW OF LITERATURE 4

- Factors contributing to foodborne disease outbreaks associated with produce 4
  - Consumption of spinach in the U.S. 6
  - Outbreaks of foodborne disease associated with leafy greens and spinach 7
  - Preharvest and postharvest sources of pathogens 8
  - Survival and growth of pathogens on leafy green vegetables 11
- Microbial attachment on produce 12
  - Factors that influence attachment 14
  - Cell surface charge 15
  - Hydrophobicity 17
  - Bacterial structures 18
  - Plant surfaces 20
  - Biofilms 21
  - Internalization 22
- Microscopy 24
  - Confocal scanning laser microscopy (CSLM) 24
  - Scanning electron microscopy (SEM) 26
- Decontamination methods for fresh produce 28
  - Chlorine (Hypochlorite) 29
  - Chlorine dioxide (ClO_2) 31
  - Organic acids 32
  - Peracetic acid 34
  - Ozone 35

Page

Irradiation of food 37
Types of ionizing radiation 38
Dosimetry 39
Mechanism of ionizing radiation 41
Food composition and microbial resistance to irradiation 42
Irradiation of produce 43
Microbial, sensorial, and chemical quality of irradiated produce 43
Pathogen reduction of irradiated produce 46

MATERIALS AND METHODS 50

Microorganisms 50
Media preparation 52
Preliminary experiments 53
Growth curves 53
Acid sensitivity 54
Irradiation resistance 54
Inoculation method 55
Optimum packaging material for spinach leaves 55
Growth and survival of *E. coli* O157:H7 on spinach leaves 56
Preparation of inoculum 56
Product obtainment and inoculation 56
Survival studies 57
Bacterial enumeration 57
Use of CSLM to identify the attachment sites for *E. coli* O157:H7 and *Salmonella* on the leaf and stem of spinach 58
Preparation of inoculum and sample preparation 58
Attachment 58
Confocal microscopy 59
Effectiveness of chemical sanitizers on the reduction of *E. coli* O157:H7 and *Salmonella* inoculated on spinach 59
Preparation of inoculum 59
Preparation of spinach leaves 59
Application of treatments 60
Microbiological analysis 62
Efficacy of e-beam irradiation for the reduction of *E. coli* O157:H7 and *Salmonella* on spinach 64
Inoculum preparation 64
Product obtainment and inoculation 64
Irradiation 65
Bacterial enumeration 65

Page

D_{10}-value for *E. coli* O157:H7 on irradiated spinach leaves 66
Microorganisms 66
Dosimetry 66
Bacterial enumeration 67
Microbiological and sensory characteristics of spinach 67
Sample preparation 67
Irradiation 67
Microbial quality 68
Respiration rates 69
Gas composition 69
Sensory evaluation 69
Data analysis 73

RESULTS AND DISCUSSION 76

Preliminary studies 76
Growth curves 76
Acid sensitivity 76
Irradiation resistance 80
Inoculation method 80
Optimum packaging material for spinach leaves 82
Growth and survival of *E. coli* O157:H7 on spinach 82
Growth and survival 82
Use of CSLM to identify the attachment sites for *E. coli* O157:H7 and *Salmonella* on the leaf and stem of spinach 88
Effectiveness of chemical sanitizers on the reduction of *E. coli* O157:H7 and *Salmonella* inoculated on spinach 92
Lactic acid 94
Peroxyacetic acid 95
Chlorine 96
Ozone 98
Chlorine gas 99
Efficacy of e-beam irradiation for the reduction of *E. coli* O157:H7 and *Salmonella* on spinach 101
D_{10}-value for *E. coli* O157:H7 on irradiated spinach 106
Apparent D-values 108
Microbiological and sensory characteristics of spinach 109
Aerobic plate count (APC) 109
Lactic acid bacteria (LAB) 113
Yeasts and molds counts 116
Respiration rate and gas composition 118

Page

Sensory evaluation and objective color... 126
Quality changes 135

CONCLUSIONS 139

REFERENCES 141

VITA 174

LIST OF FIGURES

FIGURE | Page

1 Growth curves for *E. coli* O15:H7 cultures grown at 37°C in TSB 77

2 Growth curves for rifampicin-resistant *E. coli* O15:H7 cultures grown at 37°C in TSB 78

3 Gas composition of stomacher and cryovac vegetable storage film containing different amounts of spinach after storage at 4°C for 8 days 83

4 Survival and growth of *E. coli* O157:H7 on spinach leaves stored at 21°C for 24 h 84

5 Survival and growth of *E. coli* O157:H7 stored at 10°C on spinach leaves for 7 days 85

6 Survival and growth of E. coli O157:H7 on spinach stored at 4°C for 7 days 86

7 Confocal scanning laser microscopy (CSLM) photomicrographs of spinach leaves inoculated with GFP-expressing *E. coli* O157:H7 and RFP- expressing *Salmonella* 89

8 Confocal scanning laser microscopy (CSLM) photomicrographs showing GFP-expressing *E. coli* O157:H7 and RFP-expressing *Salmonella* in the interior of spinach stems 90

9 Effects of irradiation dose on the populations of *E. coli* O157:H7 (white) and *Salmonella* (grey) after e-beam irradiation 103

10 Survival of *E. coli* O157:H7 and *Salmonella* stored at 4°C for 8 days exposed to 0.4, 0.79, 1.07, 1.16, 2.04 or 2.48 kGy e-beam irradiation 104

11 Regression line for *E. coli* O157:H7 counts on spinach leaves treated with increasing doses of irradiation 107

12 Aerobic plate counts of spinach leaves over 35 days of storage at 4°C after e-beam irradiation 111

FIGURE Page

13 Growth of lactic acid bacteria (LAB) on irradiated spinach leaves stored at 4°C over 35 days 114

14 Growth of yeasts and molds on irradiated spinach leaves stored at 4°C over 35 days 117

15 Respiration rates of spinach leaves exposed to 0, 1.2, 2.1 and 3.2 kGy e-beam irradiation 119

16 Percentage of CO_2 and O_2 levels in spinach samples treated with 0.0 kGy e-beam irradiation 122

17 Percentage of CO_2 and O_2 levels in spinach samples treated with 0.7 kGy e-beam irradiation 123

18 Percentage of CO_2 and O_2 levels in spinach samples treated with 1.4 kGy e-beam irradiation 124

19 Mean O_2 concentrations as affected by interactions between storage days and 0 kGy, 0.7 kGy, and 1.4 kGy 127

20 Mean CO_2 concentrations as affected by interactions between storage days and 0 kGy 0.7 kGy, and 1.4 kGy 128

21 Diagram summarizing the effects of e-beam irradiation and storage on the microbial quality, gas composition and sensory quality of spinach samples 136

LIST OF TABLES

TABLE Page

1 Texture, basic tastes, aromatics, and mouthfeel attribute definitions, references and intensity based on a 15-point scale established after consensus 71

2 Counts (log CFU/ml) of *E. coli* O157:H7 and their rif-resistant derivatives on Tryptic Soy Agar (TSA) at different pH values 79

3 Estimated log reductions (ELR) (log_{10} CFU/g) for 5 strains of *E. coli* O157:H7 and their rif-resistant derivatives in gelatin discs as affected by e-beam irradiation 81

4 Estimated log reductions (ELR) (log_{10} CFU/g) in *E. coli* O157:H7 and *Salmonella* inoculated onto fresh spinach leaves 93

5 Estimated log reductions (ELR) (log_{10} CFU/g) for *E. coli* O157:H7 and *Salmonella* inoculated onto fresh baby spinach leaves as affected by dose of e-beam irradiation 102

6 Survival of APC, LAB and yeasts and molds on fresh spinach leaves as affected by dose of e-beam irradiation immediately after treatment 110

7 Means for gas composition in fresh bagged spinach as affected by treatment dose, storage days and treatment by storage days 121

8 Means for gas composition in fresh bagged spinach as affected by treatment dose, storage days and treatment by storage days 129

9 Least squares means for fresh spinach for main effects for trained sensory descriptive attribute 131

10 Least squares means for fresh spinach for main effects for trained sensory descriptive attributes 132

INTRODUCTION

The number of reported foodborne illness outbreaks associated with fresh produce has increased in the past thirty years. This increase can be attributed not only to changes in consumption patterns but also changes in production and processing technologies, new sources of produce as well as the manifestation of pathogens that have not been previously associated with raw produce. Fruit and vegetable growers and packers use chemical decontamination methods as interventions which are effective in reducing counts of bacteria as well as yeasts and molds that cause spoilage; however, chemical decontaminates are only successful when they come into direct contact with the microorganisms. There are several factors and circumstances in which microorganisms including pathogens, such as *E. coli* O157:H7 and *Salmonella,* can survive chemical decontamination treatments. These circumstances may include irregular surfaces of fruits and vegetables which may have locations where the decontaminants cannot reach the pathogens, or the ability of certain fruits and vegetables to internalize pathogens via contaminated irrigation water, or leafy greens sticking together during washing procedures. These limitations have lead researchers to investigate alternative intervention technologies that are more successful in reducing or eliminating pathogenic microorganisms from fruits and vegetables. Spinach has become

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This dissertation follows the style of the *Journal of Food Protection.*

a concern because of its involvement as the vehicle of *E. coli* O157:H7 in the 2006 multistate outbreak originating in California. In an effort to reduce the number of foodborne illnesses associated with fresh produce, continued 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.

Ionizing irradiation is one possibility that has had extensive research over the past 100 years and has been applied to foodstuffs for decades. Ionizing radiation kills microorganisms by causing irreparable damage to cell biomolecules. Electron beam (e-beam) technology uses high-energy electrons to destroy pathogens. While not being a novel concept, this technology may now be utilized more frequently and help prevent the numerous outbreaks associated with produce and other commodities. Recently, the FDA approved the use of ionizing radiation for the control of foodborne pathogens and extension of shelf-life of fresh iceberg lettuce and fresh spinach. This approval offers great opportunities for research on the development of irradiation treatments for lettuce and spinach.

The goals of the present investigation were: (a) to determine the growth and survival of *E. coli* O157:H7 on spinach leaves; (b) to observe attachment for *E. coli* O157:H7 and *Salmonella* on the leaf and stem of spinach using confocal microscopy; (c) to determine the effectiveness of chemical sanitizers on the reduction of inoculated *E. coli* O157:H7 and *Salmonella* counts on spinach; (d) to study the effects of e-beam

irradiation on the reduction of inoculated *E. coli* O157:H7 and *Salmonella* counts on spinach; (e) to determine the D_{10} value for *E. coli* O157:H7 on spinach irradiated with e-beam; and (f) to determine the effectiveness of low dose e-beam irradiation on the microbiological and sensory characteristics of spinach during refrigerated storage.

REVIEW OF LITERATURE

Factors contributing to foodborne disease outbreaks associated with produce

There has been an increase in the number of foodborne disease outbreaks associated with fresh produce in the past thirty years *(7, 152, 295)*. Researchers have attributed this increase to several factors including changes in dietary habits such as increases in the amount of fresh produce consumed as well as the number of meals eaten away from the home *(7, 28, 32, 45, 55, 57, 89, 152, 154, 202, 237, 295, 332)*. With the encouragement of the United States government, as well as independent nutrition and health authorities, Americans are eating more fresh produce which therefore increases the risk of exposure to fruits and vegetables that may have been contaminated with pathogenic microorganisms *(32, 55, 57, 89, 295, 321)*. However, based on consumption patterns, the number of outbreaks reported is increasing faster than increases in consumption. Consumption of leafy greens in the U.S. increased 17.2% during the years 1986-1995 compared to the previous decade; however, the amount of all foodborne disease outbreaks associated with leafy greens increased 59.6% *(154)*. Similarly, from 1996-2005 consumption of leafy greens rose 9.0% and foodborne disease outbreaks associated with leafy greens increased 38.6% *(154)*. Therefore, while the proportion of foodborne disease due to leafy greens has increased, it cannot be attributed solely to increases in leafy green consumption *(154)*. In addition, contamination of produce by food service workers has been a significant factor in foodborne illnesses associated with the consumption of fresh produce *(89)*. This may be attributable to the amount of direct

hand contact during the preparation of meals, poor personal hygiene, as well as the lack of application of any treatment meant to reduce or eliminate pathogens *(89)*. Other suggestions for the increase in produce associated outbreaks include new production and processing technologies, intensification and centralization of production, and more sources of produce *(13, 57, 62)*. In addition, increases in the size of at-risk population, enhanced epidemiological surveillance, improved methods of identifying and tracking pathogens as well as the manifestation of pathogens previously not associated with raw produce have also contributed to the increases in produce associated outbreaks *(321, 332)*. With the advent of bagged salads and leafy greens, centralized processing from multiple farms can now facilitate washing, typically with wash flumes, processing and mixing, and packaging of ready-to-eat salads. This creates a longer food chain potentially increasing the growth of pathogens and their distribution to more people in more geographically disperse areas *(62, 89)*. To compensate for the increase in consumption of fresh produce, imported produce is now more common and provides a year-round supply to the U.S.; however, this potentially exposes consumers to more risk of foodborne illness should a single grower or processor distribute their product nationwide *(28, 89, 152)*. Improvements in healthcare have contributed to an increasing proportion of the elderly as well as immunocompromised *(89)*. Improved technology and communication systems such as FoodNet (which uses active surveillance for identifying and tracking pathogens) have given better information regarding the actual number of annual foodborne illnesses. Examples of produce related outbreaks where the etiological agent was determined include: raspberries, basil, lettuce, alfalfa sprouts, radish sprouts,

carrots, salad vegetables, cabbage, tomatoes, watermelon, cantaloupe, green onions, parsley, and spinach *(57)*.

Consumption of spinach in the U.S. The benefits of a diet rich in fruits and vegetables and their contribution to good health and healthy eating has been conveyed and encouraged by the U.S. Department of Agriculture (USDA), the Centers for Disease Control and Prevention (CDC), the National Cancer Institute (NCI) as well as the healthcare community and numerous other organizations. Native to Iran, spinach (*Spinacia oleracea)* is a leafy green vegetable, cultivated during the Greek and Roman civilizations and first appeared in American seed catalogs in 1806 *(248)*. It is a member of the Chenopodiaceae family along with Swiss chard, and sugar beets. Spinach is well recognized as a good source of nutrients which include high levels of vitamin C, lutein, iron, folic acid and magnesium *(332)*. Savoy (wrinkled), semi-savoy and smooth leaf are the three types of spinach produced in the U.S. with savoy and semi-savoy being the most popular for bagged salads *(332)*. Production in the U.S. occurs mainly in California (64%), Texas (15%) and Arizona (10%) with 16 other states reporting production of over 100 acres *(201, 332)*. The per capita consumption of all spinach has risen 66% between 2000-2002 with the consumption of fresh spinach averaging 1.4 lb per person *(201, 334)*. The majority (80%) of fresh spinach consumed in the U.S. is purchased in retail stores and prepared in the home with only 14% of fresh spinach being consumed in full service restaurants *(62)*. Geographically, the Northeast consumes the most fresh spinach (2.04 lb per capita) followed by the West (1.9 lb), with both the South (1.16 lb) and the Midwest (1.08 lb) consuming below the national average (1.49) *(62)*.

Demographically, women consume more spinach than men, and men and women over the age of 60 consume more fresh spinach than any other age group *(62)*. Recent trends toward healthier diets have lead to the increase in demand for a variety of fresh vegetables however, due to busier lifestyles, consumers prefer convenient packaging. Washed, packaged fresh baby spinach has increased in popularity over the past decade and is one of the fastest growing segments of the packaged salad industry *(201)*. Spinach is often categorized as "baby" or "teen" although there are no regulatory standards for the terms, and the difference may be based on leaf size rather than the age of the spinach at the time of harvesting *(62)*. The explosive demand for bagged salads has lead to many challenges for the produce industry to improve the quality, extend the shelf life and ensure the safety of the product.

Outbreaks of foodborne disease associated with leafy greens and spinach. Associated to a culmination of factors listed above, the incidence of foodborne disease outbreaks linked to fresh produce has increased. Epidemiologically, there are different bacteria, viruses and protozoan implicated with fresh produce associated disease. Where the etiological identity was known, most outbreaks associated with fruits and vegetables were of bacterial origin and *Salmonella* was the most commonly reported pathogen *(89)*. For outbreaks associated specifically with leafy greens, between 1973 and 2006 there have been approximately 502 (4.8%) outbreaks, 18,242 (6.5%) illnesses, and 15 (4.0%) deaths and *E. coli* O157:H7 has been the most frequently reported pathogen *(73, 74, 75, 154, 202)*. In 1995, two separate foodborne disease outbreaks associated with *E. coli* O157:H7 and leaf lettuce occurred; 29 people in Montana and 30 people attending a Boy

Scout convention in Maine were affected *(71, 72)*. A multistate outbreak of *E. coli* O157:H7 associated with mesclun lettuce was reported in 1996 with 21 cases in Connecticut and 28 reported cases in Illinois *(156)*. In 2003 there was an outbreak (16 cases) of *E. coli* O157:H7 implicated with the consumption of spinach in a retirement community in Portola Valley, California *(267)*. During this outbreak, 10 patients were hospitalized, 3 developed a type of renal failure known as hemolytic uremic syndrome (HUS) and 2 died *(75)*. In 2006 there was a multi-state outbreak (71 cases) of *E. coli* O157:H7 related to the consumption of iceberg lettuce from a national quick service restaurant chain where 53 consumers were hospitalized and 8 developed HUS *(76)*.

Recently spinach has become a concern because of its involvement as the vehicle of *E. coli* O157:H7 in the large 2006 multistate outbreak originating in California. Twenty-six states were involved in the outbreak with highest concentrations in Ohio, Utah and Wisconsin followed by New York *(62)*. This outbreak resulted in 205 confirmed cases and 3 deaths. Thirty one of the 103 hospitalized case patients developed HUS *(62)*. By conducting tracebacks of spinach product codes from the bagged spinach obtained from the ill consumers, investigators were able to identify the potential sources of field contamination which identified 4 fields in the California counties of Monterey and San Bonito *(62)*.

Preharvest and postharvest sources of pathogens. Spinach can be contaminated with pathogens anywhere along the preharvest and postharvest route *(28, 55, 57, 89)*. Potential preharvest sources include irrigation water, water used to apply insecticides and fungicides, manure, feces, soil, wild and domestic animals including birds, reptile,

insects, and mammals including humans *(28, 55, 57, 89)*. Water quality plays an important role in the extent of contamination caused by irrigation and due to the large amount of surface areas, leafy greens such as spinach generally obtain the greatest amount of contamination *(89)*. Direct contact methods of irrigation such as spraying increases contamination as compared to either drip irrigation or flood irrigation *(89)*. Irrigation water may become contaminated either through direct contact or indirectly as in the case of drainage of rain water from animal pens *(89)*. Feces may contaminate the fields either through water runoff, the use of improperly composted manure or from free roaming wild or domestic animals *(45, 89)*. While certainly not a novel process, there has been a recent trend in consumer preference toward natural or organically cultivated fruits and vegetables. This preference has lead to an increase in the use of manure rather than chemical fertilizers. Improperly treated manure may contain enteric pathogens such as *Salmonella, Shigella* and *E. coli* O157:H7 which may serve as the source of contamination on produce *(89)*. Spinach grows close to the ground making it vulnerable to contamination from the soil, water or animal waste such as feces. In the 2006 spinach outbreak environmental samples containing *E. coli* O157:H7 were found in cattle feces, wild pig feces, river water and soil samples *(62)*. *E. coli* O157:H7 can remain viable in bovine feces for up to 70 days *(346)*. In addition, the lack of proper sanitary facilities and poor personal hygiene of field workers may also be a source of pathogens to preharvest produce *(45, 89)*.

Postharvest factors include the use of water or ice, handling by field workers, animals such as birds or rodents in the packing sheds, the potential contamination from

equipment or containers and transporting vehicles, as well as non-optimal processing methods, cross-contamination, and temperature abuse during storage, packaging and displaying *(68, 89)*. Microbiologically, processing creates several challenges: the process has the potential to cause damage such as cuts or bruises to spinach or other vegetables which then supply nutrients to microorganisms; the process does not include a lethality step minimizing or eliminating microorganisms; and, the bagging process may comingle products from multiple locations increasing the risk for contamination *(55, 359)*. Even if no cutting is involved, the quality of minimally processed produce is influenced by handling and may be more susceptible to contamination than intact fruits or vegetables *(89, 349)*. Despite the use of proper hygiene, Good Agricultural Practices (GAPs), and Good Manufacturing Practices (GMPs), under specific conditions, contamination of fresh produce may occur at any point along the farm to table continuum (*23*). In an effort to improve the safety of produce, the U.S. Department of Health and Human Services, U.S. Food and Drug Administration (FDA), and the Center for Food Safety and Applied Nutrition (CFSAN) published voluntary guidelines in 1998 entitled *Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables (329).* The primary purpose of this guide was to provide a structure for the identification and implementation of practices that would reduce the risk of pathogenic microbiological contamination of produce, based on GAPs and GMPs. Studies have been conducted to identify critical points in preharvest conditions as well as production and post harvesting *(69, 100, 128, 129, 168, 169, 225, 226, 258, 283)*. GAPs include water, the use of manure and municipal biosolids, worker health and hygiene, sanitary

facilities including toilets and hand washing stations, field sanitation, packing facility sanitation and transportation. Critical points associated with GMPs focus on the personnel working at a facility including hygiene as well as education and training, the building facilities, equipment, production and process controls, and policies and procedures for defects including action levels. Johnston et al. *(173)* monitored the microbiological quality of fresh produce from the field through the packing process as well as evaluated the prevalence of *Listeria monocytogenes, E. coli* O157:H7, and *Salmonella* on fresh produce**.** The total aerobic bacteria ranged from a geometric mean of 4.5 to 6.6 log CFU/g. *Enterococcus* levels ranged from 1.3 to 4.3 log CFU/g with cantaloupe and mustard greens having the highest levels and the geometric mean total coliform counts ranged from 1.0 to 3.4 log CFU/g. The overall geometric mean *E. coli* counts were low for most produce items (<1.0 log CFU/g) and highest for cantaloupe (1.5 CFU/g). *L. monocytogenes* and *E. coli* O157:H7 were not detected in any of the 398 produce items tested. However, *S.* Montevideo was detected on 3 cantaloupe samples, resulting in a prevalence of 0.8% for all produce items and 3.3% for cantaloupe alone.

Survival and growth of pathogens on leafy green vegetables. Bacteria found on and internalized in fruits and vegetables can survive for long periods of time, and given the proper conditions, can grow *(3, 34, 140, 165, 195, 351)*. Solomon et al. *(304)* reported that *E. coli* O157:H7 could survive on lettuce plants for 20 days following spray irrigation with contaminated water. Several of these microorganisms are psychrotrophs capable of growing at refrigerated temperatures which has significant implications because produce is often imported to the U.S. from overseas creating relatively longer

storage times which may promote the growth of psychrotrophic pathogens such as *L. monocytogenes* or *Aeromonas* spp. *(24, 70, 160, 308)*. Farber et al. *(111)* reported that *L. monocytogenes* population levels remained constant on a variety of leafy greens stored at 4°C for 9 days. Francis and O'Beirne *(124)* demonstrated that *L. innocua* and *L. monocytogenes* populations stored at 3°C for 14 days would gradually decrease by 1-1.5 log cycles. Steinbruegge et al. *(308)* reported that *L. monocytogenes* grew on lettuce stored at 5°C and 10°C producing 10^6 CFU/g after 14 days of storage at both temperatures. Jacxsens et al. *(170)* described the behavior of *L. monocytogenes* and *Aeromonas* spp. on fresh-cut produce packaged under equilibrium-modified atmosphere. Kakiomenou et al. *(175)* reported on the survival of *Salmonella* and *L. monocytogenes* on salad vegetables. Other studies have established that *E. coli* O157:H7 can survive and/or grow on lettuce *(3, 92, 95, 195)*. Beuchat *(30)* described the survival of *E. coli* O157:H7 in bovine feces applied to lettuce leaves and viable cells were detected after 15 days even at inoculum levels of 10^0 to 10^1 CFU/g. Islam et al. *(164)* reported on the persistence of *E. coli* O157:H7 on leaf lettuce and parsley in fields contaminated by either manure or irrigation water. In a separate yet similar study, these same authors reported on the survival on *S.* Typhimurim on lettuce and parsley under similar contaminated growth conditions *(165)*.

Microbial attachment on produce

Bacteria can be introduced to fruits and vegetables at any step from planting to consumption and once they are introduced, their colonization can have a tremendous

effect on both the quality and safety of the product. As consumer demands for variety increases, many different types and fruits and vegetables are available with each possessing unique surface attributes contributing to the attachment and or infiltration capabilities of microorganisms. There are many forms of fruits and vegetables which include roots, stems, florets and leaves; all of which create unique ecological recesses for microorganisms. The attachment and colonization of microorganisms on fresh produce have significant public health implications due to the fact that these processes may be related to the inability of sanitizers and decontamination treatments to remove or inactivate human pathogens *(31, 126)*. Bacteria attach to fruits and vegetables in pores, indentations and natural irregularities on the produce surface where there are protective binding sites as well as cut surfaces, puncture wounds, and cracks in the surface *(286, 290)*. Damage may also occur as a result of insects, mechanical injury during post-harvesting handling and the formation of cracks occurring at weak areas on the surface which may entrap microorganisms protecting them from decontamination treatments *(57)*. Damaged plant cells may leak nutrients that would normally not be available to microorganisms on healthy plant surfaces promoting pathogen growth *(30)*.

The role of seeds as an agent in foodborne disease has been established. Several studies have reported that *E. coli* O157:H7 could contaminate lettuce through contaminated manure or irrigation water and reported that the seedlings most likely became contaminated as the seedlings grew and broke through pathogen inoculated soil *(164-166, 304)*. Solomon et al. *(305)* reported that *E. coli* O157:H7 was capable of entering the roots of mature lettuce plants and could be transported within the edible

portions of the plant. Decontamination treatments for seeds have been suggested however, several challenges exist including the fact that the treatment doses must inactivate the microorganism without negative affects to the seedling, treatments need to contact bacteria that may be located in protected seed tissue and the seeds themselves may inactivate certain treatments making them less effective *(55)*. Warriner et al. *(348)* described how spinach seedlings inoculated with non-pathogenic *E. coli* could be established both on the exterior and interior of the roots and further migrate into the vascular system and into edible leaves. Their results showed that in mature spinach, *E. coli* was restricted to only the roots and not the vascular system however, cross-contamination from the roots to the inner leaves could occur during harvesting. In addition to seedlings, roots and the plant's vascular system, fruits and vegetables can be contaminated directly on the surface. A number of factors may be involved with the adhesion of enteropathogenic bacteria on plant tissue including strategies that may be related to those utilized for attachment to animal and human epithelial cells or those used for pathogenic bacteria to attach to plants *(45, 277)*.

Factors that influence attachment. A microorganism's ability to survive, especially if the environment is low in nutrients, is based on its ability to attach to different surfaces *(155)*. Bacteria attach to plant surfaces by different mechanisms such as Ca^2 – binding proteins, lectin-mediated attachment, nitrogen fixing, virulence genes, bacterial surface polysaccharides, and accumulation and anchoring of the bacteria to the host surface *(12, 14, 15, 87, 88, 127, 137, 158, 210, 217, 262, 270, 271, 282, 296-300, 341, 342)*. Attachment is influenced by factors such as the cell's surface charge,

hydrophobic effects, bacterial structures, excreted extracellular polysaccharides, and the nearby environment *(97, 126, 212, 228, 277, 337)*. Detachment also plays an important role in the survival of microorganisms should they need to detach if the surrounding environment present unfavorable conditions *(126, 338)*. Conversely, detachment may not be possible due to physical entrapment of cells by the capillary action of the food tissue or if the cell has produced significant amounts of extracellular binding polymers known as glycocalyx *(126)*.

Cell surface charge. Bacterial cells exhibit a net negative charge on the cell wall similar to most plant surfaces. For bacteria to attach to a plant surface, it must first overcome the electrostatic forces through bridging divalent cations, ionic bridges between local positively charged groups on one surface, hydrogen bonding, and using van der Waals forces *(20, 276)*. In addition, hydrophobic interactions of chemical bonds (i.e. electrostatic, ion-dipole interactions) can contribute to the adhesion strength *(54)*. Van Loosdrecht et al. *(339)* suggested that when an organism approaches a surface from a far distance, the physicochemical attractions occur primarily and then will utilize specific interactions. Piette et al. *(250)* reported that the adhesion of *Pseudomonas fluorescens* increased as the ionic strength of the solution increased regardless of the cation present. Ions in solution reduce the thickness of the electrical diffuse double layer on each surface which allows negatively charged cells to move closer to a negatively charged surface so that van der Waals forces or bacterial surface appendages can overcome the electrostatic repulsion forces *(126)*. The Derjagium, Landau, Verway and Overbeek (DLVO) theory of colloid stability explains that a balance between van der

Waals forces and electrostatic forces (which are typically repulsive) results in a weak attachment at a distance called the secondary minimum (>10 nm) *(54)*. Van der Waals attraction forces and electrostatic forces are part of the initial weak interaction between the bacterial cell and the plant surface which are referred to as reversible adhesion. Bacteria that are reversibly attached to surfaces can be easily removed by fluid shear forces such as washing *(208)*. Bacterial surface charge can also play a role in bacterial interaction with solid surfaces *(196)*. The importance of electrostatic interactions in attachment can be related to eletrokinetic (zeta) potential of the attaching cell *(337)*. Dickson and Koohmaraie *(97)* stated that the magnitude of the bacterial cell surface charge is an important factor in attachment to meat surfaces. Dickson and Daniels *(96)* reported that motile *L. monocytogenes*, which has a greater surface charge than nonmotile cells, attaches more readily to glass. Hassan and Frank *(151)* investigated the effect of growth in Tryptic Soy Broth (TSB) and Nutrient Broth (NB) on the ability of *E. coli* O157:H7 to attach to lettuce and apple surfaces as well as determining the charge on cells grown in these media. These authors reported that similar zeta potential values of cells grown in TSB and NB indicated that electrostatic interactions were not responsible for differences in attachment ability of the cells grown in either media. Ukuku and Fett *(326)* reported that both negative and positive charges are correlated with the strength of attachment of *Salmonella, E. coli* O157:H7, and *L. monocytogenes* to cantaloupe rind. Hassan and Frank *(150)* found that the number of cells attached to lettuce leaf increased significantly in the presence of calcium ions which indicate that electrostatic interactions may play a more important role in attachment of *E. coli* O157:H7 to lettuce than to apple

surfaces. The presence of extracellular structures can influence a cell's physicochemical properties *(114)*.

Hydrophobicity. Hydrophobicity also plays a role in the attachment and colonization of bacteria to a plant surface. A plant surface consists of an epidermis, followed by a pectin layer and then multiple layers of a hydrophobic cuticle (1-15 µm thick) which provides the principal barrier against fungal invasion, insect and physical damage desiccation *(57, 278)*. The hydrophobic cuticle is composed of cutin which is made of high-molecular-weight lipid polyesters of long-chain substituted aliphatic (having carbon atoms linked in open chains) acids and within the cutin are crystalline and amorphous wax molecules which are responsible for the water repellant nature of plant surfaces *(57)*. Hydrophobic plant surfaces and hydrophobic regions of bacterial surface proteins pull cells closer together to facilitate further interactions *(20)*. Van Loosdrecht et al. *(338)* demonstrated that hydrophobic cells adhered to a greater amount to negatively charged sulfated polystyrene than hydrophilic cells by measuring the contact angle of water on a bacterial layer. Ukuku and Fett *(326)* demonstrated that *Salmonella* bound the strongest to the hydrophobic, intact surface of a cantaloupe as compared to *E. coli* O157:H7 and *L. monocytogenes.* Lindow *(198)* suggested that differences in hydrophobicity on leaves, as a result of varying patterns of wax structures and composition, could affect the locations that bacteria may select for attachment which may result in preferential colonization of the most hydrophobic sites by bacteria which have the most hydrophobic cell surface. In contrast, Fett *(114)* reported that there was not a clear correlation between hydrophobicity and adherence potential despite the fact

that he did find differences in hydrophobicity among bacterial species which occurred on leaf surfaces. Takeuchi et al. *(317)* evaluated the attachment of *E. coli* O157:H7, *L. monocytogenes, S.* Typhimurium, and *P. fluorescens* on iceberg lettuce by plate count and confocal scanning laser microscopy (CSLM). *E. coli* O157:H7 and *L. monocytogenes* have a nonhydrophobic surface *(94, 204, 317)*. Although both *E. coli* O157:H7 and *L. monocytogenes* preferentially attached to the cut edge rather than the surface, *L. monocytogenes* showed greater difference in attachment at two sites than did *E. coli* O157:H7. One possible reason offered by the authors was that *L. monocytogenes* may have a more hydrophilic surface than does *E. coli* O157:H7, favoring attachment to the cut edge *(203, 317)*. Both cell surface charge and hydrophobicity may contribute to attachment however, it may be difficult to predict the surface properties of pathogenic bacteria because of environmental conditions that can alter the surface properties of these bacteria when they are first exposed to a plant surface *(50, 94)*. Hassan and Frank *(151)* concluded that *E. coli* O157:H7 cells with greater hydrophobicity attached better to apples and lettuce. Boyer et al. *(41)* stated that curli increased the overall hydrophobicity of the *E. coli* O157:H7 cells but did not appear to have a strong influence on cell charge.

Bacterial structures. Many epiphytic bacteria are surrounded by layers of extracellular polysaccharide (EPS) when observed on leaf surfaces *(198)*. EPS contributes to the bacterial cells ability to anchor itself to the leaf surface and may also alter both the physical and chemical environment around the cell to improve growth and survival and prevent cell desiccation *(19, 191, 198)*. The EPS fibers are involved with

the initial attachment and become thicker in time creating a biofilm matrix *(117, 186)*. EPS has also been described by additional terms such as capsule, glycocalyx, sheath, and slime *(79, 130)*. Within this group are S-layers (subunit surface layers), capsules and slime layers *(40)*. Capsule polymers may contain acidic residues which can contribute to the negative charge to the cell surface *(85)*.

Some bacteria have proteinaceous, threadlike, nonflagellar attachment structures such as fimbriae or pili *(118, 242)*. The primary purpose of pili and fimbriae is attachment and the direct effect of these appendages is the adhesion of bacterial cells onto surfaces *(277)*. These structures range in length from 0.2 to 20 µm and range in width from 3 to 14 nm *(242)*. Common pili are adhesins that are often the initial step in establishing infection when attaching to the susceptible tissues of an animal host *(123)*. They are more commonly found on gram-negative bacteria, especially members of the Enterobacteriaceae family however, similar structures have been observed on gram-positive bacteria such as *Corynebacteria* and *Streptococcus (40, 277)*. Pili contribute to the hydrophobicity of the cell as the amino acids on some of the proteins contain multiple nonpolar side chains *(84)*. Curli, which are long, thin, amyloid aggregative fimbriae made by pathogenic *E. coli* and *S.* Enteritidis, are adhesive and bind to animal surface proteins *(45, 172)*. Jester and Matthysse *(172)* described how pathogenic *E. coli* have multiple mechanisms for binding to plant surfaces and that different strains may have different genes involved in binding, therefore, having varying binding characteristics. Boyer et al. *(41)* studied the effect of curli expression on the cell's overall hydrophobicity, charge and ability to attach to cut and whole iceberg lettuce

surfaces. They reported that curli increased the overall hydrophobicity of the *E. coli* O157:H7 cells but did not appear to have a strong influence on cell charge. Lapidot et al. *(189)* reported that the ability to produce cellulose or curli does not provide any advantage during the initial step of adhesion.

Plant surfaces. Bacteria do not colonize on plant surfaces evenly. On leaf surfaces, bacteria are typically seen in depressions and invaginations in the cuticular layer of the epidermis and at the bases of trichomes *(190, 198, 206)*. Tubular protuberances (microscopic hair-like protrusions) called trichomes are on the surfaces of some fruits and vegetables which aid in preventing mold or insect invasion *(57)*. Stomata are pores in the epidermis where gas exchange occurs are protected by guard cells that open and close in repose to changes in interior turgor pressure caused by the environment *(57)*. Bacteria are also found at stomata and at epidermal cell wall junctions, in particular, the grooves along the veins and on cut surfaces, and broken trichomes *(206, 290)*. Babic et al. *(13)* reported that during storage conditions, spinach leaves were colonized primarily in areas where the waxy cuticle was broken. Adams et al. *(5)* reported that microorganisms were protected in hydrophobic pockets or folds in the leaf's surface and were not exposed to disinfectants in wash solutions. Wilson and Lindow *(353)* reported that cells in inocula with high cell concentrations exhibited up to 100-fold improved survival on bean plant leaves than cells in inocula of low cell concentration implying that leaves that were colonized by bacteria provide a habitat that is different than uncolonized leaves. Takeuchi and Frank *(315, 316)* used confocal scanning laser microscopy (CSLM) to quantify the role of plant structure in protecting

cells against chlorine disinfection. They observed differences in survival among *E. coli* O157:H7 cells attached at the surface, stomata, and damaged tissues of lettuce leaves after treatment with 200 mg/liter chlorine, showing that the quantification of the role of lettuce leaf structure in protecting *E. coli* O157:H7 cells from sanitizers can be achieved using CSLM.

Biofilms. The population density appears to affect the attachment and survival of bacteria. Once a microorganism becomes attached to a plant surface, growth in protected areas can result in the formation of biofilms which are an extracellular polysaccharide matrix that binds multiple cells together and fastens them to the surface of the plant *(286)*. Basic properties of biofilms include that they are composed of multiple layers of microbial cells which create physical and chemical barriers, they may contain multiple species of microorganisms which may promote metabolic and genetic exchanges, and the surface they attach to may include the surface of other microorganisms which is a requirement for the expression of certain genes *(223)*. Pathogens such as *E. coli* O157:H7, *Salmonella, L. monocytogenes,* as well as the spoilage bacteria *Pseudomonas* and *Erwinia* spp. all form biofilms *(286)*. Iturriaga et al. *(167)* demonstrated that environmental conditions during growth of tomatoes in greenhouses or during postharvest handling could promote the biofilm development of *S.* Montevideo on the surface of the fruit. In two separate studies, Fett *(115)* and Fett and Cooke *(116)* illustrated that biofilms are present on a variety of plant surfaces including the roots, hypocotyls and cotyledons. It has been suggested that native biofilms found on mung bean sprouts allow microbes that are pathogenic to plants and to humans potential

colonization sites and protection from both physical removal and destruction by antimicrobials *(115)*. An additional concern presented by these authors was the possibility of human pathogens becoming part of heterogeneous biofilms produced by nonpathogenic bacteria, making them more obstinate to the inhibitory effects of antimicrobial compounds *(115)*. However, Seo and Frank *(290)* indicated that *E. coli* O157:H7 may not preferentially colonize biofilms produced by natural microflora, at least not on lettuce leaves. The majority of biofilms on the leaf surface was associated with trichomes, was heterogeneous in nature and was composed of both gram-negative and gram-positive bacteria as well as yeast and filamentous fungi up to 20 µm in depth and 1 mm in length *(223, 224)*. Morris et al. *(223, 224)* demonstrated the presence of naturally occurring biofilms on the surface of a variety of commercially produced leafy greens including spinach, lettuce, broadleaf endive, and parsley. Koseki et al. *(185)* reported that biofilms created by aerobic bacteria and coliforms could not be disinfected by acidic electrolyzed water, ozone or sodium hypochlorite and residual bacteria were detected after treatments. Lapidot et al. *(189)* found that biofilms are likely to influence the effectiveness of strategies to control food-borne pathogens on parsley. However, they suggested that other protective mechanisms afford more significant protection effects. In addition, they suggested that biofilm formation strengthens the adhesion and provides protection against disinfection after storage of contaminated produce, and not immediately after contamination.

Internalization. Several studies have demonstrated that human bacterial pathogens have the ability to penetrate the interior of cut leaf edges or become

internalized within lettuce tissue *(290, 305, 316, 317, 344)*. Takeuchi and Frank *(315)* suggested that temperature may have an effect on cell penetration due to active respiration of tissues which causes gas movement creating a counterforce to penetration. There have been multiple reports that bacteria can become internalized in fruit through immersion when there is a negative temperature differential *(52, 58, 362)*. For example, if a warm commodity that contains internal air space is placed in colder water such as in a flume, the internal gas cools and contracts creating a partial vacuum which will permit water (and any subsequent microorganism within the water) to be infiltrated into pores, channels or punctures within the product *(16, 17, 162, 286)*. Even fruits and vegetables that are not bruised can experience microbial internalization if the wash water is contaminated *(52, 332)*. Menely and Stanghellini *(215)* detected enteric bacteria within the internal tissue of healthy cucumbers. Internalization of microorganism can also occur naturally during flowering or fruit development *(286)*. Bacteria can be localized in the stomata, the stem-scar, or the calyx areas of different fruits and vegetables *(12, 140, 304)*. Injury to the product can be caused by insects, hail damage, handling, packaging etc. *(332)*. Bacterial soft rot caused by spoilage microorganisms can also increase the likelihood of contamination of fruits and vegetables with pathogens. Wells and Butterfield *(349)* demonstrated that *Salmonella* was present in 18-20% of soft-rotted vegetables including lettuce. Much of what is currently known about attachment of microorganisms as well as biofilms has been gained through the use of laser scanning confocal microscopes and scanning electron microscopes which will be discussed below.

Microscopy

With the use of various types of microscopy, researchers have been able to determine how bacteria associate with plant tissue, attach, or form biofilms often with non-pathogenic bacteria and non-edible plants and then confirm these discoveries on pathogenic microorganisms and fruit and vegetables *(18, 61, 206, 216, 222, 272, 275, 280, 313)*. Schwach et al. *(288)* used scanning electron microscopy (SEM) to describe the effects of sodium hypochlorite on attachment of bacteria to stainless steel. Morris et al. *(223)* examined the spatial relationships among microorganisms on leaf surfaces to help understand their ecology by using SEM and confirmed that biofilms could be easily observed on naturally contaminated leaf surfaces. Fett and Cooke *(116)* described native biofilms on sprout surfaces and hypothesized that they may provide protected sites for plant and human bacterial pathogens. Tolker-Nielsen et al. *(324)* reported observations of bacterial movement occurring in developing biofilms using laser scanning confocal microscopy.

Confocal scanning laser microscopy (CSLM). CSLM has two main objectives: point by point illumination of the sample and rejection of out of focus light *(257)*. By obtaining optical thin sections of the specimen using focused laser light which scans the field and a pinhole detector to remove out-of-focus light, the resulting image has little depth of field but is highly focused. However, depth of image can be achieved by collecting optical sections at multiple sample depths and “stacking” them to project a three dimensional image *(42)*. This 3D capability is one of the main advantages to confocal microscopy. Another benefit is the ability to observe movement of live bacteria

through and their association with tissue by using strains transformed to produce fluorescent proteins *(336)*. Delaquis et al. *(90)* were the first to observe microorganisms on food surfaces by using CSLM. It is important to determine separately from the CSLM if the pathogens on surfaces are attached because when using CSLM, due to the nature of the microscopy, microorganisms appear on the surface of the sample and one cannot determine attachment based on the visualization. Seo and Frank *(290)* were able to show *E. coli* O157:H7 attachment to the surface, trichomes, stomata and cut edges of lettuce leaves using CSLM. They also reported that the pathogen preferred to attach to the cut edges as compared to the intact surfaces. Solomon and Matthews *(303)* suggested that entry of *E. coli* O157:H7 into plant roots is governed by the plant rather than by specific bacterial factors by observing FluoSpheres, as bacterial surrogates, using CSLM. In addition, Solomon et al. *(305)* were able to demonstrate that lettuce grown in soil containing contaminated manure or contaminated irrigation water results in contamination of the edible portions of a plant through the use of CSLM. Takeuchi and Frank *(315)* described the penetration of *E. coli* O157:H7 into lettuce tissue as affected by inoculum size and temperature. Hassan and Frank *(150)* studied the nature of detachment of *E. coli* O157:H7 on lettuce and as a result recommended that for effective treatments for detaching *E. coli* O157:H7 there will need to be simultaneous disruption of the multiple cell/tissue interactions. Takeuchi et al. *(317)* compared the attachment of *E. coli* O157:H7, *L. monocytogenes, S.* Typhimurium, and *P. fluorescens* to lettuce leaves.

Scanning electron microscopy (SEM). Scanning electron microscopy (SEM) uses electrons instead of light to form an image creating a much higher resolution, therefore specimens can be magnified at much higher levels (10x to 100,000x) *(134)*. Unlike CSLM, when using SEM microorganisms must be fixed onto a surface, for example, a spinach leaf in order to be observed. Mafu et al. *(203)* revealed using SEM that *L. monocytogenes* could attach to low and high energy surfaces after short contact times. Blackman and Frank *(39)* demonstrated the ability of *L. monocytogenes* to accumulate as a biofilm on materials commonly found in food-processing plants. Pao et al. *(244)* used SEM to confirm that sanitizing oranges can be effective in microbial reduction during fresh juice production by observing that bacterial contamination occurred near the surface which could be reached by sanitizing treatments. Ronner and Wong *(279)* reported that *L. monocytogenes* biofilm cells and extracellular matrices could remain on sanitized surfaces from which no viable cells were recovered. Iturriaga et al. *(167)* described *S.* Montevideo biofilm development using SEM. Dewanti and Wong *(94)* demonstrated that *E. coli* O157:H7 can adhere and form biofilms to stainless steel. Babic et al. *(13)* used a variation of SEM referred to as low temperature scanning electron microscopy (LTSEM) which allowed the researchers to observe spinach leaves and associated microorganisms in their frozen-hydrated condition. LTSEM was used to examine tissues that appeared undamaged and those which exhibited broken and necrotic areas. The tissue samples that appeared undamaged at the time of sampling were free of microorganisms, however; for the segments that exhibited necrotic areas, the upper epidermis and the palisade parenchyma were covered with a continuous layer of

bacteria. In addition, the bacteria did not appear to penetrate the leaf through stomata but rather seemed to concentrate in areas where the cuticle and underlying tissues had been physically damaged *(13)*. Several studies have shown that mucoid strains of *P. fluorescens* found in spinach leaves produced exopolysaccharides involved with the attachment of bacteria to the host plant which protected them from adverse environmental conditions such as desiccation *(114, 241, 265)*. Rayner et al. *(265)* investigated the prevalence of microbial biofilms on salad vegetables and household surfaces using cryostate scanning electron microscopy (CSEM). Biofilms were observed on carrots, tomatoes, mushrooms and lettuce. These authors suggested that there may be a need for fresh produce and surface sanitizing products that specifically target microbes present within this specialized mode but did not name specific products or treatment methods. Rico et al. *(269)* used cryo-scanning electron microscopy to observe the effects of wounding stress on fresh-cut iceberg lettuce leaves after treatments of chlorinated water and electrolyzed water (EW). In chlorinated samples the cells acquired polyhedral shapes. This phenomenon was observed to a lesser degree in EW treated samples in which cells remained more rounded. The authors suggested that this might be explained by a higher turgidity or water content in the samples treated with EW compared to the samples treated with chlorine. Warner et al. *(347)* observed biofilms produced by *S.* Thompson on spinach and watercress leaves using episcopic differential interference contrast (EDIC) microscopy without the need for fixation or staining. They reported that spinach leaves had a greater proportion of the surface covered by debris with an appearance of individual microbial cells and clusters, whereas on watercress the

contamination was confined almost exclusively to the margins between epidermal cells. This debris was present whether the leaves were unwashed or processed and packaged as "ready-to-eat" suggesting that it was tightly attached. Niemira *(230)* used SEM to explain that *E. coli* O157:H7 could be found throughout the vascular and intercellular spaces in romaine lettuce and baby spinach. SEM revealed that the bacterial cells within the spinach leaves were more aggregated than those within the lettuce leaves. Niemira et al. *(230)* concluded that their results confirmed the finding of the FDA that surface treatment is ineffective in reducing internalized microbial populations and that concentrations of chlorine commonly used in the processing of leafy vegetables may be inadequate to address contamination *(230, 331, 332)*.

Decontamination methods for fresh produce

The initial processing stages for minimally processed or bagged produce are critical for the removal, inactivation or controlling of pathogens because if they are not addressed at this preliminary period, they can potentially contaminate additional produce throughout processing *(333)*. Conversely, based on the structure of spinach leaves and the attachment factors described above, it is unlikely that washing and sanitizing will eliminate all pathogens once it is contaminated. A common assumption made by both processors and consumers is that washing and sanitizing fruits and vegetables will reduce the microbial load; however, they are not capable of reducing microbial populations on produce by more than 90% to 99% *(44)*. Typical washing practices were originally developed for the removal of soil from produce and not

microorganisms *(286)*. Using the current methods for decontamination, there appears to be a population reduction ceiling of 1-2 log units when realistic inoculation and treatment conditions are used *(11)*. The Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables defines the word sanitize as "to treat clean produce by a process that is effective in destroying or substantially reducing the number of microorganisms of public health concern, as well as other undesirable microorganisms, without adversely affecting the quality of the product or its safety for the consumer" *(329)*. Many treatments have been developed to decontaminate leafy green vegetables including aqueous sanitizing agents however, the effectiveness of these products depend on many factors including the treatment conditions such as the temperature of the water, pH, water hardness, contact time, amount and rate of product throughput, type of vegetable, the water to produce ratio, the amount of organic material present in the water and the resistance the pathogen has to the specific antimicrobial agent *(329)*. Therefore, it is preferable to avoid microbial contamination by utilizing GAPs and GMPs rather than relying on decontamination treatments *(246)*.

Chlorine (Hypochlorite). Chlorine remains to be the most common sanitizer used at levels of 100-200 mg/liter. Chorine is often used to sanitize produce, contact surfaces and facilities, as well as to diminish microbial loads in water used during cleaning and packaging *(80)*. Despite its popularity and convenience, the effects of chlorine on pathogens inoculated onto produce are inconsistent. Bershing et al. *(26)* described the efficacy of chlorine for inactivation of *E. coli* on lettuce and broccoli. Li et al. *(195)* reported that chlorine levels below 200 mg/liter were not effective in reducing *E. coli*

O157:H7 on cut lettuce pieces at 20 mg/liter chlorine at 20 or 50°C. Beuchat et al. *(35)* compared spraying apples, tomatoes and lettuce (inoculated with *Salmonella* and *E. coli* O157:H7) with water or 200 mg/liter chlorine and allowed them to soak in water for various times to see if pathogens would detach. Zhang and Farber *(361)* reported that 200 mg/liter for 10 min could reduce *L. monocytogenes* artificially inoculated onto shredded lettuce and cabbage by 1.7 and 1.2 log CFU/g, respectively. Takeuchi and Frank *(316)* reported that 200 mg/liter chlorine treatment at 22°C for 5 min achieved a significant reduction in the number of viable *E. coli* O157:H7 attached on leaf surfaces and in cut edge tissue (0.3 and 0.4 log CFU/g respectively) but high numbers of viable cells remained both at the cut edge and the surface. Delaquis et al. *(92)* reported on the fate and survival of *E. coli* O157:H7 and *L. monocytogenes* in ready-to-eat cut lettuce washed in cold and warm chlorinated water. Washing in cold chlorinated water after inoculation immediately reduced *E. coli* O157:H7 populations by 1 log CFU/g and the antimicrobial effect of chlorinated water was enhanced at 47°C resulting in reductions in excess of 2 log CFU/g. Burnett et al. *(56)* studied whether variations in methodology influenced the efficacy of chlorinated (200 µg/ml free chlorine) water and a commercial product in killing *L. monocytogenes* spot inoculated onto iceberg lettuce. Brackett *(43)* reported that the reduction in numbers if *L. monocytogenes* on brussel sprouts changed from 90% (dipped 10 s in sterile water without chlorine) to 99% with the addition of 200 mg/liter chlorine. When inoculated into cracks of mature green tomatoes, *S.* Montevideo survived treatment with 100 mg/liter chlorine *(348)*. Escudero et al. *(105)* reported that 100 to 300 mg/liter of chlorine could reduce *Yersinia enterocolitica* on shredded lettuce

by 2 to 3 log. Bershing et al. *(26)* reported populations of *E. coli* inoculated onto lettuce leaves and broccoli florets were generally reduced <1 log CFU/g after 5 min dip in 100 mg/liter free chlorine. Pathogens present on the surface of produce are likely to be enmeshed in organic matter, e.g., *E. coli* O157:H7 in bovine feces or *Salmonella* in avian feces as well as *L. monocytogenes* in decaying vegetation *(29, 35)*. Treatment of alfalfa seeds and sprouts with chlorine to control salmonellae and *E. coli* O157:H7 has been studied *(171, 318, 319)*. One of the biggest challenges with using chlorine as a disinfectant is that it diminishes quickly upon contact with organic matter such as decaying vegetation which can be expected in the harvesting process *(35, 195, 304, 316)*.

Chlorine dioxide (ClO_2). Chlorine dioxide (ClO_2) has been proposed as a possible sanitizing agent. There are several advantages of using ClO_2 rather than hypochlorous acid (HOCl) such as ClO_2's reduced reactivity with organic material, it has a greater activity at neutral pH, however; its stability may be problematic *(23)*. One of the disadvantages is that as a water rinse, blanching, cooking or canning treatments must be used on produce that have been treated with chlorine dioxide *(78)*. Reina et al. *(266)* reported that total bacterial counts and Enterobacteriaceae were reduced in cucumbers by 2 and 6 logs, respectively, by washing the cucumbers with a solution containing 1.3 mg/liter ClO_2. Han et al. *(148)* investigated the use of ClO_2 gas treatments for the decontamination of strawberries and reported a 5 log reduction in microbial counts however, it is important to note that they also stated that the effectiveness of ClO_2 gas treatments for reducing pathogens varies for different types of produce. In addition, Han et al. *(149)* compared the effectiveness of ClO_2 gas treatment and water washing on

inactivation and removal of *E. coli* O157:H7 from green peppers. Du et al. *(98)* described the efficacy of ClO_2 on *L. monocytogenes* spotted onto different apple surfaces. Wisniewsky et al. *(354)* reported that ClO_2 gas was ineffective in reducing populations of *E. coli* O157:H7 on artificially inoculated apples while Lee et al. *(194)* reported that *E. coli* O157:H7 inoculated on lettuce leaves were reduced by 3.4, 4.4, and 6.9 CFU/g after treatment of ClO_2 gas for 30 min, 1 h and 3 h, respectively.

Organic acids. Many pathogens cannot grow below pH levels of 4.5 thus; acidification by using organic acids may help prevent bacterial growth. The antimicrobial mechanism by which organic acids reduce microbial numbers is due to several factors. These include a reduction in the environmental pH, disruption of cell membrane transport system and permeability, accumulation of ions, or a reduction in the internal cellular pH by the dissociation of hydrogen ions from the microorganism as it attempts to maintain homeostasis *(245, 343)*. The antimicrobial effect of organic acids increases with temperature *(83, 307, 343)*. Numerous studies have been conducted to study the effects of organic acids, with the most commonly studies being acetic acid, citric acid, lactic acid, tartaric acid and orthophosphoric acid *(6, 27, 63, 104, 176, 259, 356)*. Wu et al. *(357)* studied the effects of dipping parsley leaves inoculated with *Shigella sonnei* into 2% acetic acid and reported a reduction in populations of *S. sonnei* of greater than 7 logs/g. Castillo and Escartin *(63)* studied the efficacy of citric acid in the form of lemon juice for the reduction of *Campylobacter jejuni* on watermelon and papaya and reported that lemon juice appeared more destructive to *C. jejuni* on papaya than on watermelon, although a total elimination of *C. jejuni* was not achieved on either

commodity. Delaquis et al. *(91)* tested the efficacy of gaseous acetic acid for disinfecting bean seed inoculated with *S.* Typhimurium, *E. coli* O157:H7, or *L. monocytogenes.* A 2 min dip in 5% acetic acid at room temperature was the most effective treatment of several investigated for reducing populations of *E. coli* O157:H7 inoculated onto apple surfaces *(356)*. Shapiro and Holder *(291)* reported that citric acid was much less effective than tartaric acid in preventing growth of microorganisms on salad vegetables. Lactic acid has been successfully used as a sanitizer for animal carcasses and may be a possible sanitizer for produce surfaces *(66, 245)*. Torriani et al. *(325)* reported that coliforms and fecal coliforms were reduced about 2 and 1 log CFU/g respectively, on mixed salad vegetables treated with 1% lactic acid. Alvarado-Casillas et al. *(9)* reported that lactic acid sprays can reduce bacterial pathogens by almost 3 log CFU/g on cantaloupes and 3.6 log CFU/g on produce with smooth surfaces such as bell peppers. A study conducted by Zhang and Farber *(361)* on the effects of various disinfectants against *L. monocytogenes* reduction in lettuce and cabbage showed that lactic acid was more effective than acetic acid. These authors also described the use of lactic acid, acetic acid and chlorine in combinations and reported that lactic acid or acetic acid and chlorine mixture was more effective in reducing *L. monocytogenes* than alone. Other studies have combined multiple organic acids or chemicals. Escudero et al. *(105)* demonstrated that 0.5% lactic acid in combination with 100 mg/liter chlorine reduced *Y. enterocolitica* inoculated onto shredded lettuce by more than 6 log cycles. Lin et al. *(197)* studied the anti-bacterial efficacy of hydrogen peroxide with and without lactic acid at elevated temperatures on lettuce and reported that the combination of lactic acid

and hydrogen peroxide was able to obtain reductions of >4 log CFU of *E. coli* O157:H7 and *Salmonella* per lettuce leaf and about 3 log CFU of *L. monocytogenes* per lettuce leaf was inactivated. Venkitanarayanan et al. *(340)* studied the effectiveness of lactic acid with hydrogen peroxide for inactivating *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* on apples, oranges, and tomatoes. Packers have expressed concerns about using organic acids due to the potential presence of acid-adapted microorganisms. Ryu et al. *(284)* compared different acids for their antimicrobial activity against acid-adapted and non-adapted *E. coli* O157:H7. However, Castillo et al. *(64)* reported that acid resistance could be overcome by combining other factors such as applying the treatment of organic acid at 55°C.

Peracetic acid. Peracetic acid (also known as peroxyacetic acid) is produced by a reaction between acetic acid with hydrogen peroxide. It is a colorless liquid with a low pH (2.8) and it oxidizes the outer cell membrane of microorganisms. The oxidation mechanism consists of electron transfer; when a stronger oxidant is used, electrons transfer to the microorganism quickly causing apoptosis. Peracetic acid is commonly used as a disinfectant treatment for fresh-cut vegetable process water for reducing yeast and mold counts which can improve the finished product's quality and extend the shelf-life. When used at 40 and 80 mg/liter, a sanitizer that contained peracetic acid (Tsunami™ Ecolab, St. Paul, MN) significantly ($P<0.05$) reduced salmonellae and *E. coli* O157:H7 populations on cantaloupe and honeydew melon surfaces *(246)*. Beuchat et al. *(33)* studied the efficacy of peroxyacetic acid in killing *L. monocytogenes* on iceberg and romaine lettuce with reductions not significantly different from lettuce

leaves washed only with water. Hilgreen and Salaverda *(157)* studied the effects of peracetic acid on fresh-cut vegetable process water and reported that peracetic acid combined with octanoic acid reduced yeasts and molds faster than peracetic acid alone. Wisniewsky et al. *(354)* reported that peroxyacetic acid achieved a 5 log reduction of *E. coli* O157:H7 inoculated on whole fresh apples, however; this was accomplished when the treatment was used at 2.1 to 14 times its recommended concentration depending on the length of the wash time. Rodgers et al. *(273)* reported a 4.4 log reduction *E. coli* O157:H7 and *L. monocytogenes* inoculated onto whole and shredded lettuce exposed to 80 mg/liter peracetic acid for 5 min. Hellstrom et al. *(153)* studied the efficacy of peracetic acid solution (0.05%) on precut iceberg lettuce inoculated with *L. monocytogenes.* These authors were able to reduce *L. monocytogenes* by 1.7 log CFU/g however, lettuce samples stored for 6 days were able to reach the initial inoculation level.

Ozone. Ozone has been proven as an effective treatment for disinfection of drinking water where ozone decomposes spontaneously to nontoxic products *(184, 268)*. Ozonated water has been applied to fresh-cut vegetables for sanitation purposes reducing microbial populations and extending the shelf-life of some of these products *(21, 22)*. Ozone is a highly reactive form of oxygen (O_3), which destroys microorganisms by reacting with oxidizeable cellular components *(263)*. Sharma et al. *(292)* reported a 0.4 to 1.75 log reduction of *E. coli* O157:H7 inoculated onto alfalfa seeds using 4 mg/liter and 21 mg/liter of ozonated water. Wade et al. *(345)* treated alfalfa sprouts with 23.2 µg/liter of ozone for 2 min resulted in 0.91 log reduction of *L. monocytogenes.* Kim et al.

(182) reported that an increased ozone exposure time increased the inactivation rates of microorganisms on lettuce, showing that 3-min exposure time was effective to reduce the mesophilic bacterial population on lettuce by 1.2 logs. In addition, they reported that bubbling ozone treatment combined with stirring followed by stomaching or high-speed stirring showed the greatest reduction (1.9 logs). However, Achen and Yousef *(4)* argued that long exposure times for sanitizing produce were impractical for industrial facilities. Rodgers et al. *(273)* compared different chemical sanitizers including ozone for reducing *E. coli* O157:H7 and *L. monocytogenes* on apples, lettuce, strawberries and cantaloupes. They reported reducing populations of both pathogens by ca. 5.6 log cycles using 3 mg/liter. Conversely, Yuk et al. *(359)* studied the effects of ozone alone and in combination with organic acid treatments (acetic, citric or lactic acid) to reduce *E. coli* O157:H7 and *L. monocytogenes* on lettuce and reported that ozone alone at 3 mg/liter for 1 min reduced the population of *L. monocytogenes* by 0.53 log CFU/g, while treatment with 1% acetic, citric or lactic acid resulted in population reductions of 0.59, 1.03 or 0.93 log CFU/g, respectively. The authors did mention that their reductions were minimal and not industrially important for application to lettuce. Selma et al. *(289)* reported that 1 mg/liter of ozonated water reduced the microbial population of *S. sonnei* inoculated onto lettuce by 0.7 log units after 3 min. After 5 min, *S. sonnei* counts were reduced by 0.9, 1.4, and 1.8 log units in lettuce samples treated with 2 mg/liter of ozonated water with or without ultraviolet activation and 5 mg/liter, respectively *(289)*. There are several limitations to its use. Physical injury of produce may occur due to

ozone's strong oxidizing activity and it produces toxic vapors, therefore to ensure employee safety, adequate ventilation is necessary *(273)*.

Irradiation of food

X-rays were discovered in 1895 and the use of ionizing radiation in food preservation began shortly thereafter with experiments beginning in 1896 *(293)*. Ionizing radiation can be described as a physical phenomenon in which energy travels through space or matter that produces electrically charged particles, or ions *(260)*. The first patent in the U.S. for food irradiation was awarded in 1905 for a device to irradiate to control *Trichinella spirallis (131)*. After World War II which introduced the use of atomic bombs, and the impending nuclear arms race between the U.S. and the Soviet Union, President Dwight D. Eisenhower established the "Atoms for Peace" program *(187)*. While considered a "process" in many nations, in 1958 the U.S. Congress deemed all sources of irradiation as a "food additive" under an amendment to the Federal Food, Drug and Cosmetic Act of 1938 *(93, 247)*. The U.S. Army sponsored many experiments from the 1950's-1980's which included long-term multi-generational studies on dogs, rats and mice fed irradiated chicken meat and concluded that foods irradiated up to 10 kGy were safe to eat, however, many other studies conducted including those conducted by universities and non-military laboratories reported that food irradiated up to 40 kGy was safe to eat *(199)*. Internationally, during the early 1980's the United Nations' Food and Agricultural Organization (FAO), the International Atomic Energy Agency (IAEA) and World Health Organization (WHO) published the recommendation that food

products irradiated up to 10 kGy are wholesome and present no toxicological hazards and toxicological testing of foods treated as such are no longer necessary *(355)*. Currently, the FDA has approved the use of irradiation on spices, dry vegetable seasonings and wheat for the purpose of disinfecting insects, white potatoes to prevent sprouting, pork for controlling *Trichinella spirallis*, fresh fruits and vegetables to reduce insects, and poultry, red meat and fresh shell eggs to control pathogens *(293, 328, 330)*. Recently, the FDA amended the food additive regulations to provide for the safe use of ionizing radiation for the control of foodborne pathogens and the extension of shelf-life in fresh iceberg lettuce and fresh spinach *(334)*.

Types of ionizing radiation. Radiation is the physical occurrence in which energy passes through matter or space and irradiation is the process in which this energy is applied to materials such as food or medical devices to remove microorganisms, parasites, insects, extend a food product's shelf-life or to sterilize it *(260)*. Ionizing radiation refers to the electrically charged particles (ions) produced which has higher energy than non-ionizing radiation such as light waves, microwaves and radiowaves. Types of ionizing radiation used for irradiating food include gamma rays, x-rays and electron beams. Radioactive isotopes such as Cobalt-60 (^{60}Co) or Cesium-137 (^{137}Cs) produce gamma rays which are used for radiotherapy, medical devices and the irradiation of foods *(320)*. ^{60}Co is the most common radioisotope which is produced in the form of metal "pencils" and contains energy of about 1 to 2 MeV *(86)*. Electron beams can be produced by either a linear accelerator powered by electricity or Van de Graff generators. Linear accelerators can accelerate electrons to 99% the speed of light

and when these highly accelerated electrons penetrate a thin foil of metal such as tungsten or tantalum x-rays are produced *(260)*. Electrons have less penetrating power than gamma rays but can be beneficial for irradiating large volumes of small food items such as ground beef patties. One of the advantages of using a linear accelerator over a radioisotope is the speed in which a product can be treated on a conveyor passing through the beam as apposed to gamma rays that are produced under a water source *(227)*. The absorbed dose of ionizing radiation determines the effects of the food product. Goresline et al. *(136)* developed terms used to describe various levels of irradiation that might be applied to food. Low-dose irradiation (up 1 kGy) is used predominantly to kill insects infesting fresh fruit; medium dose (up to 10 kGy) is used to kill microorganisms and extend food shelf-life and high dose (> 10 kGy) to sterilize food *(260)*. The absorbed dose of irradiation is a measure which expresses the amount of energy with the ionizing radiation imparted on a food item and the International System of Units (SI) recognizes the term “gray” (Gy) which can be defined as the absorption of 1 joule of energy per 1 kg of food. *(139)*. The dose administered to a food product can be controlled by the amount of time exposed to irradiation in the case of gamma rays and by the speed of the conveyor belt for electron beams or x-rays *(227)*. Products exposed to gamma rays for longer periods of time will absorb higher doses and products that move slowly across the conveyor for electron beam and x-ray will receive higher doses of irradiation.

Dosimetry. When food products are exposed to irradiation, chemical changes occur, producing radiolytic products just as when food products are exposed to other

processes such as cooking. Therefore there is no method to chemically differentiate food products that have been irradiated versus those that have not been irradiated *(238)*. Because there is no chemical means of measuring the dose received by a product, a radiation sensitive material called a dosimeter must be added with the product. The main objective of dosimetry is to determine the dose to achieve a desired affect. There are many different techniques suggested for the identification of irradiated foods but they can be classified under 3 broad categories: physical (a), chemical (b), and biological (c) *(143)*. The two most common methods for the detection of irradiated food are the physical techniques of electron spin resonance (ESR) and thermoluminescence (TL); the chemical approach of cyclobutanones and hydrocarbons; and the biological assays known as direct epifluorescent filter technique/ aerobic plate count (DEFT/APC), deoxyribonucleic acid (DNA) comet, Enzyme Linked ImmunoSorbent Assay (ELISA) and limulus amoebocyte lysate (LAL) *(310)*. ESR spectroscopy is one of the most reliable approaches for the detection of certain irradiated foodstuffs, and this is well illustrated by the many research papers on this topic *(37, 81, 99, 192, 240, 253, 309, 310)*. Thermoluminescence is another popular method. For this detection method, heat is applied to release trapped energy and has also been documented *(143, 179, 183, 252, 253, 310)*.

Because foodstuffs have different chemical compositions and physical states, the irradiation doses which can be applied vary in intensity and even within the same type of food, there are differences due to biological variability, growth conditions, state of maturity, or those caused by processing methods and storage, therefore it is suggested

that a combination of analytical methods should be able to solve any detection problems. *(281)*. Miyahara et al. *(220)* reported that irradiated food products experience dose gradients on the surface or within the food product and it is important to consider the dose-depth profile of various foods.

Mechanism of ionizing radiation. Each of the major irradiation technologies (e-beam, x-ray, and gamma ray) are penetrating and have the same effect on microbial cells; single or double stranded breaks in DNA and ribonucleic acid (RNA) double helix render the microorganism unable to reproduce or grow *(301)*. Nucleic acid damage is caused by a direct impact of high energy electrons from the beam or indirectly when neighboring molecules are ionized and impact the DNA or RNA. Destruction of microorganisms is logarithmic therefore, one can predict the dose that will be necessary to destroy a specific number of microorganisms in a food, which permits processors to design processes so that all cells of a selected organism are killed including in the equation a margin of approximately 2 to 3 decimal reductions to ensure that the product is free of the organism *(228)*. High energy electrons from the linear accelerator are the main source of electrons although secondary electrons are produced as a result of ionization of molecules inside a food product matrix *(251)*. Water is the main source of secondary electrons and ionizing radiation causes water molecules to lose an electron which creates a hydroxyl radical and hydrogen peroxide, both of which are highly reactive with nucleic acids and cause bond breakage within the nucleic acid strands as well as those holding the strands together *(320)*. Both primary and secondary electrons damage microorganisms. Microorganisms differ in their sensitivity to irradiation,

depending on morphological variations similarly to how they differ in sensitivity to heat, drying and freezing. Ingram and Farkas *(163)* reviewed the relative resistance of microorganisms to irradiation. Viruses are typically more resistant than bacterial spores, which are more resistant than vegetative bacteria, which are more resistant than yeasts and molds *(221)*. Some microorganisms are more vulnerable to irradiation during the logarithmic phase rather than during the stationary phase due to the fact that during the logarithmic phase, microorganisms contain multiple copies of their genomes in one cell; therefore, chances of survival are greater despite damage to their nucleic acid *(251)*.

Food composition and microbial resistance to irradiation. When considering the proper dose distribution, the food composition must be taken into account. When individual electrons or photons strike a target, their reaction depends on the geometry and physical properties (for example, density) of the target (food) *(135)*. When low to medium doses (<1.0 to 10 kGy) are applied, there are mild affects on carbohydrates which do not significantly alter the functionality or nutritional value of a food. High doses of irradiation are known to change pectin and cellulosic substances in plant tissues resulting in softening, *(161, 178, 211, 306, 358)*. Damage to cellular membranes can lead to loss of turgor *(113)*. In addition, the temperature of the food product affects the survival of microorganism to irradiation; the lower the temperature, the higher the thermal death value (D_{10} value) *(227)*. Matsuyama et al. *(209)* conducted studies to determine D_{10} values for *Pseudomonas* spp. at subfreezing and room temperatures. They observed an 8.5-fold higher dose was required for a 90% reduction in the microbial population at sub-freezing temperature compared to treatment at room temperature.

Irradiation doses of 50 and 20 kGy were required to kill similar populations of irradiation-resistant *Moraxella-Acinetobacter* in ground beef at 30°C and 35°C, respectively *(51)*.

The amount of water present in a food commodity can also affect the irradiation dose needed. Increased irradiation resistance of microorganisms at sub-freezing temperatures has been attributed to a decrease in a_w. Bruns and Maxcy *(51)* compared the effects of temperature and cellular moisture content on the survival of highly irradiation-resistant bacteria. Removal of water by lyophilization resulted in cells with irradiation resistance similar to control cells treated at sub-freezing temperatures. Snyder and Maxcy *(302)* investigated the growth response of irradiated *Moraxella-Acinetobacter* in media with various a_w values. Growth was retarded at a_w values of 0.99 or less.

Irradiation of produce. Pathogenic bacteria can be internalized within fruits and vegetables, making surface decontamination ineffective; however, the penetrating and subsurface antimicrobial efficacy of irradiation may play an important role in produce sanitation *(231)*. Fresh produce can be irradiated for several reasons in addition to the reduction of microbial loads including inhibition of sprouting, delay of senescence, and disinfection of insects. The reduction of microbial loads typically require a higher irradiation dose (0.5 kGy or greater) than for the other purposes mentioned (less than 0.3 kGy) *(231)*.

Microbial, sensorial, and chemical quality of irradiated produce. Due to the fact that fresh produce is living tissue that respires, metabolizes and ripens, it is sensitive to

ionizing radiation and excessive treatments can lead to detrimental sensory characteristics in texture, aroma, color or taste *(231)*. Because of the numerous possible fruits and vegetables that can be irradiated, each commodity must be studied separately *(207)*. Several studies have been conducted investigating the microbial quality of various fruit and vegetables *(106, 142, 243)*. Hagenmaier and Baker *(141)* reported that iceberg lettuce receiving 0.2 kGy had a reduced microbial load, a small increase in shelf-life and shear force was virtually the same among irradiated samples and controls. In a similar study, Prakash et al. *(254)* reported that irradiating romaine lettuce with 0.35 kGy did extend the shelf-life of modified atmosphere packaged lettuce leaves, the texture of the samples decreased with time but were not apparent to trained sensory panelists, and other sensory characteristics such as off flavors were not evident. Fan and Sokorai *(109)* reported on the effects of ionizing radiation on frozen corn and peas with results suggesting that doses as high as 4.5 kGy had minimal effects on color, carotenoid and chlorophyll concentrations or antioxidant capacity of the frozen vegetables; however, the shear force value of frozen peas and the ascorbic acid concentration in frozen corn were reduced. Additionally, the microbial loads were reduced and it increased the display life of the peas. Fan et al. *(107)* reported that immediately after irradiation, a dose of 1 kGy reduced aerobic plate counts (APC) >2.5 log CFU/g on fresh-cut green onions. Prakash et al. *(254)* found that a dose of 0.35 kGy of gamma irradiation decreased APC by 1.5 logs on cut romaine lettuce and the difference was maintained for 22 days of storage at 4°C. Farkas et al. *(112)* showed that ionizing radiation at 1 kGy reduced loads of bacteria, improved microbial shelf-life and extended sensory quality of precut peppers

and carrots. Khattak et al. *(180)* reported that when minimally processed cabbage was irradiated, the counts ranged from 1.78 (2.5 kGy) to 3.8 (0.5 kGy) log CFU/g at 0 days of storage and increased after 7 days. According to Eleftheriadou et al. *(102)*, a trend of decreasing followed by increasing microbial populations takes place in the first few days of storage of unpasteurized orange juice at low temperatures (<7°C). Niemira et al. *(234)* could not detect any microorganisms in broccoli and lima beans irradiated at 3 kGy for any of the temperatures studied. Howard et al. *(161)* reported that 1.0 kGy doses of irradiation reduced total aerobic and lactic acid bacteria in pico de gallo over 42 days of refrigerated storage after irradiation, thereby extending the shelf-life.

In addition to reducing microbial loads to improve a product's quality, it is also important that any treatment that is used preserves the appearance and sensory attributes of the product. Fan et al. *(106)* reported that the overall visual quality of cilantro was not affected by irradiated on days 0 and 3 however, samples that received irradiation at 3 kGy had poorer visual scores than non-irradiated samples at day 7 and 14. Typical aroma and off-odor were not affected by irradiation at day 0 or 3. Zhang et al. *(360)* reported that the sensory quality of fresh-cut lettuce irradiated with 1.0 kGy appeared to be best, which was explained by the fact that non-irradiated and 0.5 kGy irradiation were not effective in controlling the microbial population. Foley et al. *(121)* reported significant changes in the aromas of irradiated orange juice which increased in irradiation dose however, no change in color was perceived by the panelists. Electrolyte leakage is generally considered as an indirect measure of plant cell membrane damage. Fan and Sokorai *(108)* described the electrolyte leakage of broccoli, red cabbage, endive, parsley,

green leaf lettuce, romaine lettuce, iceberg lettuce, spinach, cilantro, carrots, red leaf lettuce, celery and green onion irradiated with 0.5, 1.0, 1.5, 2.0, 2.5, and 3 kGy gamma irradiation at 5°C. Electrolyte leakage of all fresh-cut vegetables increased linearly with higher irradiation doses. Kim et al. *(181)* reported similar finding that electrolyte leakage of green onions treated with irradiation gradually increased with time.

Low doses of irradiation can inactivate some radiation-sensitive pathogens however, to achieve a 5 log reduction, higher doses (>1 kGy) of radiation are required. At higher doses, irradiation may induce quality changes such as softening and browning as well as loss of vitamin C *(110)*. Fan et al. *(110)* reported that vitamin C content of lettuce samples treated with 0.5, 1 or 2 kGy decreased during storage but primarily during the first week of storage.

Pathogen reduction of irradiated produce. Irradiation effectively eliminates *E. coli* O157:H7 from lettuce *(25, 236)*. Niemira *(230)* described how lettuce and spinach leaves which are seemingly comparable, may a have a significantly different response to *E. coli* O157:H7 internalized and a different response to irradiation. In addition to salad greens, the effects of ionizing radiation on *E. coli* O157:H7 contaminated cilantro has been investigated. Foley et al. *(120)* showed that irradiation of cilantro at doses as high as 3.85 kGy did not affect product quality over its shelf-life of 13 days postharvest and doses as low as 1 kGy were sufficient for reducing *E. coli* O157:H7 by 5 log cycles or more. In a separate study, Foley et al. *(119)* reported that chlorination plus irradiation at 0.55 kGy on lettuce leaves inoculated with *E. coli* O157:H7 produced a 5.4 log reduction. Goularte et al. *(138)* reported that inoculated lettuce exposed to 0.7 kGy

reduced the population of *Salmonella* by 4.0 log and *E. coli* O157:H7 by 6.8 log without impairing the sensory attributes. Niemira *(230)* demonstrated the efficacy of irradiation for the elimination of internalized *E. coli* O157:H7 in spinach and romaine lettuce and with 1 kGy resulted in an approximately 3 log reduction. The inactivation of *E. coli* O157:H7 in apple juice by irradiation was investigated by Buchanan et al. *(53)* who reported that radiation resistance varied among different brands of juices and while there are other differences among the juices that may have influenced radiation resistances, *D*-values increased with increasing juice turbidity. Similar to other decontamination processes, a fraction of the microbial population may be expected to survive with varying degrees of injury *(2, 59, 60, 264)*. Microorganisms that are sublethally damaged and not capable of repairing die. Other injured microorganisms may be better equipped to recover especially if the post-treatment conditions are favorable for their repair. Lucht et al. *(200)* reported that *E. coli* at 37°C directly after treatment promoted permanent damage which was essentially completed within 90 min.

The radiation sensitivity of *L. monocytogenes* and *L. innocua* inoculated onto endive has been studied *(233)*. In addition, Niemira et al. *(232)* reported that irradiation and modified atmosphere packaging (MAP) of endive influences the survival and regrowth of *L. monocytogenes* and product sensory qualities. A dose of 0.6 kGy resulted in reductions of 3.09, 2.41 and 2.53 log CFU/g of *L. monocytogenes* in 3 different MAP packages (Air-o, 5/5 and 10/10), respectively. Mintier and Foley *(219)* reported that gamma irradiation at 1.15 kGy followed by refrigerated storage at 4°C resulted in a >5 log reduction compared with control samples and the difference was maintained during

the 10 day storage period. This finding is unusual because re-growth of *L. monocytogenes* on various products has been widely reported *(112, 122, 232, 233, 255, 322)*. Lee et al. *(193)* inoculated cucumbers, blanched and seasoned spinach and seasoned burdock with *S.* Typhimurium, *E. coli*, *Staphylococcus aureus,* and *L. ivanovii.* The *Salmonella* was reduced by 3 log CFU/g at 1 kGy for all 3 types of vegetables. *E. coli* was reduced below the detection limit for all vegetables. *S. aureus* was reduced 5.4 CFU/g on the cucumber and a reduction of 4 log CFU/g on the spinach and the burdock. Schmidt et al. *(287)* reported that e-beam irradiation reduced counts of *S.* Montevideo and *S.* Agona artificially inoculated onto tomato cubes and stem scars. For tomatoes inoculated with *S.* Montevideo and treated with 0.7 and 0.95 kGy, populations on cubes were reduced by 1.8 and 2.2 log CFU/g, respectively and 2.4 log CFU/g on stem scars at both doses. *S.* Agona populations were reduced by 1.3 and 1.5 log cycles on tomato cubes and by 1.3 and 2.2 log cycles on stem scars. Niemira *(229)* studied the influence of lettuce type on the radiation sensitivity of *Salmonella* and *L. monocytogenes* and reported that the radiation sensitivity of *L. monocytogenes* was not significantly influenced by lettuce type (D_{10} value for *L. monocytogenes* was 0.19 kGy). In contrast, *Salmonella* was significantly sensitive to irradiation when treated on green leaf lettuce than any other type (D_{10}=0.23 to 0.25 kGy). Prakash et al. *(256)* calculated D-values ranging from 0.26 to 0.39 kGy for *Salmonella* spp. in diced tomatoes dipped in 1% calcium chloride. Several studies have addressed the use of gamma irradiation to inactivate viruses in various biological fluids and food products *(103, 205, 311, 312, 323, 350)*. Bidawid et al. *(38)* inoculated lettuce and strawberries with hepatitis A virus

(HAV) and treated them with doses of gamma irradiation ranging between 1 to 10 kGy. D_{10} values of 2.72 and 2.97 kGy were required to achieve a 1 log reduction in HAV titre in lettuce and strawberries respectively.

MATERIALS AND METHODS*

Microorganisms

For the growth and survival studies, bacterial suspensions were prepared from microorganisms obtained from the Texas A&M Food Microbiology Laboratory (College Station, TX) culture collection consisting of three strains of *E. coli* O157:H7. For ease of counting, *E. coli* O157:H7 isolates from the culture collection previously transformed by electroporation to express green fluorescent protein (GFP), red fluorescent protein (RFP) or yellow fluorescent protein (YFP) were used. The strains were stored at -80°C on CryoCare™ Bacterial Preservers (Key Scientific Products, Round Rock, TX). When needed, the frozen cultures were revived in TSB (Becton Dickinson, Sparks, MD) and incubated for 24 h at 37°C. 0.1 ml of each broth culture was plated on Tryptic Soy Agar (TSA; Becton Dickinson, Sparks, MD) supplemented with 100 mg/liter ampicillin (Sigma Chem. St. Louis, MO; TSA + Amp). The plates were incubated at 37°C for 24 h. A single colony of each strain was picked from the plates and transferred to TSA slants. Slants were incubated at 37°C for 24 h. Three days prior to the experiment the microorganisms were resuscitated by two consecutive transfers to TSB and incubated at 37°C for 24 h. Antibiotic resistance was confirmed by streaking TSB cultures onto plates

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*Portions of this section reprinted with permission from "Reduction of *Escherichia coli* O157:H7 and *Salmonella* on baby spinach using electron beam irradiation" by Neal, J. A., E. Cabrera-Diaz, M. Marquez-Gonzalez, J. E. Maxim, and A. Castillo. 2008. *J. Food Prot.* 71: 2415-2420. Copyright 2008 by the International Association for Food Protection.

of TSA + Amp and incubated at 35°C for 24 h. For the confocal scanning laser microscopy studies, one strain of *E. coli* O157:H7 harboring plasmid for the production of GFP and one strain of *S.* Typhimurium harboring RFP plasmids obtained from the Texas A&M Food Microbiology Laboratory culture collection were used for this experiment. Three days prior to the experiment the microorganisms were resuscitated by two consecutive transfers to TSB and incubated at 37°C for 24 h. A 24 h culture of GFP *E. coli* O157:H7 and RFP *Salmonella* in TSB was harvested and washed in sterile phosphate buffer saline (PBS; EMD Biosciences, Inc. La Jolla, CA) and resuspended in 0.1% peptone water.

Rifampicin-resistant mutants were derived from 5 parent strains of *E. coli* O157:H7 from the Texas A&M Food Microbiology Laboratory culture collection according to the method published by Kaspar and Tamplin *(177)*. Rifampicin-resistant mutants were isolated by spread plating 1.0 ml of a suspension containing approximately 10^{10} CFU/ml on TSA containing 100 µm of rifampicin (Sigma, St. Louis, MO; rif-TSA) per ml. Inoculated plates were incubated at 37°C for 24 h and several of the resulting colonies were streaked on rif-TSA plates. For the chemical decontamination studies as well as irradiation studies, rifampicin-resistant mutants described above were utilized. In addition, rifampicin-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 irradiation 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 TSA slants and incubated at 37°C for 24 h. Three days prior to each experiment the microorganisms were resuscitated by two consecutive transfers to TSB and incubated at 37°C for 12 h. Rifampicin resistance was confirmed by streaking TSB cultures onto plates of TSA + 100 mg/liter rifampicin and incubated at 35°C for 24 h. Enumeration of *E. coli* O157:H7 expressing fluorescent proteins were carried out on TSA + Amp.

Media preparation

Ampicillin served as the selective agent for the ampicillin-resistant *E. coli* O157:H7, which was inoculated onto spinach leaves. 0.1 g of ampicillin dissolved in 5 ml distilled water was added to 1 liter of sterilized TSA after adequate cooling and then poured into petri plates. Lactose-sulfite-phenol red-rifampicin agar (LSPR), a selective and differential medium designed for simultaneous enumeration of rifampicin-resistant *E. coli* O157:H7 and *Salmonella* was used for the chemical decontamination studies as well as irradiation studies *(65)*. Rifampicin-resistant *E. coli* O157:H7 produced yellow colonies on the medium whereas rifampicin-resistant *Salmonella* developed colonies with a black center surrounded by a pink halo. Rifampicin served as the selective agent for the rifampicin-resistant *E. coli* O157:H7 and *Salmonella*, which were inoculated onto spinach leaves. 0.1 g of rifampicin dissolved in 5 ml methanol was added to 1 liter of sterilized LSPR after adequate cooling and then poured into petri plates. APCs were conducted on Petrifilm™ Aerobic Count Plates (3M Microbiology Products, St. Paul,

MN). The plates were incubated at 35°C for 48 h and the developing red colonies were counted and reported as CFU/g. Enumeration of lactic acid bacteria was carried out using the deMan, Rogosa and Sharp broth (MRS; Difco) 2X concentration procedure *(1)*. MRS broth was prepared by adding 110 g to 1 liter of distilled water. A 1:10 dilution of the spinach sample was incubated anaerobically at 37°C for 48 h by placing AneroGen™ packets (Oxoid, Hampshire, UK) into anaerobic jars. Yeasts and molds were enumerated on Yeast and Mold Petrifilm™ (3M Microbiology Products) and were incubated for 5 days at 25°C.

Preliminary experiments

Growth curves. To ensure that the parent strains of *E. coli* O157:H7 and their rifampicin-resistant derivatives showed the same behavior under the studies using the Rif^+ markers, growth curves were compared for Rif^+ markers and their parent strains. For growth curves, 5 strains of *E. coli* O157:H7 and their rifampicin-resistant derivatives were grown on TSA slants for 24 h and then transferred to 9 ml TSB and incubated at 37°C over a 24 h period. The populations reached by the *E. coli* O157:H7 strains and the rifampicin-resistant derivatives under these conditions were determined to be ca. 9 log CFU/ml, as determined by serial dilutions and plate counts on rif-TSA incubated overnight at 37°C. Based on this information, serial dilutions were prepared in 0.1% peptone water for each culture to obtain a concentration level of approximately 5 CFU/ml and 0.1 ml of this culture was added to TSB tubes in triplicate. The tubes were then incubated at 37°C. At hourly intervals, triplicate tubes were separated for each

organism and 1.5 ml of the cultures were placed in 1.5 ml cuvettes (Brand Tech Scientific, Essex, CT) and placed in a spectrophotometer (Biomate 3, Thermo Electron Corp. Madison, WI). Absorbance was recorded at 600 nm wavelengths.

Acid sensitivity. The resistance to acid of *E. coli* O157:H7 and rifampicin-resistant mutants were tested by exposing these microorganisms to various pH levels with lactic acid. TSA (6 liters) was prepared in 6-1000 ml flasks (1 liter each) and autoclaved. After cooling (to ca. 50°C), lactic acid was added to each flask to develop a pH range from approximately pH 6.0 to 3.0. The media were stirred constantly while adding the lactic acid and the pH of the media was monitored using a portable pH meter (Model 250 A+, Thermo Orion, Beverly, MA). Plates of TSA at the various pH levels were poured and let to dry by storing overnight at room temperature. Serial dilutions of a 12-h culture of each strain (both parent and rif-resistant *E. coli* O157:H7) were spread plated onto TSA/acid plates in duplicate using sterile bent glass rods for each pH level. Plates were then incubated for 24 h at 37°C and counted.

Irradiation resistance. The resistance of the parent stains and rifampicin-resistant mutants of *E. coli* O157:H7 to electron beam (e-beam) irradiation was tested. Washed-cell suspensions were prepared by dispensing each pathogen separately in sterile 15 ml centrifuge tubes (Fisher Scientific, Pittsburg, PA) and harvested by centrifugation at 1623 x *g* in a Jouan B4i centrifuge (Thermo Electron Corp., Madison, WI) for 15 min. The pellet for each microorganism was resuspended in 5 ml of 0.1% peptone water (Becton Dickinson). Each mutant strain was at a concentration of ca. 8 log CFU/ml, as determined by serial dilution and plate count on rif-TSA incubated 24 h at 37°C. The

resuspended microorganisms were inoculated onto gelatin (Becton Dickinson) according to the method described by Rodriguez et al. *(274)*. In their study, surrogates were identified to describe the radiation sensitivity of the most common pathogens encountered in fruits. Three inoculated gelatin discs per microorganism were placed in sterile petri dishes, sealed with parafilm, and placed in stomacher bags (Classic 400, Seward, Bohemia, NY) to prevent any leakage or contamination and sealed using an Impulse Heat Sealer (American International Electric, New York, NY). Samples were irradiated at the Food Technology Facility for Electron Beam and Space Food Research at Texas A&M University (College Station, TX) by placing in a single layer inside cardboard boxes on a conveyor belt and exposing to 1.2 kGy electron beam irradiation. Colonies of both parent stains and rifampicin-resistant mutants of *E. coli* O157:H7 were counted independently and the counts reported separately as number of CFU per ml. Colony counts were converted into log value for statistical analysis.

Inoculation method. A protocol for inoculating spinach leaves was created to ensure an even distribution of the inoculum throughout the spinach leaves in the samples. 25 g samples of spinach were placed into stomacher bags and 2.5 ml of crystal violet (Becton Dickinson) was added in order for the researcher to observe areas on the leaves that may not come into contact with the inoculum. The spinach samples with the dye solution were shaken for 15, 30 or 60 s. Leaves were visually observed to determine whether all leaves became stained evenly.

Optimum packaging material for spinach leaves. To determine the optimum material for packaging spinach leaves, spinach leaves were weighed at 25, 50 and 75 g

and packed into 2 types of 15 cm x 19 cm polyethylene bags with varying thicknesses. The 2 bags investigated were stomacher bags and vegetable storage film (CPM4090596, Cryovac Sealed Air, Duncan, SC). The bags were sealed using a vacuum packaging machine (Model X180, Koch Inc., Kansas City, MO) and stored for 4°C for 8 days. At the end of the 8 days, gas samples were taken from the bag using an air-tight syringe and analyzed for percentage of carbon dioxide using an infrared gas analyzer (Horiba Model PIP-2000, Irvine, CA) and oxygen using an oxygen analyzer (S-3A/I, AEI Technologies, Inc., Pittsburgh, PA). The package and spinach weight combination that had the lowest oxygen permeation rate at the same time preventing anaerobic fermentation of the spinach was chosen for the irradiation and storage of fresh spinach.

Growth and survival of *Escherichia coli* O157:H7 on spinach leaves

Preparation of inoculum. Nine ml of a 24 h culture of each GFP, RFP and YFP *E. coli* O157:H7 in TSB grown separately was dispensed in sterile 15 ml centrifuge tubes and harvested by centrifugation at 1623 x *g* for 15 min. The pellet for each microorganism was resuspended in 5 ml of 0.1% peptone water and then 1 ml aliquots of each were combined to make a cocktail in a sterile bottle containing 97 ml 0.1% peptone water. The average initial concentration of pathogens on the spinach leaves was 3.8 CFU/ml, as determined by serial dilutions and plate count on TSA + Amp incubated 24 h at 37°C.

Product obtainment and inoculation. Fresh bagged spinach obtained from a produce distributor (Beaumont, TX) typical of leafy greens entering the U.S. food supply

was selected for use in this study. After purchasing, the packaged spinach was placed in coolers with an internal temperature of 4°C for 2 h and transported 100 miles directly to the Texas A&M Food Microbiology Laboratory, where it was stored at 4°C for up to 24 h. Spinach leaves used in this experiment were washed during packing but were not washed or decontaminated after being removed from the original retail packaging. Using aseptic technique, 25-g portions of spinach leaves were dispensed in into sterile stomacher bags. These spinach samples were inoculated by distributing 2.5 ml of the prepared cocktail over the spinach samples and shaken for 1 min to distribute throughout.

Survival studies. The inoculated spinach samples were stored at 4°C and 10°C for 7 days. At 2 day intervals during storage, samples were separated and subjected to bacterial enumeration. Additional inoculated spinach packets were stored at 22°C over 24 h. At 4 h intervals for 24 h, samples were separated for bacterial enumeration.

Bacterial enumeration. Fluorescent *E. coli* O157:H7 were enumerated by adding 225 ml 0.1% peptone water to the samples and pummeled in a stomacher (Stomacher 400, Seward, London UK) for 1 min. Appropriate serial dilutions were made from this homogenate and surface spread on TSA + Amp using a sterile glass bent rod. The TSA + Amp plates were incubated at 37°C for 24 h. GFP *E. coli* O157:H7 produced green colonies on the medium, RFP *E. coli* O157:H7 produced red colonies on the medium and YFP *E. coli* O157:H7 produced yellow colonies on the medium under ultra violet light. Colonies of each color were counted independently and the counts reported separately as number of CFU/g. Colony counts were converted into log value for

statistical analysis. Confirmation tests were conducted to verify the identities of the colonies using biochemical tests. Presumptive screening of *E. coli* O157:H7 was conducted by using BBL® Enterotube II™ (Becton Dickinson). Presumptive-positive isolates were confirmed by using the RIM™ *E. coli* O157:H7 Latex Test (Remel, Lenexa, KS). O157 positive isolates produced agglutination of finely dispersed particles and were further tested with the H7 reagent which also produced agglutination of fine particles.

Use of CSLM to identify the attachment sites for *E. coli* O157:H7 and *Salmonella* on the leaf and stem of spinach

Preparation of inoculum and sample preparation. A microbial cocktail was prepared consisting of 1 ml of GFP *E. coli* O157:H7 and RFP *Salmonella* and added to 8 ml 0.1% peptone water. Samples consisting of 4 spinach leaf pieces measuring 1 cm^2 and 4 stem samples measuring 0.5 cm^2 were placed in the cocktail for 24 h at 37°C.

Attachment. For the confocal microscopy experiments, cell attachment was desirable. To ensure that the organisms being visualized were in fact attached to the spinach regions studied, excised samples of 4 spinach leaves measuring 1 cm^2 and 4 stem samples measuring 0.5 cm^2 were inoculated by dipping in 9 ml of a suspension of GFP *E. coli* O157:H7 and RFP *Salmonella* and colony counts were conducted by surface spreading onto TSA + Amp. The inoculated samples were then washed twice in 0.1% peptone water. Bacterial counts were conducted for the washed spinach samples as well as for the wash water by surface spreading onto TSA + Amp. Bacteria growing from the

wash solution were loosely attached, whereas those that were grown from the spinach samples after washing in peptone water were considered to be strongly attached to the spinach surfaces.

Confocal microscopy. Spinach samples were observed using a BioRad Radiance 2000MP confocal microscope (Zeiss, Heltfordshire, UK) using an excitation wavelength of 488 nm. The confocal microscopy was conducted at the Image Analysis Laboratory at Texas A&M University (College Station, TX).

Effectiveness of chemical sanitizers on the reduction of *E. coli* O157:H7 and *Salmonella* inoculated on spinach

Preparation of inoculum. Nine ml of a 24 h culture of five strains each of rifampicin-resistant *E. coli* O157:H7 and rifampicin-resistant *Salmonella* Agona, Gaminara, Michigan, Montevideo, Poona and Typhimurium was dispensed in 50 ml sterile centrifuge tubes (Fisher Scientific) and harvested using the method described above. One-ml aliquots of each microorganism were combined to make a cocktail in a sterile bottle containing 89 ml 0.1% peptone water. The prepared inoculum containing each pathogen at a concentration of ca. 8 log CFU/ml, as determined by serial dilution and plate count on rif-TSA incubated 24 h at 37°C, was used within 2 h after preparation and was kept at 23-24°C during the experiment.

Preparation of spinach leaves. Fresh spinach leaves typical of leafy greens entering the U.S. food supply were selected for use in the study (Crystal City, TX) and was generously donated by the Winter Garden Spinach Producers Board. 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 not washed or decontaminated in any manner before the spinach was obtained for this study. The fresh spinach leaves were randomly separated in 25-g portions and placed in individual stomacher bags and then inoculated with 2.5 ml of the cocktail described above. The stomacher bags were closed and shaken for 1 min to distribute the organisms in the spinach sample. To allow for attachment of both the *E. coli* O157:H7 and *Salmonella,* spinach samples were stored for 2 h at 22°C before exposing them to the various treatment solutions. The initial concentrations of *E. coli* O157:H7 and *Salmonella* on the baby spinach were 6.9 CFU/g and 7.0 CFU/g respectively.

When applying the treatments, the spinach leaves were kept separated to prevent them from sticking together, which may result in entrapped pathogens not being exposed to the treatment and in an uneven application of the treatment. This was achieved by placing onto sterile open cell plastic screens (2cm x 2 cm cell, Hunter Douglas, Poway, CA) using sterile forceps.

Application of treatments. Samples of inoculated spinach leaves weighing 25 g were placed on sterile plastic screens and sprayed with distilled water using a non-corrosive 2 liter hand-held sprayer (Scotts Miracle Grow, Marysville, OH). The sprayer was calibrated to deliver 250 ml of solution at 9 psi in 15 s and the treatments were carried out for 30 s. Washed samples were allowed to dry for 15 min and were then subjected to antimicrobial treatments. The treatments tested included spraying with 55°C lactic acid, calcium hypochlorite and peroxyacetic acid solutions; dipping in

ozonated water, and treatment with chlorine dioxide gas. The spinach receiving only the water wash described above was used as control. For lactic acid treatment, a 2% L- lactic acid solution (Purac, Linconshire, IL) was prepared by diluting 88% L-lactic acid with distilled water. The temperature of the solution was heated and maintained at 55°C at the time of treatment. Peroxyacetic acid solution (Sigma) was prepared by adding 0.2 ml of the acid to 999.8 ml of distilled water to obtain a concentration of 80 mg/liter. The temperature of the solution was maintained at 25°C at the time of treatment. Chlorinated water was prepared by dissolving calcium hypochlorite (Sigma) in distilled water to a concentration of 200 mg/liter free chlorine. The amount of free chlorine was determined using a Hatch chlorine test kit (Model CN-DT, Hatch Company, Loveland, CO). The pH of the solution was adjusted with sodium hydroxide to 6.5 $\pm$ 2 and measured with a portable pH meter (Model 250 A+, Thermo Orion, Beverly, MA). The temperature of the solution was maintained at 25°C at the time of treatment. All these treatments were applied by spray using a hand-held sprayer (Scotts Miracle Grow). The hand-held sprayers were calibrated to deliver 250 ml of solution in 15 s. The treatment was applied for 15 sec on one side then turning the spinach leaves using sterile forceps to treat the opposite side of the leaves for an additional 15 s. Uniform application of water washes and antimicrobial treatments were conducted by the same person throughout the study.

For ozone treatment, ozone was obtained using a Lotus® ozone generating system (Tersano, Buffalo, NY). Treatments with approximately 6.5 mg/liter ozone were performed by submerging inoculated spinach into 2 liters of distilled water in the sanitizing system's bowl attachment. Ozone was generated by creating an electrical

discharge to super-oxygenate distilled water, creating O_3. Ozone treatment was tested at 15 min and 30 min treatments. The concentration of ozone in the sanitizing system's bowl was measured using a spectrophotometer immediately after ozone was produced, at one half of the total treatment time, and at the end of the treatment time. Ozone absorbance was recorded at 255 nm wavelengths. Concentration of ozone in the water was measured using the formula:

$$\text{Ozone [ppm] in water} = \text{absorbance} \times 160.5$$

After treatment, spinach samples were allowed to dry and then sampled for microbial analysis.

Chlorine gas treatment was conducted in a model gas cabinet was made using a desiccation chamber to duplicate storage and/or shipping. A Scentrex® DTS 1.05 ClO_2 gas generating sachet (Aldrich, Milwaukee, WI) was activated by shaking it and enclosing it within the gas chamber along with 10 ml of distilled water (to maintain high humidity) and the inoculated spinach leaves. Concentration of the chlorine gas was measured by placing the sachet in a 1 liter screw top glass container (Kerr, Jarden Home Brands, Daleville, IN) containing 50 ml of potassium iodide for 1 h and then placed in multiple jars over for an additional 30 min and 1 h. The contents of the jar were analyzed for chlorine dioxide in mg by iodometric titration *(101)*. Inoculated spinach leaves were exposed to 1.2 and 2.1 mg/liter ClO_2 gas for 30 min and 1 h respectively. All experiments, accounting for each condition and organism were replicated two times.

Microbiological analysis. After the treatments were conducted, 225 ml of sterile 0.1% peptone water was added to each of the spinach samples in stomacher bags and

pummeled in a laboratory blender for 1 min. Serial dilutions were made and spread-plated on LSPR. Plates were incubated for 24-28 h at 35°C. Counts of *E. coli* O157:H7 and *Salmonella* were made independently. Colony counts were reported as CFU/g.

For each trial of sample analysis, 10 representative colonies of both *E. coli* O157:H7 and *Salmonella* were picked from LSPR plates for further confirmation. Presumptive screening of *E. coli* O157:H7 was conducted by using BBL® Enterotube II™ (Becton Dickinson). Presumptive-positive isolates were confirmed by using the RIM™ *E. coli* O157:H7 Latex Test. O157 positive isolates produced agglutination of finely dispersed particles and were further tested with the H7 reagent which also produced agglutination of fine particles. For *Salmonella,* confirmation was conducted using triple sugar iron (TSI; International BioProducts, Bothell, WA) and lysine iron agar (LIA; International BioProducts) slants and serologically using the agglutination reaction with *Salmonella* O Poly A-I and Vi antiserum (Becton Dickinson). On the TSI slant, typically *Salmonella* displayed an alkaline slant (red) and acid butt (yellow) with H_2S production in the form of black precipitation near the base of the slant. When using LIA, *Salmonella* produced an alkaline reaction (purple) with hydrogen sulfide (H_2S) production. The serological test was carried out by adding 1 drop of 0.85% saline solution to a glass slide. Bacterial growth was combined with the saline drop and 1 drop of *Salmonella* O Poly-A-I and Vi antiserum was added and mixed. A positive reaction for *Salmonella* was confirmed by agglutination in the drop.

Efficacy of e-beam irradiation for the reduction of *E. coli* O157:H7 and *Salmonella* on spinach

Inoculum preparation. The method used for inoculating the rifampicin-resistant strains of *Salmonella* and *E. coli* O157:H7 during the irradiation studies was identical to the method described above for determining the effectiveness of chemical sanitizers on the reduction of these pathogens on spinach.

Product obtainment and inoculation. Fresh baby spinach typical of leafy greens entering the U.S. food supply were 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 a bacterial cocktail including five strains each of mutant *E. coli* O157:H7 and mutant *S.* Agona, Gaminara, Michigan, Montevideo, Poona and Typhimurium 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 and 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 CFU/g and 7.3 CFU/g respectively.

Irradiation. All e-beam irradiation treatments were conducted at the Food Technology Facility for Electron Beam and Space Food Research at Texas A&M University. A pit and tower system with two 10 MeV and 15 kW linear accelerators was used for this experiment (Varian Linac, L3 Communications, San Leandro, CA) using 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, NY) 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 (HDPE; King Plastic Corporation, North Port, FL) 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 electron beam irradiation from a linear accelerator. Dose absorption was calculated from the dosimeters strips using an electron paramagnetic resonance instrument (EMS 104 EPR Analyzer, Bruker Instruments, Karlsruhe, Germany). Non-irradiated spinach served as control for this experiment. All experiments, accounting for each condition and organism were replicated three times.

Bacterial enumeration. After e-beam irradiation, 90 ml of sterile 0.1% peptone water was added to each of the spinach samples in stomacher bags and pummeled in a stomacher (Seward) for 1 min. Serial dilutions were made and spread-plated on LSPR. Plates were incubated for 24-28 h at 35°C. Rifampicin-resistant *E. coli* O157:H7 produced yellow colonies on the medium whereas rifampicin-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 g samples of irradiated, inoculated spinach were enriched in 225 ml TSB plus 22.5 mg rifampicin, incubated at 37°C and streaked for growth onto LSPR after days 0, 2, 4, 6 and 8.

D_{10}-value for *E. coli* O157:H7 on irradiated spinach leaves

Microorganisms. Rifampicin-resistant *E. coli* O157:H7 was prepared and inoculated onto 10 g spinach samples as described above. Since it was determined during preliminary experiments that there was no significant difference in the resistance of all strains to irradiation, a single strain was selected for determining the D_{10} value to prevent any variation in the results**.** The initial concentration of *E. coli* O157:H7 on the baby spinach was 6.4 CFU/g.

Dosimetry. 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 the spinach packets and placed alongside the spinach

samples. Dosimeters were not placed inside the sealed samples due to the presence of pathogens.

Bacterial enumeration. After irradiation, each sample was mixed with 90 ml sterile 0.1% peptone water in stomacher bags and pummeled for 1 min. Aliquots of the homogenate were serially diluted 10-fold and spread-plated onto rif-TSA. Counts of *E. coli* O157:H7 were calculated as CFU/g. Rifampicin resistance was confirmed by streaking TSB cultures onto plates of rif-TSA and incubated at 35°C for 24 h.

Microbiological and sensory characteristics of spinach

Sample preparation. Fresh spinach leaves typical of leafy greens entering the U.S. food supply were selected for use in the study (Crystal City, TX). The spinach leaves were unwashed and boxed in the field. After transporting to the Texas A&M Food Microbiology Laboratory, spinach leaves were sorted to remove leaves that were bruised, cut or had decay. The spinach was then washed for approximately 1 min under running tap water to remove dirt and debris. The spinach was then allowed to dry for approximately 15 min and then spun in a salad spinner (Oxo International, Ltd., El Paso, TX) for 2 min to remove additional moisture. Samples were weighed to 75 g in stomacher bags, sealed, labeled and returned to the refrigerator until needed.

Irradiation. E-beam irradiation treatments were conducted at the Food Technology Facility for Electron Beam and Space Food Research. Prior to treatments, dose mapping was conducted to determine the appropriate attenuation scheme and conveyor speed to achieve the target doses using alanine pellet dosimeters. HDPE sheets

were used as attenuators to reduce the energy of incident electrons in order to achieve the desired doses. Spinach samples were exposed to doses of 0.7 and 1.4 kGy electron beam irradiation from a linear accelerator. Dose absorption was calculated as described above. Non-irradiated spinach served as control for this experiment. Spinach packets were placed in a single layer inside cardboard boxes on a conveyor belt and exposed to either 0.7 or 1.4 kGy electron beam irradiation. The dose of 0.7 kGy corresponds to the highest D_{10} value for non-sporeforming pathogens *(239)* and the higher dose was chosen to investigate the effects on quality of spinach at double the dose intensity. Zero-day samples were analyzed immediately and the remaining samples along with the controls were refrigerated at 4°C for up to 35 days. Samples were removed for microbial, sensory evaluation, color analysis and gas composition periodically.

Microbial quality. APC was carried out to determine the microbial load of the samples. At each sampling time, spinach packets were aseptically opened using a scalpel sterilized by dipping in 95% ethanol and then igniting in the flame of a bunsen burner. A 25-g sample was weighed out from each packet and placed in a stomacher bag with 225 ml 0.1% peptone water. The mixture was then pummeled in a stomacher for 1 min. Appropriate serial dilutions were made from the homogenate and inoculated onto Petrifilm™ Aerobic Count Plates and enumerated. Ten characteristic LAB colonies were randomly picked from the Petrifilm Aerobic Count Plates for LAB and transferred to TSA slants for further confirmation using gram staining, catalase reaction and the oxidation/fermentation test using Hugh-Leifson medium (Becton Dickinson) .

Respiration rates. An additional experiment was conducted to determine the effect of irradiation 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 irradiation 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 non-irradiated controls and placed in separate 1 liter gas-tight glass containers 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 one-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_2 and CO_2. Gas samples were withdrawn from the jars using an airtight syringe and analyzed for percentage of oxygen using an oxygen analyzer (S-3A/I AEI Technologies Inc., Pittsburgh, PA) and carbon dioxide using an infrared gas analyzer (Horiba Model PIR-2000, Irvine, CA). Respiration rates were estimated from the O_2 and CO_2 concentrations.

Gas composition. Gases composition (O_2 and CO_2) inside the packages were measured for the duration of the experiment (37 days). Spinach samples were stored at 4°C during the length of the study. Internal gases were withdrawn using an airtight syringe and analyzed for percentage of carbon dioxide using an infrared gas analyzer and oxygen using an oxygen analyzer.

Sensory evaluation. Objective color was determined by measuring the color of three leaves within each spinach packet using a Hunter colorimeter (Hunter Assoc, Reston, VA). The L^*, a^* and b^* color space values were recorded and hue and chroma

values were calculated. Hue was calculated using the formula arctan (b^*/a^*) and chroma was calculated using the formula $[(a^*)^2 + (b^*)^2]^{1/2}$.

The sensory analysis for the spinach was carried out by an expert, trained 5-member descriptive attribute sensory panel at the Sensory Testing Facility at Texas A&M University (College Station, TX). Panelists were selected as described by the American Meat Science Association *(10)* and Meilgaard et al. *(214)*. Training and ballot development sessions were conducted to determine color, texture, aromatics, basic tastes and mouthfeel attributes of spinach. Products for training represented spinach from the treatments and storage times defined in the study. Terms from Ceville and Lyon and Meilgaard et al. *(77, 214)* were used to assist in identifying attributes. For color, paint cards ranging from yellow to dark green were obtained. A color reference ballot was developed so that 1 = pale green and 15 = dark green. Color standards used were; SW6413, SW6715, SW6414, SW6407, SW6716, SW6718, SW6719, SW6720, SW6733, SW6445, SW6734, SW6446, SW6440, SW6433, and SW6426 (Sherwin-Williams Co., Cleveland, OH). Spinach samples were evaluated for aromatics (green/grassy, earthy, musty, nutty and decay); textural attributes (surface wetness, roughness, juiciness, tooth packing, hardness, slimy); basic tastes (sweet, bitter and sour); and mouthfeel factors (astringent and burn) as defined in Table 1. Descriptive attributes were evaluated using the 15-point Universal Spectrum™ scale where 0 = none and 15 = extremely intense. After training, panelists evaluated 12 samples per day while seated in individual booths separated from the sample preparation area. Samples were identified with random 3-digit codes and served treatments in random order. Warm-up

TABLE 1. *Texture, basic tastes, aromatics, and mouthfeel attribute definitions, references and intensity based on a 15-point scale established after consensus.*

Attribute	Definition	Reference
Texture		
Surface wetness	The amount of moisture, due to an aqueous system on the leaf surface	Carrots = 3.0
Surface roughness	The amount of particles on the surface	Orange peel = 5.0
Juiciness	The amount of juice/moisture perceived in the mouth	Mushroom = 3.0
Tooth packing	The degree to which product sticks on the surface of teeth	Mushroom = 3.0
Hardness	The force to attain a given deformation such as force to compress between molars	Cheddar cheese = 4.5
Slimy	Of the nature of slime, viscous, moist	Boiled carrots = 5.0
Basic tastes		
Bitter	Basic taste stimulated by bitter agents such as caffeine, quinine, etc.	Caffeine (0.15% solution) = 10.0
Sweet	Basic taste stimulated by sugars	Chocolate bar (The Hershey Co., PA, US) = 10.0
Sour	Basic tastes stimulated by acids	Lemon juice(Real Lemon®, Mott's Inc. Stanford, CT, US) =8.0

TABLE 1 (*Continued*).

Attribute	Definition	Reference
Aromatics		
Green/grassy	Aromatic associated with fresh vegetation and green vegetables	Freshly broken green beans = 15.0
Earthy	Aromatic characteristic of damp soil or wet foliage	Potato peels = 8.0
Musty	Aromatic characteristic of damp/wet basements or turned soil	Peat moss = 5.0
Nutty	Aromatic associated with nuts or nut meats	Swiss cheese = 5.0
Decay	Aromatic associated with decaying vegetation	Water containing floral/greenery stems (2-3 days) = 10.0
Mouthfeels		
Burn	Feeling factor associated with high concentrations of irritants to the mucous membrane of the oral cavity	White vinegar (H.J. Heinz CO., Pittsburg, PA, US) = 15
Astringent	The shrinking or puckering of the tongue surface caused by substances such as tannins or alum	Cooked 1.27 cm sample of liver = 15

samples taken from the controls were given to the panelists each sensory evaluation day to standardize the panelists within a day. Panelists were provided double distilled deionized water, saltless saltine crackers and fat-free ricotta cheese to cleanse their palates between samples. Samples were served within a minimum of 4 min apart to reduce halo effect or palate fatigue. Panelists were given 6 samples per session with a 20 min break in between each session. On each day of sampling, spinach samples were removed from the refrigerator at least 1 h prior to sampling in order for them to equilibrate to 23-24°C. Panelists were served the spinach leaves on disposable 6 in. plates. Panelists evaluated the samples for color first and then bit into the leaves to evaluate the flavor and texture attributes.

Data analysis

Colony counts were calculated as CFU/g and converted into log values for statistical 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 data recorded for each parameter was analyzed by analysis of variance using the ANOVA procedures of SAS (SAS 9.1, Statistical Analysis System Institute, Cary, NC). When ANOVA indicated that there was a significant difference ($P<0.05$), mean separation was carried out using the Duncan's multiple range test. For the effect of aqueous sanitizers and irradiation doses on the reduction of pathogens, the ANOVA procedure of SAS was utilized. For the D_{10}-value study, the numbers of surviving *E. coli*

O157:H7 against increasing doses of irradiation were plotted and a linear regression chart was created.

The D_{10}-value was determined from the reciprocal of the regression line as the dose in kGy required to reduce the population of *E. coli* O157:H7 by 1 log cycle. Microbiological data for APCs, LAB and yeast and mold counts were analyzed by linear regression to establish the effect of different treatments on the bacterial counts over time. For the sensory evaluation, the PROCGLM Procedures of SAS was utilized. The data were first analyzed to determine panelist effects in a split-split plot design. Least square means were separated when effects were significant in the ANOVA table ($P<0.05$). Panelist and panelists by main effect interactions were tested. Panelists by main effect interactions were not significant; therefore, these were pooled into the error term. For the gas composition study, the ANOVA procedure of SAS was utilized and least square means was obtained and separated when effects were significant in the ANOVA table ($P< 0.05$). The whole plot included the main effects of irradiation treatment and field as a block. The whole plot error was treatment by field. The split plot included storage day as a main effect and storage day by treatment interaction. The split plot was storage day by treatment by field. The second split included panelist, panelist by treatment and panelist by storage day. The error term was defined as storage day by treatment by field by panelist. Interactions with panelist were examined and if significant ($P<0.05$), data indicated that panelists did not consistently rate spinach within the effect consistently. If panelist interactions were not reported, data were averaged across panelist. For the final analysis, data were analyzed by analysis of variance using a repeated measures, split-plot

model with PROCGLM. The whole plot included field as a block and irradiation treatment as a main effect. The whole plot error term was defined as treatment by field. The split included storage day as a repeated measure and irradiation by storage day interaction was also included in the split. The split error term was defined as irradiation treatment by storage day by field. When significance was determined in analysis of variance ($P<0.05$), least squares means were calculated and differences between means were determined using the pdiff function ($P<0.05$).

RESULTS AND DISCUSSION*

Preliminary studies

Growth curves. To ensure that the parent strains of *E. coli* O157:H7 and their rifampicin-resistant derivatives showed the same behavior under the studies using Rif^{+} markers, growth curves were compared for Rif^{+} markers and their parent strains. The growth curves obtained for *E. coli* O157:H7 and their rif- resistant derivatives had no significant differences in the growth rates of the parent strains and their corresponding rif-resistant strains developed for this study. The growth curves of parent strains are shown in Figure 1 and variations in growth rates among parent strains were not significant. Figure 2 demonstrates the growth curves of the rif-resistant strains. Variations in growth patterns among rif-resistant strains were not significant. These similarities in growth patterns suggested that the mutation for resistance to rifampicin did not change the growth characteristics of these markers.

Acid sensitivity. Acid sensitivity experiments were conducted with parent strains of *E. coli* O157:H7 and their rif-resistant mutants to determine if they responded similarly to acidic conditions. Results from the initial trial to evaluate the acid sensitivity of the bacteria strains used in this study (Table 2) revealed no differences between the growth of the parent and rif-resistant counterpart of *E. coli* O157:H7 when tested at

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*Portions of this section reprinted with permission from "Reduction of *Escherichia coli* O157:H7 and *Salmonella* on baby spinach using electron beam irradiation" by Neal, J. A., E. Cabrera-Diaz, M. Marquez-Gonzalez, J. E. Maxim, and A. Castillo. 2008. *J. Food Prot.* 71:2415-2420. Copyright 2008 by the International Association for Food Protection.

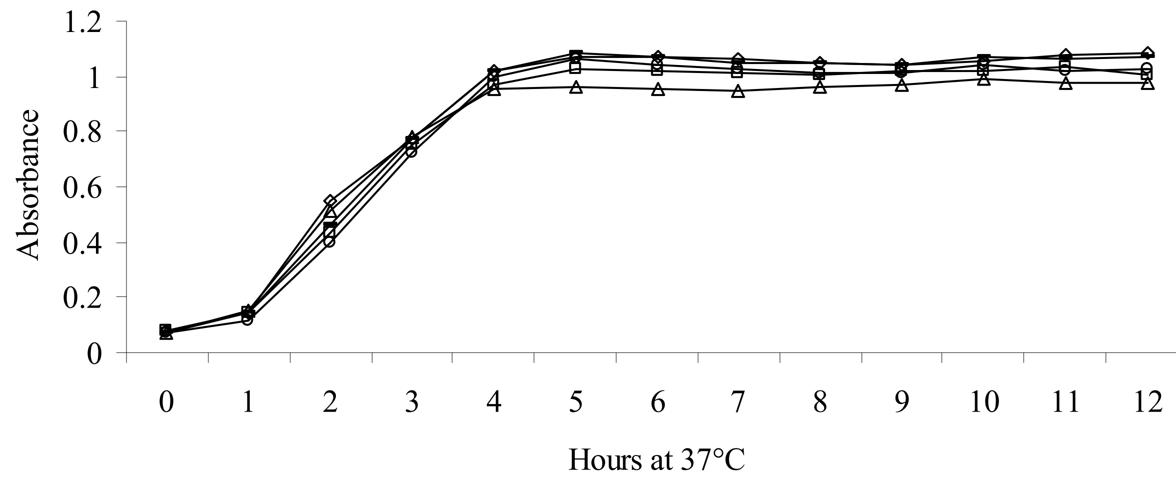


Figure 1. *Growth curves for* E. coli *O15:H7 cultures grown at 37°C in TSB.* □ *Parent strain 1,* ◊ *Parent strain 2, Δ Parent strain 3,* ○ *Parent strain 4, - Parent strain 5.*

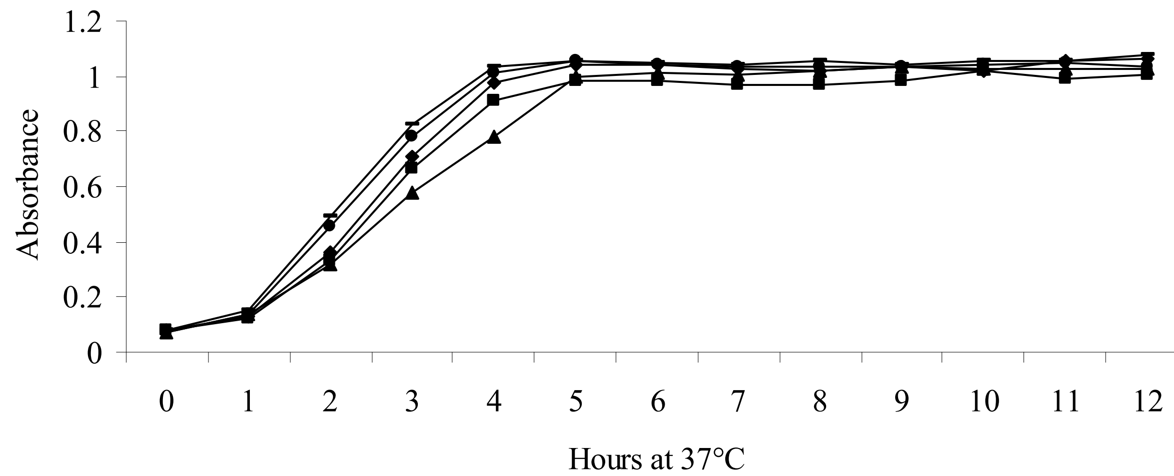


Figure 2. *Growth curves for rifampicin-resistant* E. coli *O15:H7 cultures grown at 37°C in TSB.* ■ *Mutant strain 1,* ♦ *Mutant strain 2,* ▲ *Mutant strain 3,* ●*Mutant strain 4, - Mutant strain 5.*

TABLE 2. *Counts (log CFU/ml) of* E. coli *O157:H7 and their rif-resistant derivatives on Tryptic Soy Agar (TSA) at different pH values.*

	pH			
Organism	7.01	5.92	4.95	3.53
E. coli O157:H7	1.7A[a]	1.6A	1.6A	NG[b]
rif-*E. coli* O157:H7	1.6A	1.6A	1.6A	NG

[a] Means within columns with the same letter are not significantly different (P>0.05).
[b] NG= no growth observed after incubation of plates for 24 h at 37°C.

varying pH levels using lactic acid as an acidulant. This indicates that the use of rif-resistant markers would reflect the acid sensitivity of parent strains of *E. coli* O157:H7. The use of labeled markers is advantageous since the enumeration methods may be simplified considerably.

Irradiation resistance. The resistance of the parent strain and mutant strains of *E. coli* O157:H7 to e-beam irradiation was tested to ensure that they both show the same behavior. Counts of both rif-resistant organisms and their parent strains receiving a dose of 1.2 kGy e-beam were not significantly different ($P>0.05$). This data is shown in Table 3. The similarities found in growth patterns, sensitivity to acid using lactic acid as an acidulant, and sensitivity to irradiation of the rif-resistant strains of *E. coli* O157:H7 and their parent strains validated the use of rif-resistant derivatives as markers in the present research as representing regular strains of *E. coli* O157:H7.

Inoculation method. A protocol for inoculating spinach samples was created. For the inoculation experiment, 25 g samples of spinach were placed into stomacher bags and 2.5 ml of crystal violet were shaken for 15, 30 or 60 s. For samples shaken for 15 s, the crystal violet dye remained in the center of the leaves indicating that more time was needed. Samples shaken for 30 s had more dye spread throughout the sample, but still had leaves in the corners of the stomacher bags that did not come into contact with the dye (data not shown). From these results, it was determined that the most effective way

TABLE 3. *Estimated log reductions (ELR)*[a] *(log_{10} CFU/g) for 5 strains of* E. coli O157:H7 *and their rif-resistant derivatives in gelatin discs as affected by e-beam irradiation.*

Strain	Condition	ELR
1	Parent strain	6.9A[b]
	Rif-resistant	6.8A
2	Parent strain	6.7A
	Rif-resistant	6.9A
3	Parent strain	6.3A
	Rif-resistant	6.4A
4	Parent strain	6.4A
	Rif-resistant	6.3A
5	Parent strain	6.3A
	Rif-resistant	6.2A

[a]Estimated log reduction: (log CFU/g on control cylinder – log CFU/g on cylinder after treatment).
[b] Means within columns with the same letter are not significantly different ($P>0.05$).

to inoculate the spinach samples was to inoculate the samples in the stomacher bags and shake for 1 min for even distribution on all leaves throughout the stomacher bag. This inoculation method was carried out throughout the remaining experiments.

Optimum packaging material for spinach leaves. Preliminary experiments were conducted to determine the optimum packaging material for spinach leaf samples used in the following experiments. Of the packaging materials tested, the vegetable storage film had lower oxygen levels in the packages (Fig. 3). Bags containing 75 g of spinach did not show anaerobic fermentation. However, stomacher bags were able to retain higher levels of CO_2. From these results, stomacher bags containing 75 g of spinach were chosen for the irradiation study.

Growth and survival of *E. coli* O157:H7 on spinach

Growth and survival. Populations of *E. coli* O157:H7 strains which were transformed by electroporation to express green, red and yellow fluorescent proteins (GFP, RFP AND YFP) and inoculated onto spinach leaves grew by 1.4, 1.2 and 0.9 log CFU/g respectively during the 12 h storage period held at 21°C (Fig. 4). When the inoculated spinach was stored at 10°C, *E. coli* O157:H7 did not grow, but was able to survive the 7 day storage period without any significant change in counts (P>0.05) (Fig. 5). For spinach samples stored at 4°C for 7 days, *E. coli* O157:H7 strains were not able to grow, and although not statistically significant, a decrease in counts was detected by day 5 (Fig. 6). The 3 strains used showed a similar behavior in all experiments with the

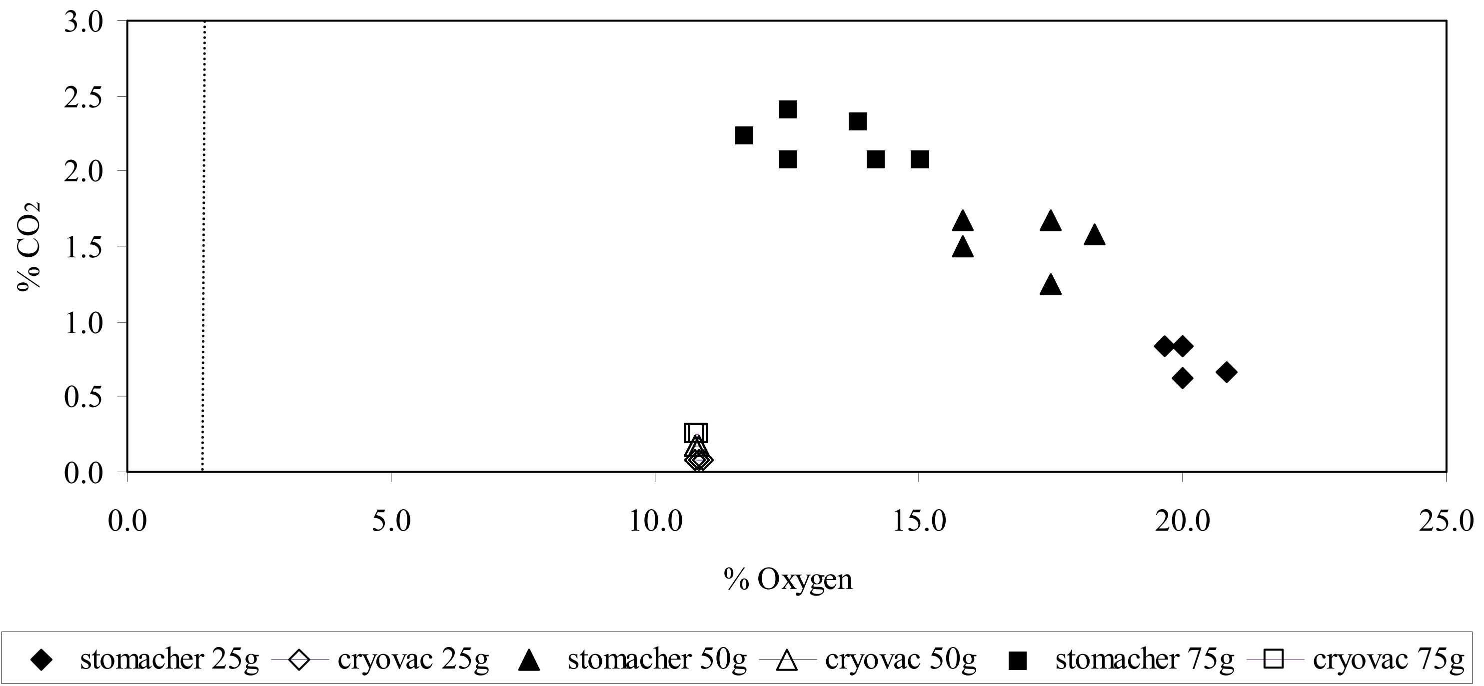


Figure 3. *Gas composition of stomacher and cryovac vegetable storage film containing different amounts of spinach after storage at 4°C for 8 days. The dotted line indicates the beginning of anaerobic fermentation.*

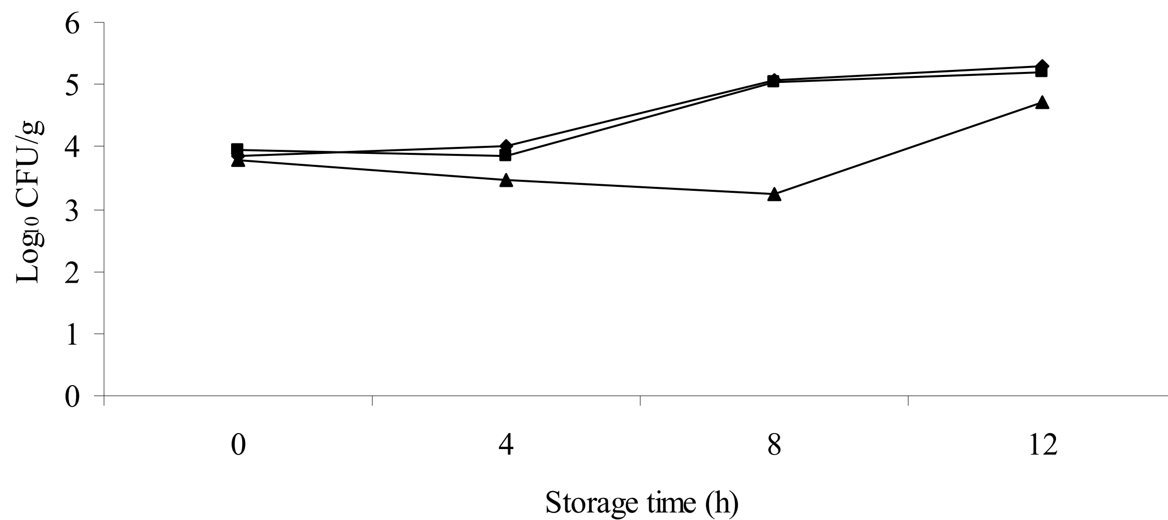


FIGURE 4. *Survival and growth of* E. coli *O157:H7 on spinach leaves stored at 21°C for 24 h*. ● *GFP*, ■ *RFP and* ▲ *YFP*.

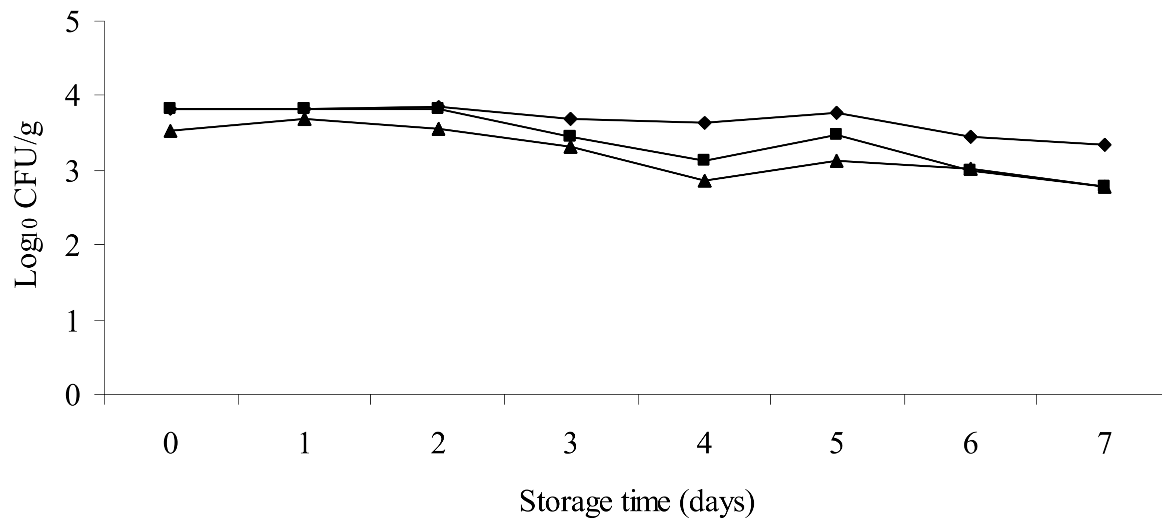


FIGURE 5. *Survival and growth of* E. coli *O157:H7 stored at 10°C on spinach leaves for 7 days*. ● *GFP*, ■ *RFP and* ▲ *YFP*.

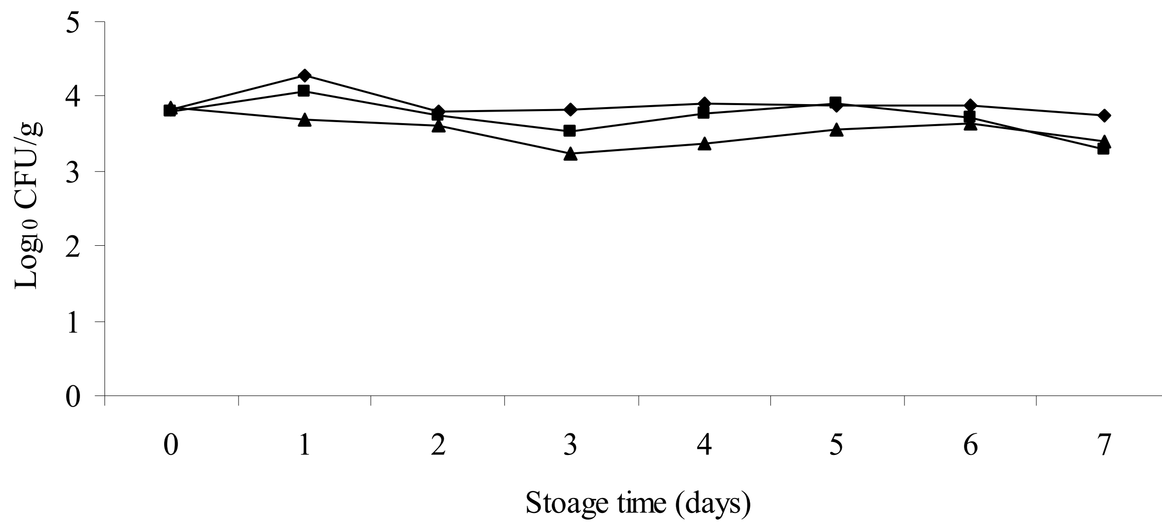


FIGURE 6. *Survival and growth of* E. coli *O157:H7 on spinach stored at 4°C for 7 days.* ● *GFP*, ■ *RFP and* ▲ *YFP.*

exception of YFP in the spinach stored at 21°C, where the YFP strain had a lag phase of 8 h, whereas the GFP and RFP showed a lag phase of 4 h.

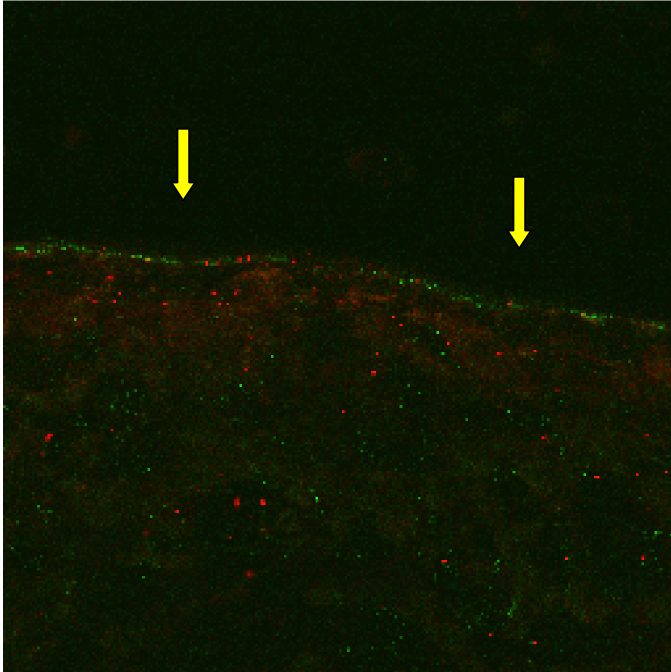
The ability of *E. coli* O157:H7 to grow and survive on ready-to-eat vegetables has previously been demonstrated. Abdul-Raouf et al. *(3)* described the survival and growth of *E. coli* O157:H7 on salad vegetables including shredded lettuce, sliced cucumber and shredded carrots. They reported significant decreases in populations of *E. coli* O157:H7 on shredded lettuce stored at 5°C for 14 days and significant increases in populations on samples stored at 12°C and 21°C. Similar results were obtained for the sliced cucumbers. Francis and O'Beirne *(125)* studied the effects of vegetable type, package atmosphere and storage temperature on the growth and survival of *E. coli* O157:H7 and *L. monocytogenes* and reported that populations of both pathogens increased significantly on lettuce stored at 8°C for 12 days, but there were no significant changes in populations of pathogens stored at 4°C. Likewise, Delaquis et al. *(92)* observed that both *E. coli* O157:H7 and *L. monocytogenes* were capable of growing at 10°C for 7 days. Our data also demonstrates the ability of *E. coli* O157:H7 to survive at 10°C and grow at 21°C. Packaging, storage and handling practices have not been able to prevent the survival and growth of pathogens on leafy greens. Therefore, precautionary methods to prevent contamination of leafy greens are needed.

Use of CSLM to identify the attachment sites for *E. coli* O157:H7 and *Salmonella* on the leaf and stem of spinach

E. coli O157:H7 and *Salmonella* were observed on the surface and the cut leaf edge (Fig. 7). During preliminary experiments it was demonstrated that bacterial pathogens that were not strongly attached to the excised spinach samples were easily removed by washing the inoculated spinach. The spinach samples were subjected to washing before preparing for CSLM. Therefore, all microorganisms visualized by CSLM were assumed to be strongly attached to the spinach surfaces. Both pathogens could be demonstrated within tissue layers of the spinach stem (Fig. 8). In our study, the spinach leaf provided a thin, relatively flat sample which produced meaningful images.

Likewise, spinach stems were sterilely sectioned with a surgical stainless steel scalpel to create flat surfaces which assisted in the observation of both pathogens. Internalization of *E. coli* O157:H7 and *Salmonella* was seen on leaves and stems by taking multiple images of the same sample at different layers. Fluorescent cells not seen on the surface layer of the sample appeared in the interior images of the same sample. Figure 7A and 7B shows fluorescent bacteria allocated in the interior of the spinach stem. Figure 8A illustrates microorganisms near the cut surface of a spinach leaf. Figure 8B demonstrates the preferential gathering of pathogens to the stomata and cracks in the cuticle. These images demonstrate the ability of pathogens to congregate in areas on the leaf surface as well as internalization within the plant possibly escaping chemical decontamination treatments. One possible reason for the congregation of pathogens in

A. **B.**



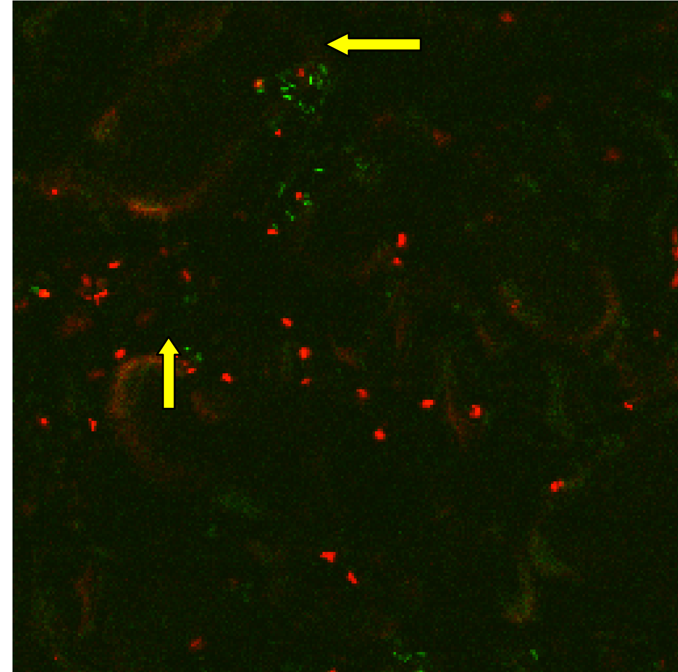
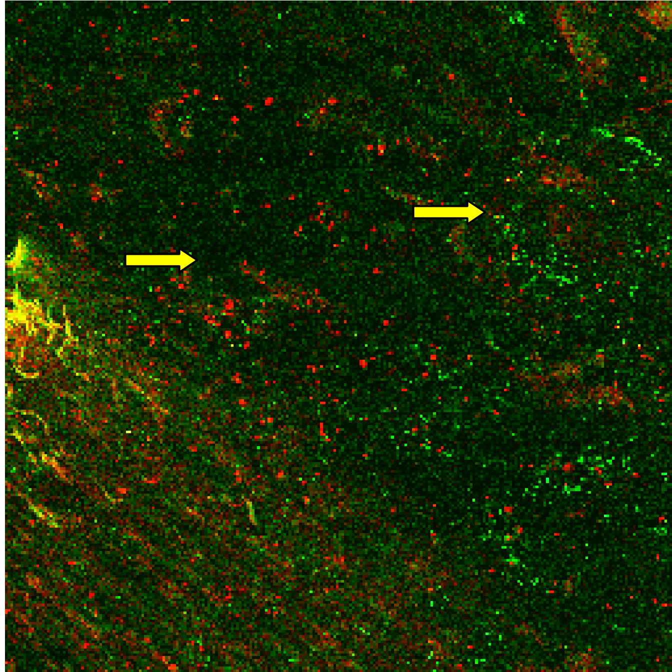


FIGURE 7. *Confocal scanning laser microscopy (CSLM) photomicrographs of spinach leaves inoculated with GFP-expressing* E. coli *O157:H7 and RFP- expressing* Salmonella*. (A) Pathogens lined along the cut edge of the spinach leaf (arrows). (B) Pathogens at the stomata and cracks (arrows).*

A. **B.**



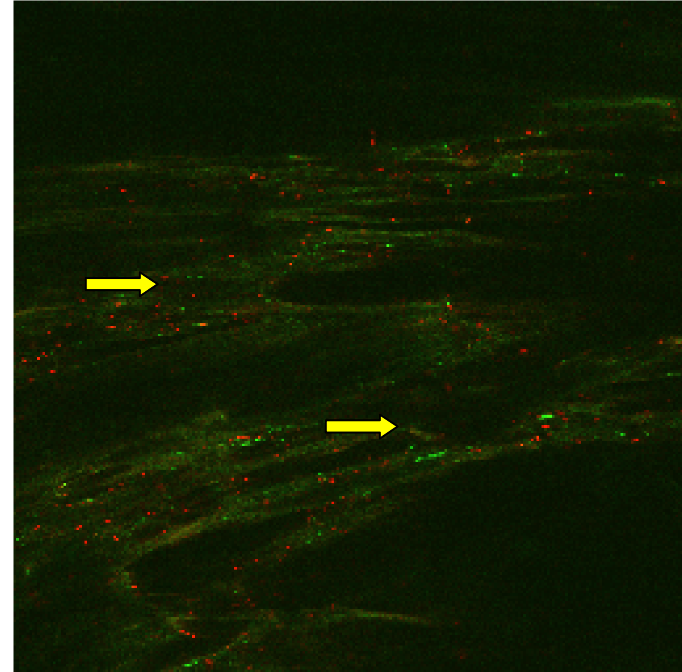


FIGURE 8. *Confocal scanning laser microscopy (CSLM) photomicrographs showing GFP-expressing* E. coli *O157:H7 and RFP-expressing* Salmonella *in the interior of spinach stems. (A) Pathogens throughout stem fissures (arrows). (B) Pathogens lodged within crevices in the stem interior (arrows).*

specific areas on the leaf surface may be due in part to high hydrophobic leaf surfaces allowing surface water to accumulate in depressions of leaf veins suggesting that more free water is available to pathogens at these locations. Brandl and Mandrell (*49*) observed *Salmonella* Thompson behaving similarly on cilantro leaves. In addition, guttation fluids containing carbohydrates, amino acids and numerous inorganic compounds including significant amounts of ammonia (NH_4) and nitrate (NO_3) ions may also accumulate in these hydrophobic areas on leaf surfaces (*133*). Brandl and Amundson (*48*) observed that young romaine lettuce leaf tissue contained more nitrogen than middle leaves. These authors suggested a scenario in which guttation fluids on young lettuce leaves contributed to increased levels of nitrogen available to bacteria that contaminate their leaves. In addition, spinach contains fructose and sucrose which may serve as carbon sources for microorganisms to metabolize. While nutrients may be available on leaf surfaces, microorganisms need moisture for survival and growth. Harvesting and processing spinach can cause tissue damage to the leaves causing plant cells to leak contents thus providing moisture creating a more hospitable environment for microorganisms. In addition, lesions on lettuce and spinach leaves provide sites for internalization of microorganisms where they may be protected from adverse conditions and provide a higher availability of substrates (*46*). Once internalized, it is not known if active movement within the vascular system is caused by the microorganism through the use of its flagellum or by passive movement caused by water flowing through the system (*47*).

Morris et al. *(222)* observed biofilms on leaves of numerous plant leaves. Seo and Frank *(290)* described the preferential attachment of *E. coli* O157:H7 to cut edges rather than intact surfaces and the penetration of the pathogen into the interior of the leaf. Takeuchi and Frank *(315)* reported similar findings and suggested that *E. coli* O157:H7 may attach to less favorable attachment sites once all of the preferred initial attachment sites were occupied. From our findings, it is apparent that pathogens like *E. coli* O157:H7 and *Salmonella* can not only lodge themselves onto exterior locations inaccessible to chemical sanitizers but can also be internalized within the plant structure.

Effectiveness of chemical sanitizers on the reduction of *E. coli* O157:H7 and *Salmonella* inoculated on spinach

The effects of the various sanitizers on the reduction of *E. coli* O157:H7 and *Salmonella* inoculated on spinach are presented in Table 4. The 2% L-lactic acid spray at 55°C produced the greatest reduction in both pathogens (2.7 log CFU/g reduction for *E. coli* O157:H7 and 2.3 log CFU/g reduction for *Salmonella*) and the only treatment whose reductions were significantly different from the reductions obtained by the water wash, which resulted in a 0.7 log reduction (log CFU/g) for both pathogens. Reductions for all other treatments were not significantly different ($P>0.05$) from the reductions obtained by water wash alone. Peroxyacetic acid reduced *E. coli* O157:H7 and *Salmonella* by 1.1 and 0.8 log CFU/g, respectively. Calcium hypochlorite was able to reduce *E. coli* O157:H7 by 1.0 log CFU/g and *Salmonella* by 0.7 log CFU/g. Spinach samples treated with ozone for 15 min reduced *E. coli* O157:H7 and *Salmonella.*

TABLE 4. *Estimated log reductions (ELR)*[a] *(log_{10} CFU/g) in* E. coli *O157:H7 and* Salmonella *inoculated onto fresh spinach leaves.*

Treatment	*E. coli* O157:H7	*Salmonella*
RMSE[b]	0.15	0.15
Water wash[c]	0.7A[d]	0.7AC
L-Lactic acid	2.7B	2.3B
Calcium hypochlorite	1.0A	0.7AC
Peroxyacetic acid	1.1A	0.8AC
Ozonated water 15 min	1.1A	0.9A
Ozonated water 30 min	0.6A	1.0A
Chlorine dioxide gas 30 min	0.7A	0.3C
Chlorine dioxide gas 1 h	0.7A	0.6AC

[a] Estimated log reduction = (log_{10} CFU/g before treatment) - (log_{10} CFU/g after treatment).
[b] Root Mean Square Error.
[c] Water: 1.5-L hand wash at 21°C (30 s, 9 psi).
[d] Means within columns with the same letter are not significantly different ($P>0.05$).

Salmonella by 1.1 and 0.9 logs CFU/g, respectively and ozone treatment for 30 min reduced the pathogens by 0.6 and 1.0 logs CFU/g. Spinach samples exposed to ClO_2 gas for two different treatment times (30 min and 1 h) had no reductions in pathogen populations.

The effectiveness of various sanitizers can be dependent on many factors such as differences in inoculation method (dip, drop, sprinkle) *(294)*, produce surface (hydrophobic, hydrophilic, intact or injured) *(145, 149)*, produce storage time before treatment *(327)*, method of sanitizer application (dipping, spraying, washing) *(9, 35, 296)*, temperature differential between produce and sanitizer *(11, 145, 361)*, sample processing (stomaching, homogenizing, washing) *(273)*, and recovery methods for surviving cells (selective versus nonselective plating media) *(146, 147)*.

Lactic acid. Lactic acid is more commonly used in the meat industry as a decontamination method; however, several researchers have reported that it can be relatively effective on fruits and vegetables in reducing several pathogens *(67, 197, 340, 361).* Ibarra-Sanchez et al. *(162)* reported that 2% L-lactic acid was effective in reducing *E. coli* O157:H7 and *S.* Typhimurium in tomatoes between 3 and >3.1 log CFU/cm^2. Alvarado-Casillas et al. *(9)* used lactic acid sprays to reduce pathogens by approximately 3 log CFU on cantaloupes and 3.6 log CFU on bell peppers. Several studies have reported the effectiveness of combining lactic acid and hydrogen peroxide for an antimicrobial treatment for fruits and vegetables. Venkitanarayanan et al. *(340)* examined the antimicrobial activity of this combined method for the inactivation of *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* on apples, oranges and tomatoes. They

determined that the treatment of apples, oranges and tomatoes with 1.5% lactic acid plus 1.5% hydrogen peroxide at 40°C for 15 min could reduce these three pathogens by $\geq$5.0 log CFU per fruit. In a similar study, Lin et al. *(197)* reported that 1.5% lactic with 1.5% hydrogen peroxide inactivated *E. coli* O157:H7 and *S.* Enteritidis more than 4 log CFU per lettuce leaf and *L. monocytogenes* approximately 3 log CFU per lettuce leaf.

Peroxyacetic acid. Peroxyacetic acid has a residual effect when acetic acid is released by the degradation of peroxyacetic acid which may help reduce yeast and mold populations however, it may not be as effective against pathogens *(273)*. In our study, we found that peroxyacetic acid reduced *E. coli* O157:H7 and *Salmonella* on spinach leaves by 1.1 and 0.8 log CFU/g, respectively. The efficacy of sanitizing agents is dependent on several factors including pH, the water hardness, contact time, and type of vegetable *(335).* One possible cause for the limited results of this study may be attributed to the amount of surface area of the spinach leaf as well as surface structure. Peroxyacetic acid kills microorganisms by oxidation and consequent disruption of the cell membrane by producing hydroxyl radicals which are highly reactive. This strong oxidizing effect results in transfer of electrons to the microorganism, quickly causing apoptosis; however, this is dependent on the ability of the decontamination treatment to reach all surface areas of the spinach leaf. Savoy and semi-savoy spinach leaves are curly and may provide protected areas for pathogens where surface sanitizers may not come into contact with the microbes. Lesions on the leaf surface may also provide microorganisms opportunities to internalize into the leaf protecting them from decontamination treatments as well. Similar to our study, Zhang and Farber *(361)*

reported that peroxyacetic acid reduced *L. monocytogenes* by 0 to 0.2 log CFU/g more than tap water. Hellstrom et al. *(153)* reported a higher reduction of *L. monocytogenes* with the difference between peroxyacetic acid and water being 1.2 log CFU/g. Rodgers et al. *(273)* reported that 80 mg/liter peroxyacetic acid reduced *E. coli* O157:H7 and *L. monocytogenes* on apples, lettuce, strawberries and cantaloupe by as much as 4.5 and 4.3 logs, respectively; however, whole produce samples were immersed for up to 5 min and sliced samples were misted for 3 min. This may be one of the reasons why there was a greater reduction in comparison to our study, because our spinach samples were misted for 30 s. Contact time does affect the efficacy of surface decontamination treatments. Wisniewsky et al. *(354)* reported 5 log reductions of *E. coli* O157:H7 counts on whole apples using peroxyacetic acid at levels two to three times the recommended amount. In addition to treatment dose, the surface of the fruit or vegetable may also be a factor. Beuchat et al. *(33)* reported that peroxyacetic acid reduced populations of *L. monocytogenes* inoculated at 3 different inoculum levels (1.55, 2.7 and 4.52 CFU/ml) on iceberg lettuce pieces by 1.25, 1.06 and 1.83 log CFU/g respectively; however, shredded lettuce showed lower reductions of 0.25, 0.85 and 1.59 CFU/g at similar inoculation levels. In addition, the authors reported that for romaine lettuce pieces with all levels of inoculums, populations recovered after treatment with peroxyacetic acid and that these levels were not significantly different from populations recovered after washing with water.

Chlorine. While chlorine remains commonly used as a sanitizer in wash, spray and flume waters in the fruit and vegetable industry, it also remains to be minimally

effective or at least unpredictable. Calcium hypochlorite reduced *E. coli* O157:H7 by 1.0 log CFU/g and *Salmonella* by log 0.7 CFU/g. One of the biggest challenges with using chlorine as a disinfectant is that it is deactivated quickly upon contact with organic matter. Spinach samples in this experiment were given a 30 s water wash prior to treatment. However; organic material such as dirt or small areas of decay may not have been removed in the washing process. The spinach surface itself may have contributed to the deactivation of the chlorine. The plant structure and lesions caused in sample preparation on the leaf surfaces may also have contributed to the limitations of this study. Li et al. *(195)* reported analogous results to our study in which their treatment of cut lettuce with 20 mg/liter chlorine at 50°C or 20°C before or after inoculation with *E. coli* O157:H7 did not influence the behavior of the pathogen. Takeuchi and Frank *(316)* observed that 200 mg/liter chlorine treatment at 22°C for 5 min reduced *E. coli* O157:H7 attached on lettuce leaf surfaces and in cut edge tissues by 0.3 and 0.4 CFU/cm^2 respectively. However, high numbers of viable *E. coli* O157:H7 cells remained at both sites with more cells attached at the cut edge than at the surface. These authors concluded that sanitizers that are easily inactivated by organic material are unlikely to be effective in eliminating *E. coli* O157:H7 cells that penetrate into the leaf tissue *(316)*. Taormina and Beuchat *(319)* reported that chlorine was able to reduce *E. coli* O157:H7 inoculated onto alfalfa seeds at concentrations ranging from 500 to 2000 mg/liter for 3 min. The authors stated similar reasoning as Takeuchi and Frank (*316)* for this limitation; the lack of available chlorine as it contacts organic material. Beuchat et al. *(33)* explained that the most rapid reduction in free chlorine concentration in solutions

used to treat shredded lettuce inoculated with *L. monocytogenes* were attributable to the release of tissue juices, which increased the concentration of organic materials accessible for reaction with and neutralization of chlorine. Similarly, Jaquette et al. *(171)* observed that chlorine was not effective in eliminating *S.* Stanley inoculated onto alfalfa seeds treated for 10 min. Hellstrom et al. *(153)* observed that chlorinated water was able to reduce *L. monocytogenes* by 0.7 CFU/g. Zhang and Farber *(361)* reported a maximum log reduction of 1.3 to 1.7 for *L. monocytogenes* on shredded lettuce using 200 mg/liter chlorine with 10 min of exposure. Escudero et al. *(105)* reported a 2.68 log CFU/g reduction for strains of *Y. enterocolitica* on lettuce by treatment with 100 mg/liter chlorine after a 10 min exposure. These studies suggest that both *L. monocytogenes* and *Y. enterocolitica* may be more susceptible to chlorine decontamination than *E. coli* O157:H7 or *Salmonella.*

Ozone. The reported efficacy of ozone has varied results. For this experiment, ozone (6 mg/liter) reduced *E. coli* O157:H7 and *Salmonella* by 1.1 and 0.9 CFU/g on spinach leaves treated for 15 min. Selma et al. *(289)* observed that *S. sonnei* counts were reduced by 0.9, 1.4 and 1.8 log units at 5 mg/liter ozone depending on the amount of time exposed. In contrast, Rodgers et al. *(273)* reported ozone to reduce *L. monocytogenes* and *E. coli* O157:H7 on multiple types of produce by >5 log CFU/g. Koseki et al. *(185)* reported that the numbers of aerobic microorganisms on lettuce decreased only 1.5 log following a 10-min exposure to 5 mg/liter ozone. One reason for the variability in results may be the difference in produce surfaces. The generation of ozone agitates the water and produces bubbles which may help microorganisms detach

from smooth surfaces such as apples; however, surfaces that have curvatures such as those of leafy greens may protect microorganisms from such agitation *(182, 359)*.

Chlorine gas. Our experiments with ClO_2 gas showed no reductions in pathogen populations which may be attributed to the ability of pathogens to penetrate into injured or cut surfaces on the spinach leaves. Han et al. *(149)* and Seo and Frank *(290)* reported that bacteria may penetrate into injured tissues protecting them from inactivation by low levels of ClO_2 gas treatments. An additional explanation for our limited results may be the ClO_2 concentration that was used. Han et al. *(148, 149)* reported that green peppers inoculated with *E. coli* O157:H7 that received a 0.60 mg /liter ClO_2 gas treatment achieved 4.37 and 1.36 more log reductions on uninjured and injured surfaces respectively than green pepper samples receiving only a 0.15 mg/liter ClO_2 treatment indicating that the concentration of ClO_2 gas played a significant role in the inactivation of *E. coli* O157:H7 on green pepper surfaces. Lee et al. *(194)* reported that when inoculated lettuce leaves were treated with ClO_2 gas, levels of *E. coli* O157:H7 were reduced by 3.4, 4.4 and 6.9 log units after 30 min, 1 h, and 3 h, respectively. In addition to the ClO_2 gas concentration, another possible reason for the difference in results may be attributed to the manner in which the samples were inoculated. In the studies by Han et al. *(147, 148)* and Lee et al. *(194)*, inoculation was achieved by depositing droplets of the pathogen cocktails onto the produce surface, while samples in this study were inoculated by distributing the inoculum on the spinach leaves and shaking the sample bags for one min. Han et al. *(148)* explained that the spot inoculation method was used to inoculate strawberries, because it allowed the application of a known amount of

bacteria onto the berry surface regardless of the berry size. Singh et al. *(294)* reported that the effectiveness of sanitizers against *E. coli* O157:H7 on shredded lettuce was affected by the inoculation method and concluded that at high inoculum populations of *E. coli* O157:H7 on lettuce, a large number of bacteria may be entrapped in injured sites and thus be minimally affected by sanitizer treatments. Other investigations have been conducted to study the influence in methodology including inoculation and time between inoculation and analysis *(56, 188)*. Relative humidity has a synergistic effect with ClO_2 gas concentration *(194)*. In this study, 10 ml of sterile deionized water was added to increase humidity in the gas chamber but relative humidity was not measured. Lee et al. *(194)* included a small electric fan to facilitate circulation of ClO_2 gas throughout the chamber. We did not include a fan in our study. By not circulating the ClO_2, higher concentrations of the gas may have floated to the top of the gas chamber while the spinach samples remained at the bottom of the chamber and possibly not received an adequate dose. Further research needs to be conducted to determine what affect the fan had on the distribution of ClO_2 gas in the chamber and if greater microbial log reductions could be obtained. Overall, this research confirmed our concerns with the efficacy of chemical sanitizers and that the chemical sanitizers have limited effectiveness in areas where the sanitizer comes into contact with the pathogen. This necessitates an intervention such as irradiation that addresses this issue.

Efficacy of e-beam irradiation for the reduction of *E. coli* O157:H7 and *Salmonella* on spinach

The populations of *E. coli* O157:H7 and *Salmonella* inoculated on baby spinach decreased significantly after e-beam irradiation. The counts of both pathogens were inversely proportional to the dose of energy applied. Estimated log reductions (ELR) for *E. coli* O157:H7 and *Salmonella* are shown in Table 5. 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. 9). When the pathogens were inoculated at levels of ca. 8.0 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, 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 logs (Table 5). During refrigerated storage (Fig. 10), 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

TABLE 5. *Estimated log reductions (ELR)*[a] *(log_{10} CFU/g) for* E. coli O157:H7 *and* Salmonella *inoculated onto fresh baby spinach leaves as affected by dose of e-beam irradiation.*

Irradiation dose (kGy)	*E. coli* O157:H7	*Salmonella*
0.4	3.7A[b]	3.4A
0.79	4.1B	4.0B
1.07	6.3C	6.1C
1.16	6.3C	>6.6C
2.04	>6.6C	>6.5C
2.49	>6.4C	>6.6C

[a] Estimated log reduction: (log CFU/g on control spinach – log CFU/g on spinach after treatment).
[b] Means within columns with the same letter are not significantly different ($P>0.05$).

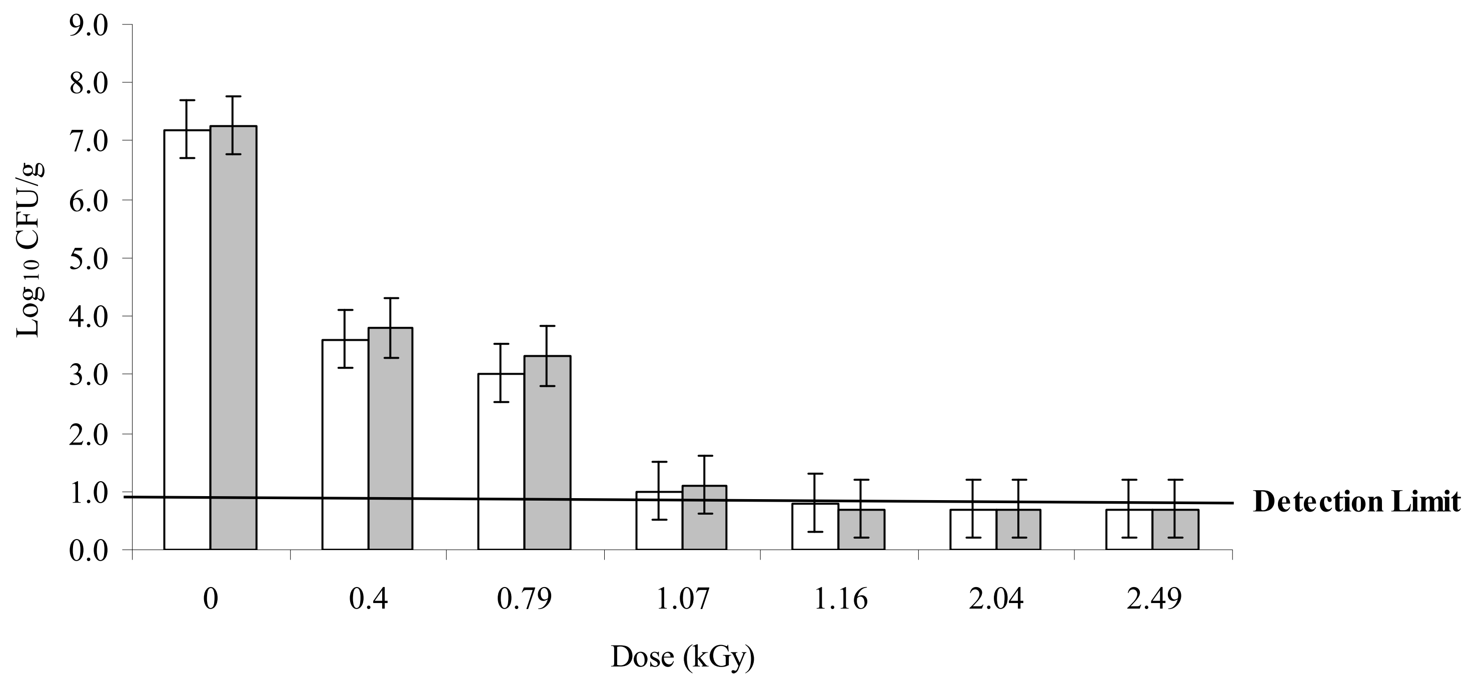


FIGURE 9. *Effects of irradiation dose on the populations of* E. coli *O157:H7 (white) and* Salmonella *(grey) after e-beam irradiation.*

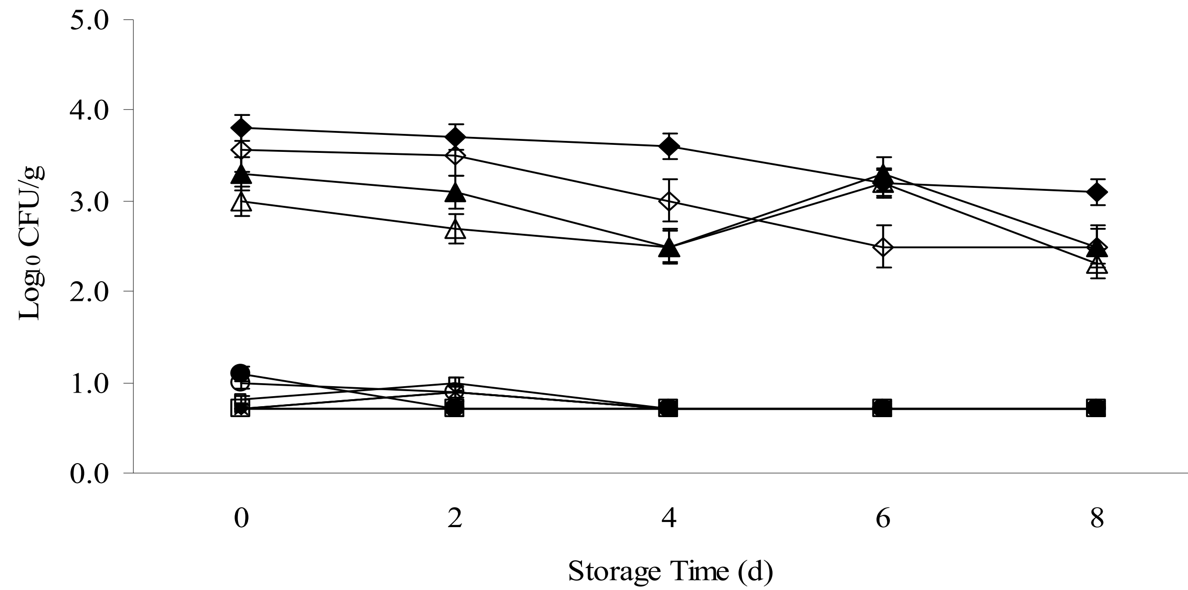


FIGURE 10. *Survival of* E. coli *O157:H7 and* Salmonella *stored at 4°C for 8 days exposed to 0.4 (◊), 0.79 (Δ), 1.07 (○), 1.16 (□), 2.04 (*) or 2.48 (□) kGy e-beam irradiation. Open data points denote* Salmonella *and solid data points denote* E. coli *O157:H7. Error bars reflect standard deviations of the mean obtained from triplicate samples.*

undetectable levels by day 4. *E. coli* O157:H7 irradiated at 2.04 kGy had 1 CFU/g on day 2 and then fell below the detection limit. *Salmonella* were not detectable at 2.04 kGy. Neither *E. coli* O157:H7 or *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* were consistently recovered after enrichment over the 8 days of storage. No *Salmonella* were recoverable after enrichment when the spinach was treated at doses of 2.04 kGy.

In this study, we found that e-beam irradiation at 1.16 kGy was successful in reducing *E. coli* O157:H7 and *Salmonella* on baby spinach to levels below the detection limit of the counting method. This finding is consistent with that of Lee et al. *(193)*, who reported that low-dose irradiation was effective in eliminating pathogens inoculated in ready-to-eat vegetables. According to these authors, gamma irradiation at doses of 1 kGy resulted in a 4 log reduction of *E. coli* inoculated onto seasoned spinach. Foley et al. *(119)* showed that chlorination plus irradiation at 0.55 kGy could achieve a 5.4 log reduction in *E. coli* O157:H7 in inoculated shredded lettuce. Goularte et al. *(138)* 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 enrichment of samples where no colonies were detected on the count plates, *E. coli* O157:H7 was not detected 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 irradiation than *E. coli* O157:H7 *(228)*. Foley et al. *(119)* also reported that *E. coli* O157:H7 decreased during storage and were undetectable after 7 days.

D_{10}-value for *E. coli* O157:H7 on irradiated spinach

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 (±0.01) was obtained for *E. coli* O157:H7 in baby spinach (Fig. 11). While D values differ based on moisture content and the matrix of a particular food item, Clavero et al. *(82)* 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. *(138)* reported D values ranging from 0.11 to 0.12 kGy for *E. coli* O157:H7 on lettuce. Niemira et al. *(235)* reported similar D-values for *E. coli* O157:H7 on different types of lettuce. Microorganisms' sensitivity to irradiation differs and certain *Salmonella* may have a higher D-value range than *E. coli* O157:H7 *(325)*. Prakash et al. *(256)* reported a D-value range of 0.26 to 0.39 kGy for *Salmonella* spp. inoculated onto irradiated diced tomatoes. Niemira et al. *(235)* found D-values ranging from 0.35 to 0.71 kGy for different *Salmonella* tested in orange juice indicating that a 5 log_{10} CFU/g reduction in *Salmonella* would require a dose of 1.75-3.55 kGy. These results suggest that *Salmonella* serotypes differ in sensitivity to irradiation.

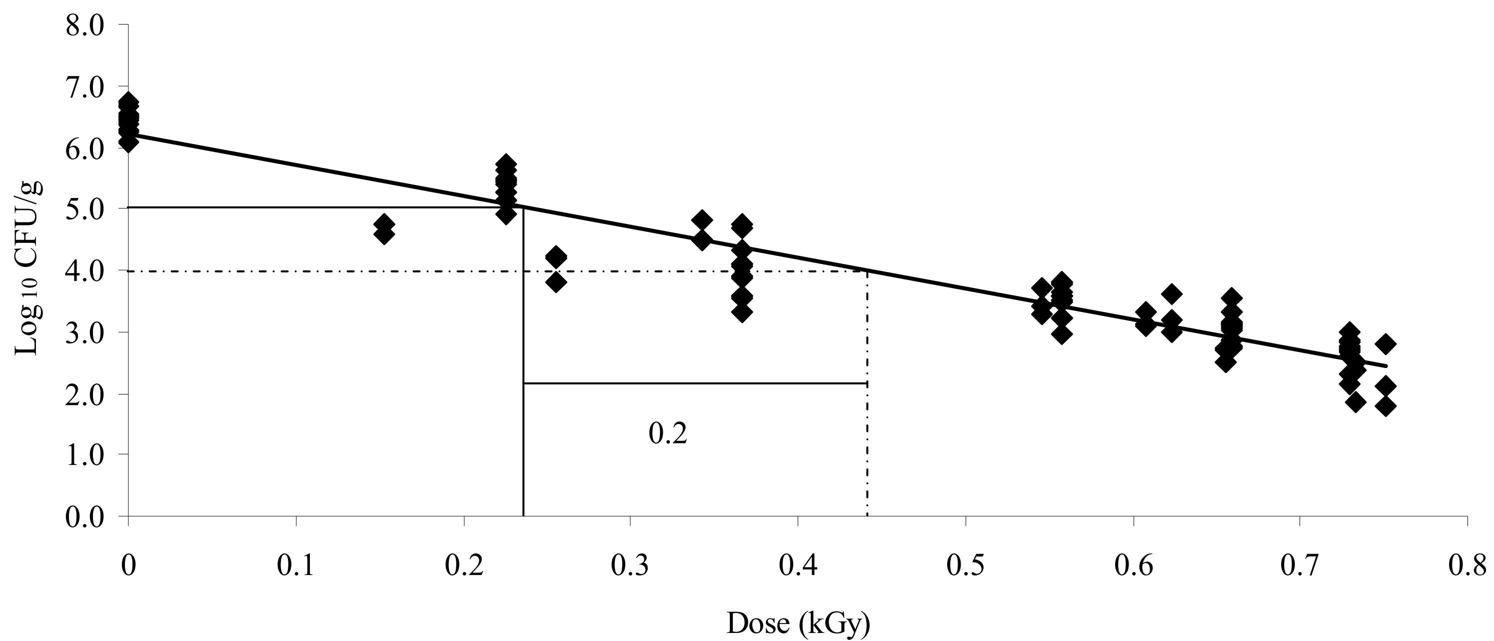


FIGURE 11. *Regression line for* E. coli *O157:H7 counts on spinach leaves treated with increasing doses of irradiation. Data points represent the average of triplicate samples. Lines drawn serve the purpose of illustrating the D-value but not for the calculation.*

Apparent D-values. Apparent D-values are estimations of D-values that can help researchers approximately predict D-values and doses needed for specific log reductions. This can be calculated from data from preliminary experiments or data from related studies that may not be under optimal conditions for determining D-values but can still give useful estimations. An apparent D-value was calculated for *E. coli* O157:H7 and *Salmonella* using the data obtained from the e-beam efficacy study. Because this data was from a previous study, high precision dosing of the sample packages was not used. Counts of these pathogens on spinach exposed to doses of 0, 0.4, 0.79 and 1.07 kGy were plotted to determine the apparent D-values. For *E. coli* O157:H7, an apparent D-value of 0.2 kGy (±0.01) was obtained (data not shown). This is in agreement with the D-value obtained for the single strain of *E. coli* O157:H7 and confirms that there were no significant differences in sensitivity to irradiation among the *E. coli* O157:H7 strains used. This D-value indicates that a 5 log CFU/g reduction of *E. coli* O157:H7 would require a dose of 1.0 kGy. For the *Salmonella* serotypes, an apparent D-value of 0.2 kGy (±0.01) was also obtained. This apparent D-value is lower than D-values previously reported for various *Salmonella* serotypes but also reiterates that sensitivity to irradiation may by serotype specific. While irradiation sensitivity was determined for the *E. coli* O157:H7 strains, individual *Salmonella* serotype sensitivity was not tested. Based on this apparent D-value, a 5 log CFU/g reduction of *Salmonella* would require a dose of 1.0 kGy. However, higher D-values for *Salmonella* have been reported and therefore it is recommended that treatment doses must be determined based on the most resistant

serotype. The recent FDA approval of irradiation for fresh spinach and iceberg lettuce permits doses up to 4.0 kGy which addresses this issue.

Microbiological and sensory characteristics of spinach

Aerobic plates counts (APC). The ELR for APC are shown in Table 6. The microbial populations over a 35 day period were observed. E-beam irradiation significantly reduced the total aerobic microbial counts of fresh spinach in comparison to non-irradiated spinach (Day 0, Fig. 12). The initial microbial counts of the control sample were 5.4 log CFU/g. After 14 days post-packaging (which is the typical "best-if-used-by" date) the control samples had counts of 6.1 CFU/g at 4°C and gradually increased by 0.5 log on day 30. The irradiation dose of 0.7 kGy produced a 2.6 log CFU/g decrease in the microbial load on day 0 and a 2 log deficit was maintained through 14 days. On day 18, samples that received 0.7 kGy had counts of 5.3 CFU/g which was 0.8 log CFU/g less than the control samples. Samples that received 1.4 kGy had a 3.2 log CFU/g reduction as compared to control samples on day 0. A deficit of approximately 3.0 log CFU/g was maintained through day 14 for samples that received a 1.4 kGy treatment as compared to non-irradiated controls. Microorganisms had continual growth for all samples through day 14. In addition, microbial populations did not exceed the initial concentration of the control over the 35 days of storage. Mean populations for control samples reached 6.5 log CFU/g on day 30 of storage. For samples treated with 0.7 kGy, APC counts reached 5.3 log CFU/g on days 18 and 30. APC counts for spinach samples that received 1.4 kGy reached 3.6 log CFU/g on day 35.

TABLE 6. *Survival of APC, LAB and yeasts and molds on fresh spinach leaves as affected by dose of e-beam irradiation immediately after treatment.*

Irradiation dose (kGy)	APC	LAB	Yeasts and Molds
0.0	5.4A[a]	3.7A	3.3A
0.7	2.8B	1.4B	2.7B
1.4	2.2C	1.0B	1.2C

[a] Means within columns with the same letter are not significantly different (*P* >0.05).

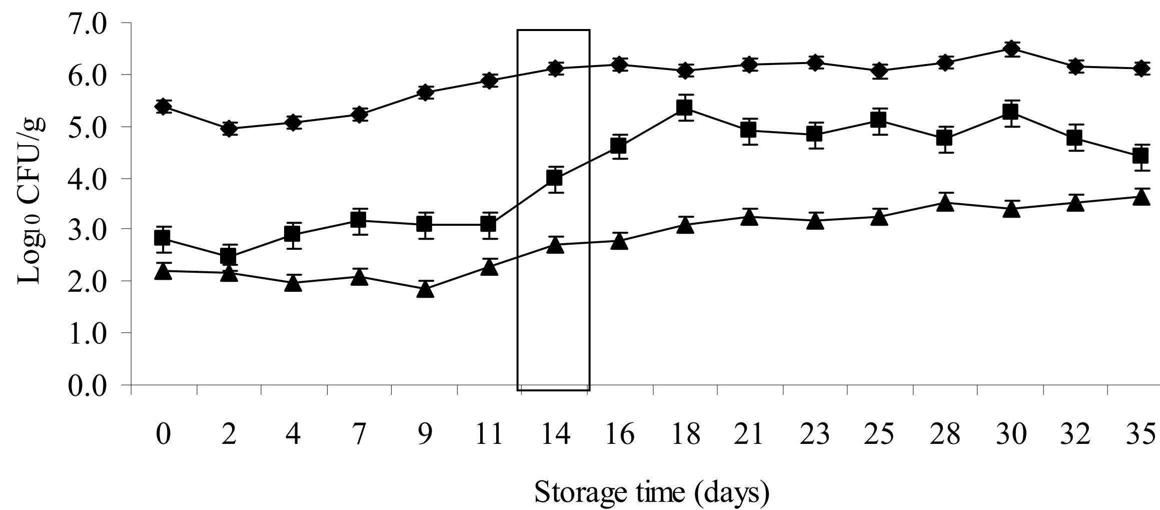


FIGURE 12. *Aerobic plate counts of spinach leaves over 35 days of storage at 4°C after e-beam irradiation. 0 kGy (♦), 0.7 kGy (■), and 1.4 kGy (▲). Day 14 is highlighted to indicate the typical shelf-life of bagged spinach.*

Multiple regression analysis was conducted and the linear model was determined to be the best model. The R-square value was 0.888 indicating that 88.8% of the variance in survival was accounted for by the irradiation treatment received. A significant reduction in bacterial populations, which is directly proportional to the dose applied, was also reported by Prakash et al. *(254)* with their work concerning the use of low-dose gamma irradiation on cut romaine lettuce under modified atmosphere. These authors also reported that when irradiated lettuce was stored at 4°C, APC increased over a 22 day storage period, but irradiated levels were always lower than the controls. Storage at 4°C is not favorable for growth of most mesophilic pathogens such as *E. coli* O157:H7 or *Salmonella*; however, psychrotrophic microorganisms can grow at refrigerated temperatures with the predominant species belonging to the Pseudomonadaceae family (especially *P. fluorescens)* and the Enterobacteriaceae family (especially *Erwinia herbicola* and *Rahnella aquatilis*) *(261).* Psychrotrophic pathogens such as *L. monocytogenes, Aeromonas hydrophila*, or *Y. enterocolitica* are also capable of growing at these temperatures. Several studies investigating the microbial quality of spinach and lettuce reported incubating samples at temperatures ranging from 25°C to 30°C, enabling psychrotrophic microorganisms to proliferate. One of the limitations of our study is that APC samples were incubated at 37°C which is still within the 3M Petrifilm recommended temperature range; however, incubation at this temperature may have limited the growth of some psychrotrophic microorganisms. Gimenez et al. *(132)* reported a dramatic increase in total aerobic bacterial counts from 3.9 log CFU/g to 6.8 log CFU/g after storage of 3 days on minimally processed artichoke stored at 4°C. Similarly, Hagenmaier and Baker *(141)* reported that microbial populations on irradiated

iceberg lettuce increased noticeably with storage time and temperature. This indicates the need for a substantial reduction of the bacterial load on spinach during decontamination steps to help maintain the produce quality and extend the shelf life. Our data suggest that irradiation doses of 1.4 kGy substantially reduce the APC load and can extend the shelf-life of bagged spinach.

Lactic acid bacteria (LAB). E-beam irradiation reduced the counts of LAB on spinach samples. The ELR for LAB are shown in Table 6. E-beam reduced LAB counts by 2.3 log CFU/g with 0.7 kGy and 2.7 log CFU/g with 1.4 kGy on day 0 (Fig.13). The initial microbial counts of the control samples was 3.7 log CFU/g. There was a significant difference in LAB counts between the control and irradiated samples, with no significant difference in counts between the 2 irradiation doses on day 0. When irradiated spinach samples were stored at 4°C, LAB counts for samples that received 0.7 kGy increased significantly higher than counts for samples that received 1.4 kGy on day 7; however, by day 14, LAB counts for samples receiving both doses were 1 log CFU/g and 2.3 log CFU/g less than control samples. After day 14, LAB counts for samples that received 0.7 kGy varied through the remainder of storage days. LAB counts for spinach samples that received 1.4 kGy did not increase until 21 days of storage. Mean populations for control samples reached 3.8 log CFU/g by day 21. For irradiated samples treated with 0.7 kGy, LAB counts reached 2.5 log CFU/g by day 32 of storage. LAB counts for samples that received 1.4 kGy reached 1.6 log CFU/g by day 32. One possible factor that may have limited the growth of the LAB was the gas composition within the sample packages. While some LAB are aerotolerant, others are strictly anaerobic *(351)*, therefore, O_2 levels within the sample packages may have contributed to LAB inhibition.

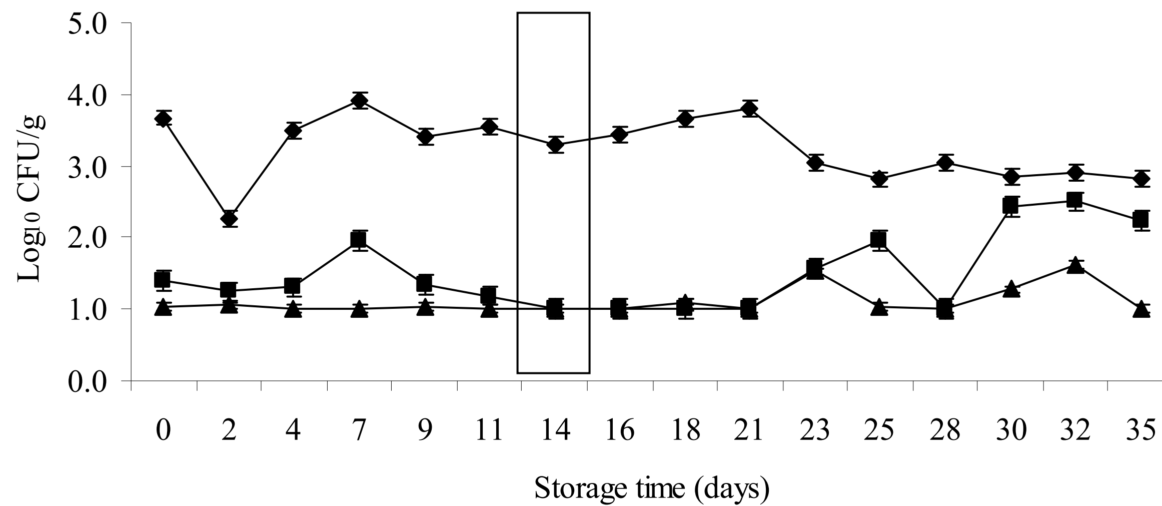


FIGURE 13. *Growth of lactic acid bacteria (LAB) on irradiated spinach leaves stored at 4°C over 35 days. 0 kGy (♦), 0.7 kGy (■), and 1.4 kGy (▲). Day 14 is highlighted to indicate the typical shelf- life of bagged spinach.*

In addition, CO_2 is commonly used to inhibit microorganisms in packaged food commodities. Vegetables continue to respire once they have been packaged and will create a natural modified atmosphere package as it consumes CO_2 and produces O_2 depending on the gas permeability of the packaging film used.

Due to their ability to grow at low temperatures, LAB are able to propagate and cause spoilage as a result of the fermentation of sugars present in the spinach leaves. Therefore, it is important to reduce their initial levels to increase the shelf-life of bagged leafy greens. Gram-positive spoilage microorganisms such as LAB, as well as yeasts and molds, are more resistant to irradiation than gram-negative organisms *(221)*. However, Babic et al. *(13)* reported that LAB typically are present on spinach at lower levels than gram-negative bacteria and are capable of growing at 10°C. Therefore, larger initial reductions in APC counts may be expected in irradiated spinach; however, stressed cells may be able to recover and begin to grow. Given time, through fermentation, LAB can reduce the pH level that can contribute to their advantage over gram-negative microorganisms; however, that action was not observed in this study. LAB has been known to inhibit the growth of pathogens on several commodities. Wilderdyke et al. *(351)* identified LAB isolates that inhibited *S.* Enterica, *E. coli* O157:H7 and *L. monocytogenes.* They reported that the primary mode of inhibition was through the production of acid by the LAB. In this experiment, LAB increased over 35 days which may suggest that gram-negative pathogens might be inhibited by the production of acid from LAB. Further research needs to be conducted to determine if there is a lethal effect by LAB on *E. coli* O157:H7 and *Salmonella* in irradiated bagged spinach.

Yeast and mold counts. The ELR for yeasts and molds are shown in Table 6. E-beam did not reduce total yeasts and molds to a significant degree at 0.7 kGy. Total yeasts and molds were reduced significantly in samples that received 1.4 kGy; however, they were able to recover to non-treatment levels by day 4 (Fig. 14). Over the remaining days of storage, controls as well as irradiated samples showed growth and no significant differences in counts. At all three doses, storage over 35 days at 4°C yeasts and molds grew similarly. The storage temperature of 4°C did not appear to suppress growth of yeasts and molds. For the 35 days of storage in this experiment, the increases in yeasts and molds counts in the control, 0.7 and 1.4 kGy spinach samples were 0.9, 1.5 and 2.8 log CFU/g, respectively. This may be a result of slower growth at 4°C or competing microflora. Hagenmaier and Baker *(141)* found that refrigerated cut iceberg lettuce that had been irradiated with 0.2 kGy after a chlorine treatment had significant reductions in yeasts and molds counts, but the fungi were able to recover with time. Prakash et al. *(254)* described the effect of gamma irradiation on the population of yeasts on cut romaine lettuce as initially being approximately 1 log and increasing with storage, however remaining lower than the control counts. They also suggested that the reason for differences in efficacy of irradiation of yeasts and molds may be attributed to the type of produce used and the headspace gas concentrations within the packaging. Foley et al. *(120)* reported a 3.7 log reduction of yeasts and molds on cilantro irradiated with 0.5 kGy. This difference between cilantro leaves and our results with spinach leaves may also emphasize the significance of the food matrix in determining the effectiveness of irradiation.

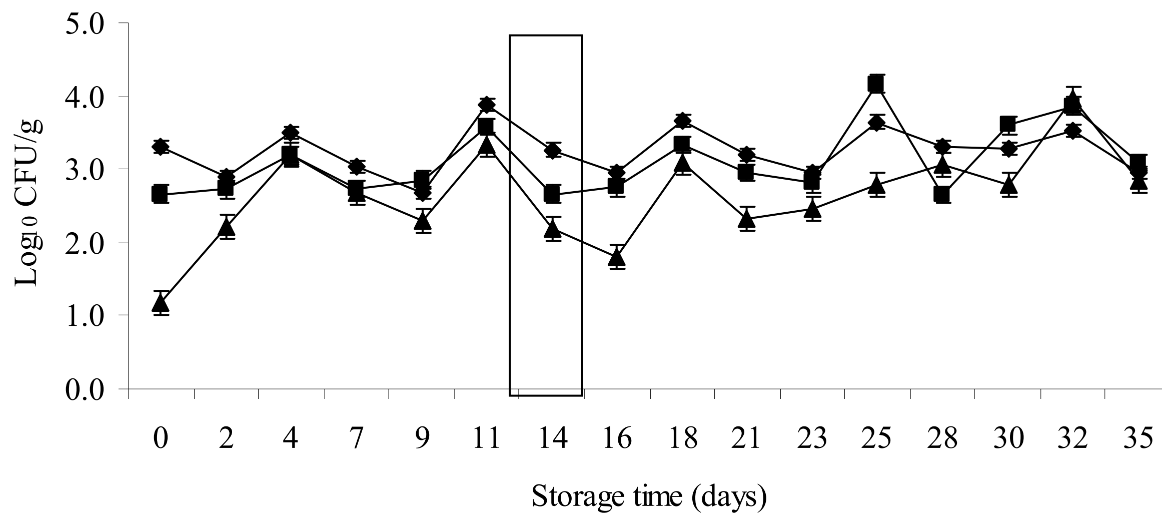


FIGURE 14. *Growth of yeasts and molds on irradiated spinach leaves stored at 4°C over 35 days. 0 kGy (♦), 0.7 kGy (■), and 1.4 kGy (▲). Day 14 is highlighted to indicate the typical shelf- life of bagged spinach.*

Respiration rate and gas composition. The respiration rates for spinach samples increased as treatment dose increased. Spinach samples treated with 1 kGy were higher however, not significantly ($P>0.05$) different from the control samples on day 1 at 4°C. While respiration rates for samples that received 2 kGy were different from the control, they were not significantly different from samples that received 1 kGy. For irradiated spinach samples receiving 3 kGy, the respiration rate was significantly different with an approximately 33% higher rate than that for sample controls 1 day after irradiation at 4°C (Fig. 15). The respiration rate for all samples decreased for each additional day of storage. During respiration, the loss of stored food reserves speeds up senescence as the reserves of energy used to maintain the spinach's living status is exhausted *(174)*. Wound-induced respiration may have increased respiration rates in response to higher doses of irradiation. Higher doses of irradiation are known to cause softening of fruits and vegetables due to its effects on the plant cell walls *(36)*. Irradiation as well as other processing techniques such as cooking, chopping or grinding produces free radicals which are atoms or molecules with unpaired electrons (*229).* The newly formed free radicals will then search for any available electrons causing a series of chemical reactions creating more free radicals which may result in the disruption of living cells *(253).* A common response to wounding is increase in respiration rates. Wound-induced respiration is an immediate response and no lag phase is present *(314).* Ideally, day 0 through day 8 measurements would have been taken for this experiment to determine if there was a lag phase and to determine a steady-state respiration value. This data would help indicate if the increase in respiration was in fact caused by wounds. Hagenmaier

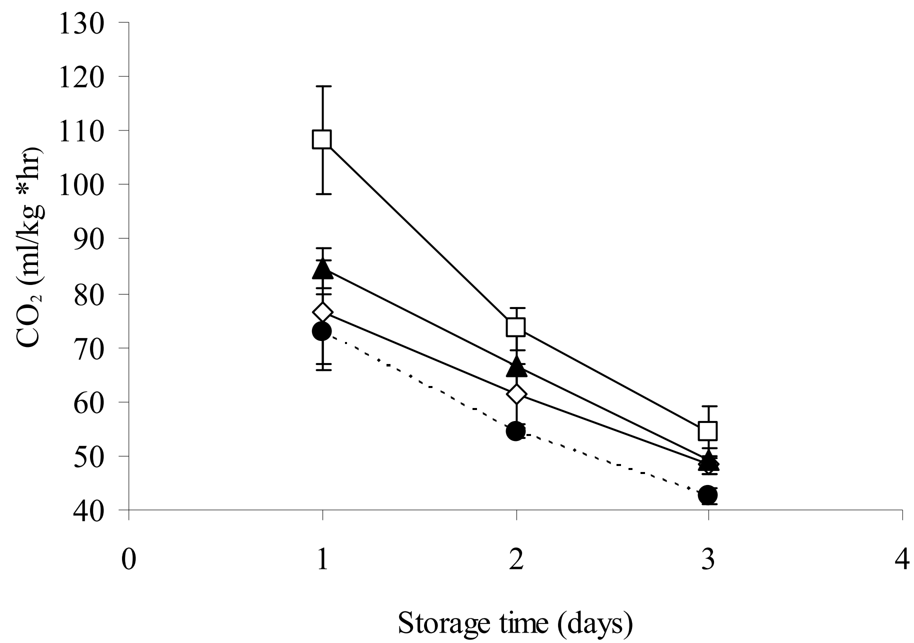


FIGURE 15. *Respiration rates of spinach leaves exposed to 0 (●), 1.2 (◊), 2.1 (▲) and 3.2 (□) kGy e-beam irradiation.*

and Baker *(142)* reported similar findings in irradiated cut iceberg lettuce in modified atmosphere packaging with a 33% higher respiration rate in irradiated samples than controls; however, these measurements were at significantly lower doses (0.2 and 0.5 kGy respectively).

Respiration rates of vegetables can be reduced by modifying or controlling the gas composition within a product's packaging. In a separate experiment, O_2 and CO_2 gas compositions were measured. Gas composition of spinach samples were affected by irradiation treatments. Gas compositions also were different over 35 days of storage. The O_2 and CO_2 gas compositions of spinach samples irradiated at 0.7 and 1.4 kGy were both significantly ($P<0.05$) different from the control on day 1 (Table 7). Oxygen levels inside the spinach sample bags decreased over time (Figs. 16, 17, 18); however, O_2 levels did not drop below 1% which might have induced anaerobic fermentation of the spinach leaves and subsequent damage. CO_2 levels for all treatments increased through day 4; samples contained 4.1%, 6.2% and 7.1% CO_2 treated with 0.0, 0.7 and 1.4 kGy respectively (Figs. 16, 17, 18). However, 7 days after irradiation, CO_2 levels were virtually the same for both control and irradiated samples which contained approximately 3.8% CO_2. This indicates that the sample bags used in this study had a sufficient CO_2 transmission rate which permitted CO_2 evolving from the spinach leaves to be transmitted out of the package. The drop in oxygen values on day 16 accompanied by a large standard error for the control samples may reflect atypical bags in that sample set where sample bags may not have been sealed properly. This may have permitted O_2 to escape out the package.

TABLE 7. *Means for gas composition in fresh bagged spinach as affected by treatment dose, storage days and treatment by storage days.*

Effect	CO_2	O_2
RMSE[x]	0.94	0.68
Treatment	0.01[z]	0.54[z]
0.0 kGy	4.4 A[a]	3.2A
0.7 kGy	4.7A	3.3A
1.4 kGy	5.7B	3.2A
Storage Day	<0.0[z]	<0.0[z]
1	6.2A	7.2A
2	9.0B	4.1B
4	5.8A	3.7B
7	6.1A	3.9B
9	3.5C	2.2C
11	4.3D	2.7CD
14	4.6D	2.9CD
16	3.8C	2.5CD
18	4.5D	2.9CD
21	4.8D	3.0D
23	4.3D	2.7CD
25	4.4D	2.7CD
28	4.0D	2.5CD
30	4.5D	2.8 CD
32	4.3D	2.7CD

[a]Mean values within a column and effect followed by the same letter are not significantly different ($P > 0.05$).

[x]Root Mean Square Error.

[z]*P*-value from analysis of variance tables.

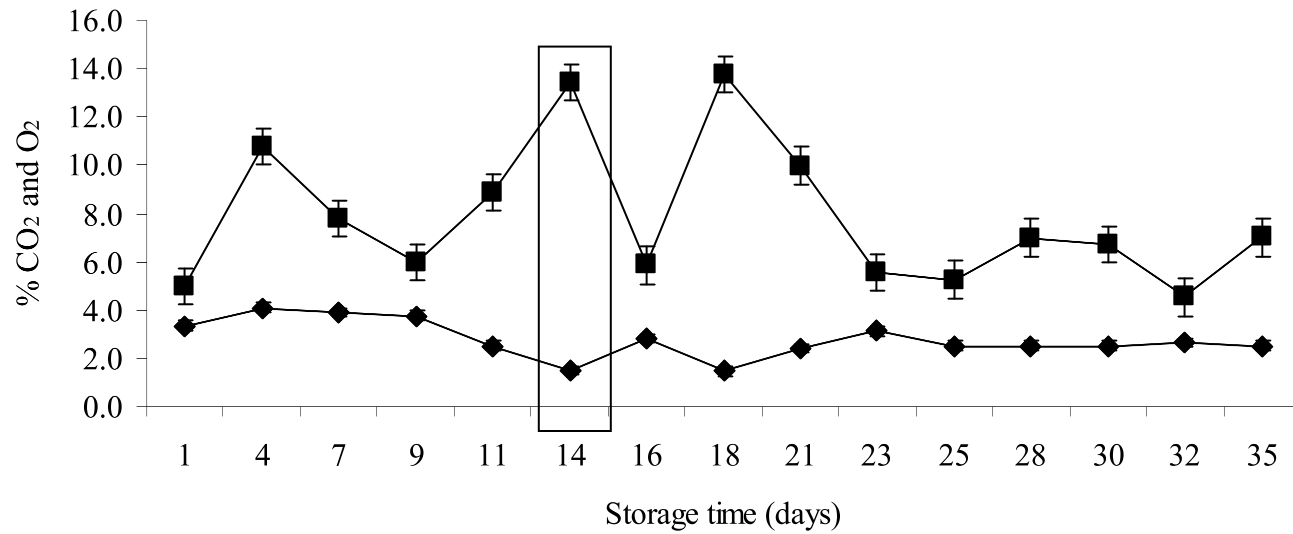


FIGURE 16. *Percentage of CO_2 (♦) and O_2 (■) levels in spinach samples treated with 0.0 kGy e- beam irradiation. Day 14 is highlighted to indicate the typical shelf-life of bagged spinach.*

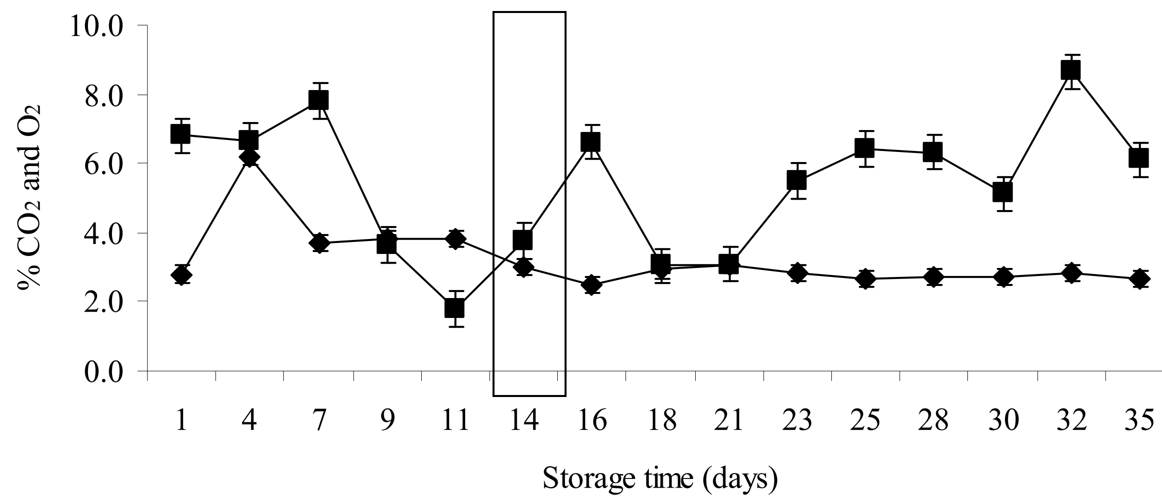


FIGURE 17. *Percentage of CO_2 (♦) and O_2 (■) levels in spinach samples treated with 0.7 kGy e- beam irradiation. Day 14 is highlighted to indicate the typical shelf-life of bagged spinach.*

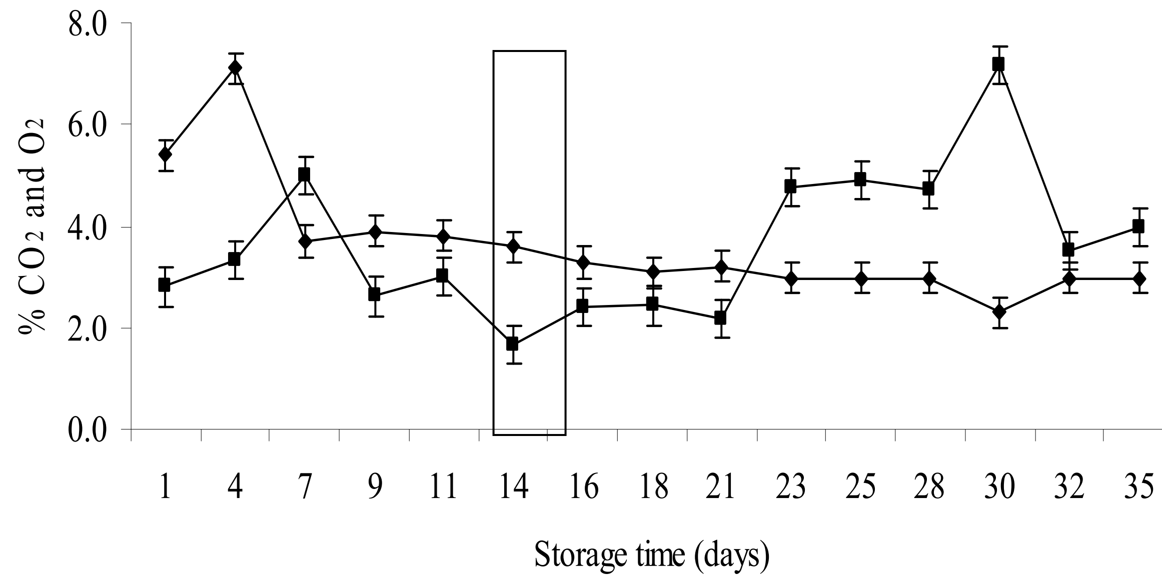


FIGURE 18. *Percentage of CO_2 (♦) and O_2 (■) levels in spinach samples treated with 1.4 kGy e-beam irradiation. Day 14 is highlighted to indicate the typical shelf-life of bagged spinach.*

This drop in oxygen value was not seen in spinach samples throughout the remainder of the experiment. Allende et al. *(8)* described the gas composition of spinach leaves stored under super atmospheric oxygen and modified atmosphere conditions and reported that samples for both treatments (except those in perforated bags) accumulated CO_2 and may have increased the production of CO_2 due to anaerobic respiration. Prakash et al. *(254)* reported gradual increases in CO_2 levels and decreases in O_2 levels in packaged cut romaine lettuce receiving gamma irradiation. McDonald and Risse *(213)* stressed the importance of packaging film gas transmission rates as well as storage temperatures for extending the shelf-life of chopped lettuce and reported that no discoloration or fermentation when CO_2 levels were below 20%. Spinach samples used in this experiment were maintained in a laboratory refrigerator that maintained a relatively constant temperature of 4°C throughout the study. Refrigeration is one of the most effective tools in extending the shelf-life of fruits and vegetables *(352)*. The constant temperature maintained in this study along with the reduction of spoilage bacteria and proper gas compositions maintained in the spinach samples all contributed to the shelf-life extension of the spinach. Hamza et al. *(144)* enriched minimally processed romaine lettuce leaves with higher levels of CO_2 and concluded that CO_2 levels up to 10% were beneficial. In addition, these authors reported that O_2 levels reduced to the minimum of 1% were beneficial for the preservation of processed romaine lettuce due to the increased CO_2 levels controlling wound browning, the development of brown stain and anaerobiosis in the tissues. There were interactions between irradiation treatments and storage days for both O_2 and CO_2 concentrations indicating that the respiration rate of spinach increased with an increase in treatment dose and that gas compositions within the

packages changed over time (Figs. 19, 20). The least squares means for these interactions are included in Table 8. Control samples and samples treated with 0.7 kGy maintained similar levels of both CO_2 and O_2 concentrations over the 35 days of storage. Spinach samples treated with 1.4 kGy had lower O_2 concentrations and higher CO_2 concentrations than the controls and 0.7 kGy irradiated samples. Microbial quality was not affected by the gas compositions of spinach samples. For gas compositions to affect microbial quality, decreases in O_2 may contribute to the reduction of APC and increases in anaerobic LAB. This was not observed. Wound induced injury from irradiation dose may have increased respiration rates due to structural damage to the spinach cell walls and pectins which provide structural rigidity to the leaf. Differences in respiration rates and gas compositions indicated that structural damage may have occurred at irradiation doses of 1.4 kGy and textural differences were identified by a trained sensory panel described below.

Sensory evaluation and objective color. Panelists by main effect interactions were not significant; therefore, data were averaged across panelists and the final model as a repeated measure split-plot design. The least squares means for each parameter analyzed by the sensory panel are included in Table 9 and Table 10. The field in which the samples were harvested did not affect sensory attributes. Irradiation dose had no affect on basic tastes, aromatics, or mouth feels. Hardness and slimy attributes did differ with irradiation dose. Spinach samples treated with 1.4 kGy were less hard. Spinach leaves have a high water content (90%) and the ionization of water is the predominant reaction occurring with irradiation. When water molecules are irradiated, they lose an electron and produce a positively charged water radical (*229).* Irradiation at higher doses is known to

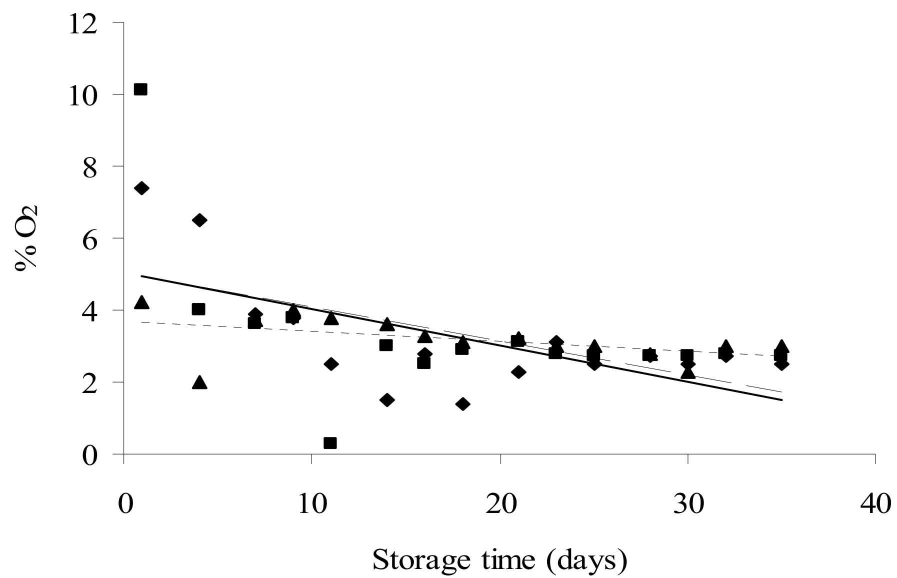


FIGURE 19. *Mean O_2 concentrations as affected by interactions between storage days and 0 kGy (♦, ▬ linear,) 0.7 kGy (■, --- linear), and 1.4 kGy (▲,▪▪▪ linear).*

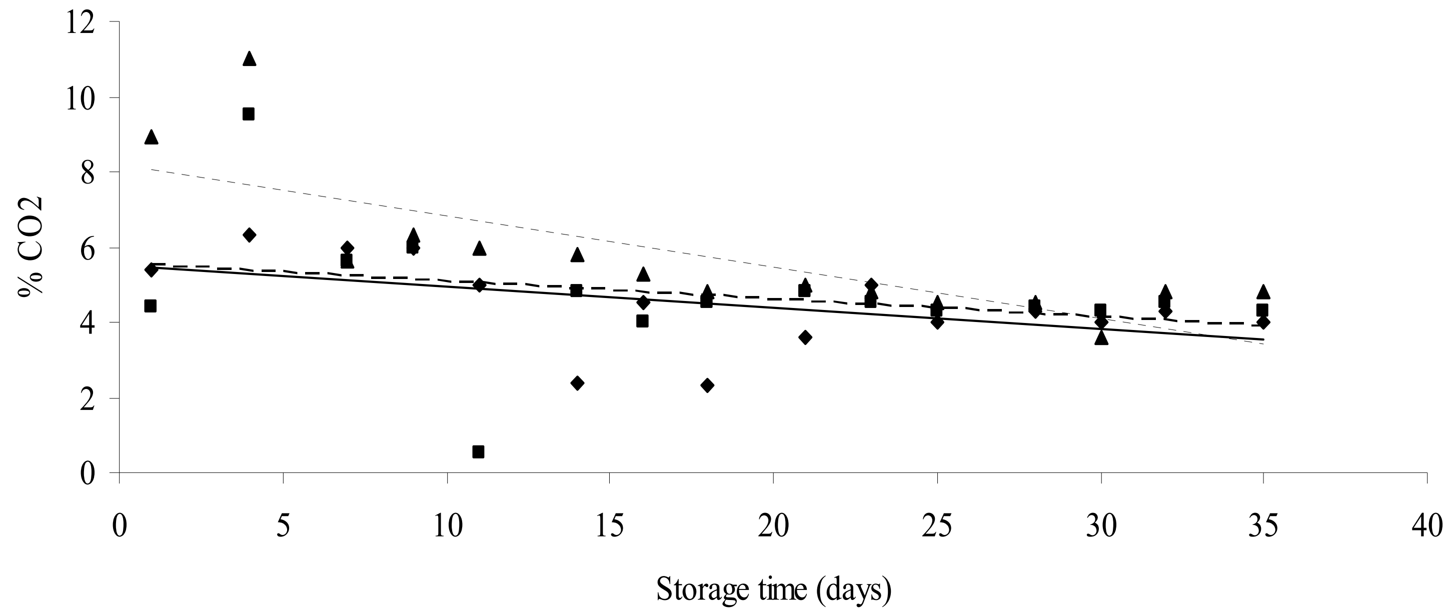


FIGURE 20. *Mean CO_2 concentrations as affected by interactions between storage days and 0 kGy (♦, ▬ linear,) 0.7 kGy (■, --- linear), and 1.4 kGy (▲, ▪▪▪ linear).*

TABLE 8. *Means for gas composition in fresh bagged spinach as affected by treatment dose, storage days and treatment by storage days.*

Effect	Day	Dose	CO_2	O_2
Dose x Day			*<0.0*[z]	<0.0[z]
	1	0.0	5.4A[a]	7.4A
		0.7	4.4B	10.1B
		1.4	8.9D	4.2C
	2	0.0	6.3C	6.5D
		0.7	9.5E	4.0CF
		1.4	11.0F	2.0E
	4	0.0	6.0C	3.9F
		0.7	5.6AC	3.6F
		1.4	5.6AC	3.7F
	7	0.0	6.0C	3.8F
		0.7	6.0C	3.8F
		1.4	6.3C	4.0CF
	9	0.0	4.0B	2.5E
		0.7	0.5G	0.3G
		1.4	6.0C	3.8F
	11	0.0	2.4D	1.5H
		0.7	4.8B	3.0I
		1.4	5.6AC	3.6F
	14	0.0	4.5B	2.8EI
		0.7	4.0B	2.5E
		1.4	5.3A	3.3FJ
	16	0.0	2.3D	1.4H
		0.7	4.5B	2.9I
		1.4	4.8B	3.1I
	18	0.0	3.6E	2.3E
		0.7	4.5B	2.9I
		1.4	4.8B	3.1I
	21	0.0	5.0AB	3.1I
		0.7	4.5B	2.8EI
		1.4	4.8B	3.0I
	23	0.0	4.0B	2.5E
		0.7	4.3B	2.7EI
		1.4	4.5B	2.8EI
	25	0.0	4.3B	2.7EI
		0.7	4.4B	2.7EI
		1.4	4.5B	2.8EI

TABLE 8 *(Continued)*.

Effect	Day	Dose	CO_2	O_2
Dose x Day			*<0.0*[z]	<0.0[z]
	28	0.0	4.0B	2.5EI
		0.7	4.3B	2.7EI
		1.4	3.6E	2.3E
	30	0.0	4.3A	2.7EI
		0.7	4.5B	2.8EI
		1.4	4.8B	3.0I
	32	0.0	4.0B	2.5E
		0.7	4.3B	2.7I
		1.4	4.8B	3.0I

[a]Mean values within a column and effect followed by the same letter are not significantly different (*P* > 0.05).
[x]Root Mean Square Error.
[z]*P*-value from analysis of variance tables.

TABLE 9. *Least squares means for fresh spinach for main effects for trained sensory descriptive attributes.*

	Texture						Basic Tastes		
Effect	Surface Wetness	Roughness	Juiciness	Tooth Packing	Hardness	Slimy	Bitter	Sweet	Sour
RMSE[x]	0.22	0.15	0.17	0.09	0.16	0.11	0.20	0.11	0.06
Irradiation	0.37[z]	0.32[z]	0.36[z]	0.74[z]	0.0004[z]	0.03[z]	0.81[z]	0.58[z]	0.37[z]
0.0 kGy	1.4A[a]	3.6A	2.7A	3.3A	3.6A	0.13B	3.1A	0.1A	0.2A
0.7 kGy	1.3A	3.6A	2.7A	3.3A	3.6A	0.05A	3.1A	0.1A	0.3A
1.4 kGy	1.3A	3.5A	2.7A	3.3A	3.4B	0.06A	3.1A	0.2A	0.3A
Storage Day	<0.01[z]	<0.01[z]	<0.01[z]	<0.01[z]	<0.01[z]	<0.01[z]	<0.01[z]	<0.01[z]	<0.01[z]
1	2.1A	4.3A	2.9AB	3.5AB	4.1A	0.1A	3.4A	0.2A	0.3A
2	2.3A	4.1A	2.9AB	3.6A	4.2A	0.1A	3.4A	0.2A	0.2A
4	1.6B	3.9A	2.7AB	3.4AB	4.1A	0.1A	3.4A	0.4A	0.2A
7	1.7B	3.6AB	2.9AB	3.4AB	4.1A	0.1A	3.0AB	0.2A	0.2A
9	1.7B	3.2BC	2.8AB	3.1AB	3.7ABC	0.1A	2.8B	0.2A	0.4A
11	1.5B	3.6AB	2.8AB	3.4AB	3.5BC	0.1[a]	3.0AB	0.1A	0.4A
14	1.5B	3.5AB	3.0A	3.5AB	3.7ABC	0.1A	3.0AB	0.2A	0.2A
16	1.4B	3.8A	3.0A	3.3AB	3.8AB	0.1A	3.1AB	0.1A	0.2A
18	0.9BC	3.6AB	2.4B	3.3AB	3.3BC	0.1A	3.4A	0.1A	0.2A
21	1.3BC	3.3BC	2.7AB	3.4AB	3.4BC	0.1A	3.1AB	0.1A	0.2A
23	1.3BC	3.4B	2.4B	3.3AB	3.4BC	0.1A	3.3AB	0.1A	0.2A
25	1.1BC	4.2A	2.8AB	3.4AB	3.2C	0.1A	3.4A	0.1A	0.2A
28	0.8BC	3.4B	2.6AB	3.2AB	3.5BC	0.2A	2.8B	0.2A	0.4A
30	0.5C	3.2BC	2.2B	3.2AB	2.8CD	0.1A	3.0AB	0.1A	0.2A
32	1.2BC	2.8C	2.3B	3.0B	2.8CD	0.3A	3.0AB	0.1A	0.4A
35	1.1BC	3.1BC	2.6AB	3.0B	3.0CD	0.3A	2.8B	0.1A	0.3A

[a]Mean values within a column and effect followed by the same letter are not significantly different ($P > 0.05$).
[x]Root Mean Square Error.
[z]*P*-value from analysis of variance tables.

TABLE 10. *Least squares means for fresh spinach for main effects for trained sensory descriptive attributes.*

	Aromatics					Mouthfeel		
Effect	Green/ Grassy	Earthy	Musty	Nutty	Decay	Burn	Astringent	Color
RMSE[x]	0.23	0.14	0.10	0.63	0.11	0.18	0.17	0.25
<u>Irradiation</u>	0.86[z]	0.56[z]	0.72[z]	0.47[z]	0.82[z]	0.43[z]	0.33[z]	0.01[z]
0.0 kGy	12.0A[a]	0.7A	0.1A	0.1A	0.1A	0.7A	3.1A	12.3A
0.7 kGy	12.0A	0.7A	0.1A	0.A	0.1A	0.6A	3.1A	12.1B
1.4 kGy	12.0A	0.7A	0.1A	0.A	0.1A	0.7A	3.1A	12.1B
<u>Storage Day</u>	<0.01[z]	<0.01[z]	<0.01[z]	0.023[z]	<0.01[z]	<0.01[z]	<0.01[z]	<0.01[z]
1	13.8A	1.0A	0.3A	0.1A	0.1A	1.2AC	3.4A	12.1A
2	14.2A	1.0A	0.1A	0.1A	0.1A	1.1AC	3.4A	12.0A
4	13.1B	0.6AB	0.1A	0.1A	0.1A	0.8AC	3.2AB	12.1A
7	12.7BC	0.8AB	0.1A	0.1A	0.1A	0.1B	3.1AB	12.2A
9	12.5C	0.7AB	0.1A	0.1A	0.1A	0.6BC	2.8B	12.4A
11	12.3CE	0.8AB	0.1A	0.1A	0.1A	0.6BC	3.0AB	12.3A
14	12.2CE	0.4B	0.1A	0.1A	0.1A	0.3BC	2.9AB	12.2A
16	12.0CE	0.6AB	0.1A	0.1A	0.1A	0.7C	3.2AB	12.1A
18	11.2D	0.6AB	0.1A	0.1A	0.1A	0.9AC	3.4A	12.3A
21	11.0DF	0.6AB	0.1A	0.1A	0.1A	0.7C	3.1AB	12.0A
23	11.8E	0.6AB	0.1A	0.1A	0.4A	1.1AC	3.3AB	12.0A
25	11.9E	0.8AB	0.1A	0.1A	0.4A	1.2AC	3.3AB	13.0B
28	11.1D	0.7AB	0.1A	0.1A	0.2A	0.4BC	3.0AB	12.1A
30	11.1D	1.0A	0.1A	0.1A	0.3A	0.6BCE	3.2AB	12.4A
32	10.5F	0.9AB	0.3A	0.1A	0.2A	0.4BC	3.0AB	12.0A
35	10.3F	0.5AB	0.1A	0.1A	0.3A	0.2DE	3.0AB	13.0B

[a]Mean values within a column and effect followed by the same letter are not significantly different ($P > 0.05$).
[x]Root Mean Square Error.
[z]*P*-value from analysis of variance tables.

cause softening of fruits and vegetables due to its effects on the plant cell walls and on the pectins, which provide the structural rigidity to plant tissue *(36)*. The slimy attribute was also affected by treatment dose. Slime production is the result of spoilage caused by bacteria such as LAB. As indicated from the microbial quality data, irradiation treatments significantly reduced the initial loads of spoilage bacteria. Control spinach samples were scored as having higher slimy attributes indicating higher levels of spoilage bacteria as compared to irradiated samples. Panelists also scored color attributes as being lighter for treated spinach compared to control samples. The storage time in which the samples were held had an affect on texture, basic tastes, aromatic attributes and mouthfeels. For the texture attributes, spinach leaves were drier on the surface, less rough and less juicy with less toothpacking and were softer after 35 days of storage. Basic tastes and flavor aromatics were affected by storage. With increased storage, spinach was less bitter, had lower green/grassy and earthy flavor aromatics. Additionally, spinach was not as astringent and had less burn mouthfeel after 35 days of storage. Surface wetness and juiciness decreased over storage days. These results indicated that water was lost from all spinach samples during storage. The water loss may be attributed to the senescence of the spinach over time. Water loss is a main cause of spinach deterioration resulting in wilting and shriveling. With water loss, limpness, loss of crispness and decreased juiciness would be expected. Surface roughness decreased in spinach after 9 days of storage. Spinach decreased in hardness after 11 days of storage. After 32 and 35 days of storage, spinach toothpacking decreased. The change in these textural attributes may be attributed to water loss and possibly spoilage microorganisms breaking down pectins giving rise to a soft consistency. The basic taste attributes of bitter, sweet and sour were measured over the 35

storage days. Bitter basic taste decreased after 9, 30 and 35 days of storage. Bitter basic taste has been reported in spinach and the bitter flavor has been attributed to oxalic acid *(285)*. Oxalic acid will break down over time which may decrease bitter basic taste. Green/grassy flavor aromatic decreased over time. Spinach is used as a reference for green/grassy aromatic attribute and the chemical compounds responsible for this aromatic (hexyl benzoate, hexyl phenylacetate, hexyl proprionate and hexyl tiglate) can be expected to breakdown over time *(159)*. Musty, nutty and decay flavor aromatics were not affected by storage day. It is interesting to note that the earthy flavor aromatic, which is typical flavor attribute of spinach, fluctuated over the 35 days of storage. The mouthfeel attributes burn and astringent fluctuated over the 35 days of storage. Burn decreased during the first 18 days; however, it increased on days 23 and 25. This may be due to sample variability. Astringent attributes decreased gradually. There were no interactions between treatment and storage days for sensory attributes. For storage days, the spinach color was darker on days 25 and 35 however, L values varied across storage day. As panelists most likely accounted for variation in color across leaves that occurred with the longest storage times. These differences were not detected by objective color measurements. Lightness, hue or chroma values for spinach were not affected by treatment, field, or storage period and interactions between treatment and storage day were not detected.

Piagentini et al. *(249)* found similar results associated with storage time. They studied the combined effects of citric acid, ascorbic acid, and the type of packaging film on sensory characteristics of fresh-cut spinach. Their data indicated that all sensory attributes studied were affected by storage time. Prakash et al. *(254)* reported that as

storage time increased, sensory panelists gave lower scores to gamma-irradiated romaine lettuce leaves for color and texture and higher scores for off-flavors. Miller et al. *(218)* observed that e-beam irradiation up to 1 kGy had no effect on the color of “Sharpblue” blueberries. Prakash et al. *(254)* also detected no difference in the color of lettuce leaves due to gamma-irradiation. Gomes et al. *(135)* reported that overall, exposure to e-beam irradiation up to 1 kGy did not affect the color attributes of spinach leaves; however, they did detect higher b^* values (more yellowish) for samples treated with 1.4 kGy. These data show that low dose electron beam irradiation, at the doses used in this study, has minimal effect on sensory attributes and objective color of spinach leaves.

Quality changes. On the basis of the results from the present study and literature information, we present in Figure 21 a diagram of the affects of ionizing radiation on the quality of fresh spinach. Influencing factors include the reductions in the microbial load, the affects of reactive oxygen species and physical and physiological deterioration over time. In general, our results indicate that e-beam irradiation elicits several responses in spinach before and during storage that are dose dependent.

Quality loss of fruits and vegetables can also be attributed to microbial growth, deterioration, moisture loss, and senescence. As mentioned previously, the APC, LAB and yeasts and molds counts were reduced depending on the dose received. APC and LAB counts were reduced at both 0.7 and 1.4 kGy. In addition to having reduced counts, the trained sensory panel reported reductions in slimy texture for irradiated samples indicating reductions in slime producing spoilage bacteria. Yeasts and molds were reduced significantly only at the higher dose of 1.4 kGy however; microbial counts were equal to those of the control samples on day 2. The storage temperature of the spinach

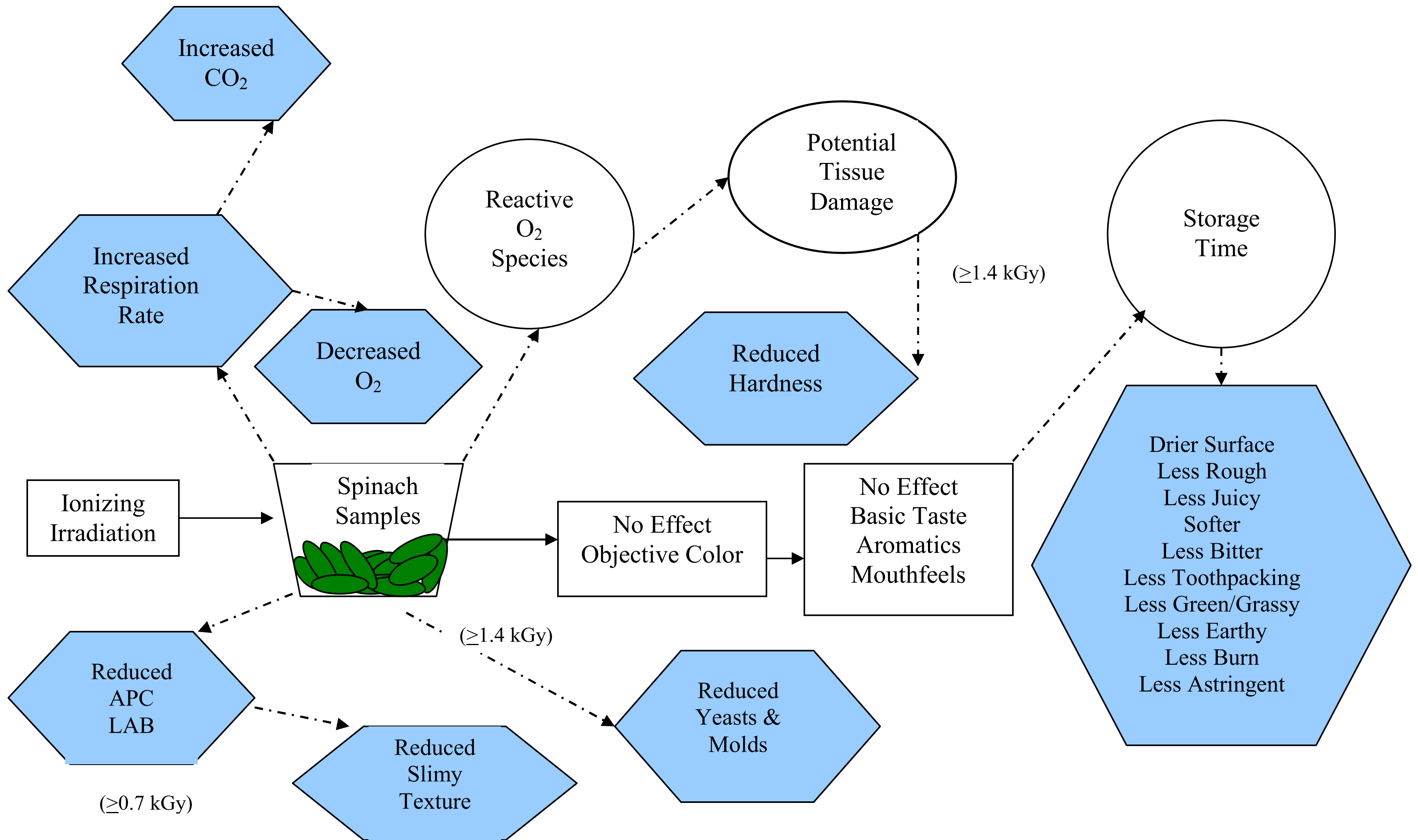


FIGURE 21. *Diagram summarizing the effects of e-beam irradiation and storage on the microbial quality, gas composition and sensory quality of spinach samples.*

samples may also have contributed to the reduced growth of microorganism over the 35 days of storage. Spinach samples were stored at 4°C throughout the study. This storage temperature would permit psychrotrophic microorganisms to grow and mesophilic microorganism may have been able to survive without growth. Gas composition measurements were taken throughout the study; however, the affect of gas composition on microbial growth appears to be limited. With increases in O_2 levels, one may expect increases in APC growth and with reduced O_2, increases in anaerobic LAB could be expected. Neither observation was detected in this study. Respiration rates increased as irradiation dose increased indicating possible wound-induced respiration. This damage may have been caused by reactive oxygen species which can cause cell damage (DNA damage and cell membrane lesions) resulting in localized tissue death. Irradiation at higher doses is known to cause softening of fruits and vegetables because of its effect on the plants cell walls and on the pectins which provide structural rigidity to the plant tissue. While localized tissue death in the form of dark spots or color changes were not observed by the sensory panel, the hardness texture was less for spinach samples that received 1.4 kGy. Respiration rates for the spinach samples declined over time. Increases in CO_2 and decreases in O_2 have been reported to improve the shelf-life of minimally processed vegetables. This can be achieved through controlled atmosphere packaging. Controlling the gas composition within the sample package retards the respiration rate of the spinach sample which also prevents moisture loss and microbial growth. Gas compositions of spinach samples were measured over the 35 day storage period. The CO_2 levels in spinach samples irradiated at both doses were higher than control samples; however, CO_2 for irradiated and control samples were equal on day 7. The O_2 levels were

significantly lower for irradiated samples over 14 days of storage. While O_2 levels decreased over time, they did not lead to fermentative metabolism. Fermentative metabolism can be identified as O_2 concentrations below 1% and by off-odors and off-tastes which were not detected by the sensory panel. Moisture loss was determined by the sensory panel by measuring surface wetness and juiciness textures. The loss in moisture may have been caused by the initial increase in respiration rate. Panelists were able to detect reductions in surface wetness on spinach samples by day 4 for all samples. Samples were less juicy after 23 days of storage. As the storage time increased, panelists were able to detect signs of deterioration. In addition to moisture loss, panelists reported textural reductions in roughness and toothpacking. Basic tastes, aromatics and mouthfeels were also reduced over time indicating senescence.

CONCLUSIONS

Electron beam irradiation was found to be an effective method for the decontamination of spinach leaves. Irradiation at doses up to 1.2 kGy followed by storage at 4°C was efficient at pathogen reduction and inhibiting proliferation of survivors for *E. coli* O157:H7; however, *Salmonella* proved to be more resistant to irradiation and survivors were recoverable at doses below 1.2 kGy. These data suggest that 1.2 kGy irradiation or higher is needed to control *Salmonella* levels. Confocal microscopy images of both *E. coli* O157:H7 and *Salmonella* on the spinach leaf edge and within the stem demonstrated the capacity of pathogens to lodge themselves in locations possibly unreachable by commonly used chemical decontamination methods. Our study showed that e-beam irradiation can also be an effective tool for reducing counts of spoilage bacteria and extending the shelf-life of fresh spinach. Irradiation was effective in reducing the aerobic microbial load of the spinach. Yeasts and molds were more resistant to irradiation than vegetative bacteria. This is in agreement with previous studies. Respiration rates increased with irradiation dose indicating wound-induced respiration. Gas compositions were measured to determine the effect on storage. The sensory data indicated that the objective color, basic tastes, aromatics and mouthfeel attributes of spinach were not affected by e-beam irradiation. Irradiation decreased the hardness and reduced the sliminess of the spinach samples. Due to the fact that spinach leaves are capable of sticking together or folding over during water wash treatments,

irradiation of bagged spinach leaves may be a reasonable option for the produce packaging industry.

Clearly, our research indicates that e-beam irradiation can be an effective tool for reducing *E. coli* O157:H7 and *Salmonella* counts in fresh spinach. E-beam irradiation also effectively reduces counts of spoilage bacteria and extends the shelf-life of fresh spinach without affecting objective color and limited affects of organoleptic characteristics.

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