INACTIVATION OF *ESCHERICHIA COLI* O157:H7 ON BABY SPINACH USING LOW-ENERGY X-RAY IRRADIATION

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ABSTRACT

INACTIVATION OF *ESCHERICHIA COLI* O157:H7 ON BABY SPINACH USING LOW-ENERGY X-RAY IRRADIATION

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Low-energy X-ray irradiation was assessed as a means of inactivating *Escherichia coli* O157:H7 on baby spinach. Round-cut (2.54 cm diameter) baby spinach samples were dip-inoculated in a 3-strain *E. coli* O157:H7 cocktail, and irradiated on each side of the leaf using a prototype low-energy (70 kV) X-ray irradiator to achieve X-ray doses of up to 0.176 kGy per side. The D_{10} value for *E. coli* O157:H7 on baby spinach was 0.033 ± 0.001 kGy. Irradiated bags of baby spinach (225 g) yielded a dose rate distribution of 1.93 ± 0.75 Gy/s at the mid-plane to 7.18 ± 3.85 Gy/s on the bag's surface. Predicted log reduction for *E. coli* O157:H7 in the bag was 4.10 log CFU, while the observed mass averaged value was 3.99 log CFU. Next, pre-irradiated, round-cut baby spinach leaves were inoculated with an *E. coli* O157:H7 cocktail pre-exposed to a peracetic acid-based (PAB), chlorine-based (CB) or quaternary ammonium compound-based (QACB) sanitizer to obtain 90 to 99% injury, and subjected to X-ray doses of up to 0.063 kGy. Compared to control values of 0.021 ± 0.001 kGy, D_{10} values for PAB-, CB-, and QACB-injured *E. coli* O157:H7 on baby spinach were 0.0136 ± 0.000, 0.0223 ± 0.001, and 0.0242 ± 0.001 kGy respectively, with exposure to PAB significantly ($P < 0.05$) enhancing *E. coli* O157:H7 susceptibility to X-ray irradiation, while exposure to CB significantly ($P < 0.05$) reduced susceptibility. Based on these findings, low-energy X-ray irradiation appears to be most effective against *E. coli* O157:H7 when spinach is flume tank-washed using a PAB sanitizer prior to irradiation.

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1. INTRODUCTION

Leafy green vegetables are among the top ten riskiest foods regulated by the U.S. Food and Drug Administration (CSPI, 2009a). This is due in large part to the potential for contamination to occur throughout all facets of production. Leafy greens are prone to contamination when grown in an open environment and undergo minimal processing before consumption. Evidence of contamination has mounted in recent years, as the number of documented outbreaks of *E. coli* O157:H7 associated with the consumption of leafy greens has increased (Soderstrom et al., 2005; Ackers et al., 1998; Hilborn et al., 1999; Ethelberg et al., 2010). Heightened consumer concerns regarding the safety of leafy greens have warranted a reassessment of the production practices for ready-to-eat leafy greens.

Ensuring the safety of leafy greens is a process that must also be balanced with maintaining product quality. Current microbial reduction practices have relied solely on chemical sanitizers during washing of leafy greens. However, this process has remained largely ineffective, demonstrating pathogen reductions of < 2 logs (Sapers, 2001; Beuchat et al., 2004; Keskinen et al., 2009; Lopez-Galvez et al., 2009). Considering the low infectious dose associated with *E. coli* O157:H7 and the increasing number of outbreaks, chemical sanitizers alone are insufficient to ensure the safety of leafy greens. In response to three nationwide outbreaks linked to bagged baby spinach and shredded iceberg lettuce in 2006, the FDA issued a rule allowing the use of ionizing irradiation as an alternative strategy to ensure the safety of fresh and bagged iceberg lettuce and baby spinach (FDA, 2008).

Ionizing radiation, in the form of X-rays, E-beam, and gamma ray, has found many food applications, including the control of insects, inhibition of sprouting, and pathogen reduction. In controlling pathogens associated with leafy greens, ionizing irradiation has been particularly effective. Using gamma irradiation, D_{10} values for *E. coli* O157:H7 on surface-inoculated lettuce, in internally inoculated lettuce, and in homogenized lettuce suspensions were 0.119-0.140, 0.30-0.45, and 0.092-0.339 kGy, respectively (Niemira et al., 2002; Niemira, 2008). E-beam irradiation (10 MeV) has shown to be equally effective, with a D_{10} value of 0.186 kGy for *E. coli* O157:H7 on spinach (Gomes et al., 2008).

Until recently, X-rays have been undervalued as an alternative source of ionizing irradiation. The technology was considered to be inefficient and less effective compared to gamma radiation and E-beam; however, recent advances in the efficiency and versatility of machine sources have been working to change perception. X-ray radiation is now being recognized for its low shielding requirement, small machine footprint, and the ability to be incorporated into an existing processing system.

Using a newly developed low-energy X-ray irradiator (Rayfresh Technologies, Ann Arbor, MI), the efficacy of X-rays as been demonstrated for ground beef, lettuce, asparagus, blueberries, almonds, walnuts, and chestnuts. X-ray irradiation has been particularly effective for inactivating *E. coli* O157:H7 on lettuce, with a D_{10} value of 0.040 kGy (Jeong et al., 2010b), which is 3.4 times lower than the previously reported value of 0.136 kGy using gamma radiation (Niemira et al., 2002).

Sublethal injury and cross-protection from one stressor to the next is always a concern when multiple microbial reduction strategies are used. Chemical sanitizers and ionizing irradiation often target similar components of an organism, with varying degrees of injury seen depending on the concentration and length of exposure to the stressor. Given similarities in the inactivation mechanisms for many chemical sanitizers and ionizing irradiation, the potential for enhanced microbial resistance from sublethal sanitizer stress needs to be addressed before implementing low-energy X-ray technology as a microbial reduction strategy in the processing of leafy greens.

This thesis aims to investigate the efficacy of low-energy X-ray irradiation for inactivating *E. coli* O157:H7 on baby spinach, with a focus on the commercialization of this technology within existing leafy green processing facilities. Assuming that X-ray radiation is used as the final kill step, pathogens on leafy greens will be exposed to X-rays following sanitizer treatment of the product, which will remain a standard industry practice. Objectives outlined in this thesis include establishing the D_{10} value for *E. coli* O157:H7 on baby spinach, determining the dose required to result in a 5-log reduction of *E. coli* O157:H7 in commercial bags of baby spinach, and to evaluate the potential for cross-protection from chemical sanitizers during production. Lastly this thesis will address further considerations for commercial applications and safety concerns of X-ray technology.

2. CHAPTER 1

LITERATURE REVIEW

2.1. Introduction

Escherichia coli O157:H7 first appeared in the public headlines in 1993 following an outbreak at Jack-in-the-Box restaurants from consumption of improperly cooked hamburgers (CDC, 1993). Since its emergence, this pathogen has been routinely associated with the gastrointestinal tract of ruminant animals, and consequently as a potential hazard during slaughter and beef production. Other outbreaks involving *E. coli* O157:H7 have been traced to apple juice (Cody et al., 1999), sprouts (Breuer et al., 2001), cucumbers (Duffell et al., 2003), and leafy vegetables (Soderstrom et al., 2005; Ackers et al., 1998; Hilborn et al., 1999; Ethelberg et al., 2010).

Initial symptoms from *E. coli* O157:H7 infections are generally quite mild and self-limiting, resulting in diarrhea and stomach cramps. Complications of the infection may occur in some cases and can be severe, beginning with bloody diarrhea (hemorrhagic colitis) and severe dehydration, with the potential to progress further into conditions such as hemolytic uremic syndrome (HUS) and thrombocytopenic purpura (TTP) (FDA, 2009). HUS is of particular concern in children under the age of 5. It is a condition that develops in up to 15% of cases and can lead to permanent loss of kidney function, with a mortality rate of 3-5% (Buchanan and Doyle, 1997). TTP, a neurological illness more common in the elderly, can have a fatality rate of up to 50%. In 2010, the hospitalization and fatality rates for *E. coli* O157:H7 infections were 41.6% and 0.45%, respectively (CDC, 2010a; CDC, 2010b). It is estimated that on average, *E.*

coli O157:H7 causes approximately 63,000 illnesses, 2,100 hospitalizations, and 20 deaths each year (Scallan et al., 2011).

From 1973 to 1997, produce accounted for a total of 190 outbreaks from all causes, 16,058 illnesses, 598 hospitalizations, and 8 deaths, with produce-associated outbreaks increasing in proportion from 0.7% in the 1970's to 6% in the 1990's (Sivapalasingam, 2004). From 1998 to 2007, the Center for Science in the Public Interest (CSPI) reported that produce as a category had accounted for 684 outbreaks, and resulted in the largest number of illnesses in the United States compared to any other food group (CSPI, 2009b). CSPI reports further identified particular risks associated with a number of produce-pathogen combinations, namely *Salmonella*, *E. coli* O157:H7 and Norovirus contamination of leafy greens. These three pathogens combined for a total of 265 outbreaks and 9,001 illnesses from consumption of lettuce and leafy green-based salads.

Concern for *E. coli* O157:H7 contamination of leafy greens came to a head in 2006 following three prominent outbreaks: one in September associated with baby spinach resulting in 205 confirmed cases in 28 states and 3 deaths (CFERT, 2007), and another two in December associated with shredded lettuce that resulted in a total of 80 confirmed cases in 5 northeastern states (U.S. FDA, 2007a; U.S. FDA, 2007b). In light of the heightened severity of illness and symptoms associated with *E. coli* O157:H7 infections, the pathogen has presented itself as the target for control on leafy green vegetables, with the primary focus on spinach and iceberg lettuce.

Control of pathogens in the processing of leafy greens has been reliant on the application of chemical sanitizers in the wash water; however, the efficacy of these agents has been less than ideal with bacterial populations decreasing only 1 to 2 logs on the surface of the produce during commercial processing (Sapers, 2001; Beuchat et al., 2004; Keskinen et al., 2009; Lopez-Galvez et al., 2009). In light of the poor performance of sanitizers and the continued outbreaks associated with leafy greens, alternative solutions such as X-ray irradiation need to be investigated.

X-ray irradiation has proven to be a highly effective means of bacterial inactivation, particularly in the treatment of leafy greens (Jeong et al., 2010b, Moosekian et al., 2010a; Mahmoud, 2010a; Mahmoud et al., 2010). The technology also provides the capability of implementation into a processing line as a final kill step. Given similarities in the inactivation mechanisms for many chemical sanitizers and ionizing irradiation, the potential for enhanced microbial resistance from sublethal sanitizer stress should be addressed before implementing low-energy X-ray technology as a microbial reduction strategy in the processing of leafy greens.

The implications of cross-protection for the food industry and public health, begins with an understanding of the actions of chemical sanitizers and their limitations, and how the bacteria use their environment (produce surface, organic load) and defense mechanisms (gene expression, protection from reactive oxygen species (ROS)) to overcome, adapt and thrive under such conditions.

2.2. Potential Sources of Contamination

Measures to prevent the interaction of *E. coli* O157:H7 with leafy green vegetables have proven to be difficult, due to the manner in which the leafy greens are grown and the uncertain origin of the initial contamination. Plants grow in the open environment, exposed to the elements of nature, and low to the ground. Leafy greens also demand plenty of water for growth, and depending on the variety and fragility of the leaf tissue, cultivation by hand may be necessary. Measures to amplify the cultivation of leafy greens have helped to elevate the potential risk of bacterial contamination by creating a process that requires larger plots of land, unskilled workers, and irrigation water to further expand growing seasons and locations. Furthermore, the raw manner in which most leafy greens are consumed adds another level of difficulty in the assessment of contamination routes. In order to maintain the leaf's visual and organoleptic qualities, leafy greens cannot be heat-treated to inactivate pathogens. Such difficulties in either preventing or reducing the contamination of leafy greens with *E. coli* O157:H7 have culminated in a reassessment of established field environment and processing conditions in a search for reliable strategies to better ensure end product safety. Furthering the understanding of these key components in the process and their role in a contamination event has been a primary focus in determining the overall risk from the field and future outbreak prevention.

2.2.1. *Irrigation practices*

Water is a bare necessity to a plant's growth and survival, and may be considered a limiting factor to the location and growing season of certain crops. Leafy greens in particular have a relatively high water requirement, and are therefore not traditionally grown in certain areas of the country due to water limitations and temperature extremes. However, demand for fresh produce is consistent year-round, driving the necessity for their growth in greenhouses or in the outdoors under less-than-favorable conditions. Irrigation, or artificially supplying land with water, is a necessary practice in locations where rainfall may be limited. By transferring water from sources where it is plentiful to locations where it is lacking, crop growth may be possible under even the driest conditions.

Irrigation has found widespread use in the U.S. and abroad, including the expansion of the growing season and growing locations. In 2008, the Farm and Ranch Irrigation Survey showed that U.S. farmers and ranchers irrigated 54.9 million acres of land, with the amount of water applied totaling 91.2 million acre-feet from 2003 to 2008 (USDA, 2008). The source of this irrigation has originated primarily from surface waters such as lakes, rivers, ponds, and streams located in either on-farm water supplies or off-farm reservoirs. Other less common sources include treated wastewater, ground water and well water. Similar trends have also been recorded in the United Kingdom, where the total irrigated area reported in 2003 was about 12,700 acres, with surface waters accounting for approximately 71% of the water used for leafy green irrigation (Tyrrel et al., 2006).

Considering the close contact of irrigation water with the edible portions of the growing plant, the quality of the water has been identified as a primary source of microbial concern. Consequently, guidelines for irrigation water quality have been set by the EPA and WHO for treated waste water (Hespanhol and Prost, 1994) and surface waters (National Research Council, 1974), requiring fewer than 1,000 fecal coliforms per 100 ml.

Testing of surface waters is difficult and unreliable, as the quality of surface water may fluctuate from day to day depending on how it is supplied and collected from the water source, which may in turn vary due to any number of interactions that the water may encounter en route to the farm. Pollution of surface waters may occur from industrial dumping or run-off from areas such as feedlots, waste piles, or known sources of fecal contamination, during events of flooding (Wing et al., 2002) or the sudden rise in river flow (Fremaux et al., 2009).

Not all methods for applying irrigation water are equal. It is estimated that the majority of farms and ranches in the United States prefer spray irrigation over other methods such as drip, hand-watered, subirrigation or gravity irrigation (USDA, 2008). Irrigation by spraying, however, potentially enhances the interaction of the growing crop with contaminated water by increasing the dissemination and survival of *E. coli* O157:H7 across the crop (Moyne et al., 2011; Fonseca et al., 2011; Solomon et al., 2002a; Solomon et al., 2003).

2.2.2. *Interaction with wildlife and animal excrement*

Plant contact with wildlife can occur directly through interactions that take place in the field, or indirectly by means of contaminated water, manure, or other items that may be used in growing crops. Direct interaction in the field from uncontrolled wild animals or insects has shown to be an uncommon and sporadic route of contamination, while indirect sources have been the focus of greater scrutiny due to their unpredictability and potential to amplify and spread contamination throughout an entire lot of product.

Animals that graze on nearby fields are seen as the greatest contributors to surface water contamination. *E. coli* O157:H7 has consistently been isolated from the hides and feces of livestock including pigs (Gill and Jones, 1998), cattle (Fegan et al., 2004; Smith et al., 2009), sheep (Ogden et al., 2005), deer (Rice et al., 2003), and wild rabbits (Scaife et al., 2006). *E. coli* O157:H7 has also been shown to survive for more than two months in feces (Beuchat, 1999; Wang et al., 1996) and manure-amended soils (Mukherjee, et al., 2006a; Duffy, 2003; Johannessen et al., 2005; Islam et al., 2004), and up to 28 days on equipment that has come into contact with feces (Williams et al., 2005), with the ability to leach out from ruminant feces (Williams et al., 2008) under rainy conditions.

Minimizing the spread of pathogens from the water source can be achieved through good grazing management practices, such as rotational grazing, portable water supply, portable shade source, and fencing animals from water access (Hubbard et al., 2004). During investigations into the spinach outbreak of 2006, *E. coli* O157:H7 was recovered from cattle feces, wild pig feces, soil, and water, with 58% of the isolates being similar to the outbreak strain by PFGE analysis (CFERT, 2007). In a report from the California Food Emergency Response Team potential risk factors included the presence of wild pigs in and around spinach fields and the close proximity of irrigation wells to surface water exposed to feces from cattle and wildlife. In a rural Virginia watershed, with the installation of fencing and in-pasture watering stations to restrict the range of cattle on the farm, fecal coliforms were reduced by an average of 94%, from pre-fencing average populations of 15,900 per 100 ml to post-fencing average populations of 960 per 100 ml (Hagedorn et al., 1999).

Air contamination by bioaerosols can also occur from nearby fields. When sprayed onto a neighboring field, *E. coli* K12-contaminated pig slurry (10^3 CFU/ml) contributed to bacterial levels recovered as far as 125 m into the field (Hutchison et al., 2008). Further reports on the spread of pathogens by bioaerosols have been collected in a review by Pillai and Ricke (2002).

Potential for insects to act as vectors of pathogen transmission, particularly from local sources of ruminant feces to the fields of edible plants, has also been raised as an area of concern. In an investigation of leafy green fields in the Salinas Valley of California, 11 of 18 flies collected from one field tested positive for *E. coli* O157:H7 (Talley et al., 2009). Further research by this group also determined experimental transmission of the pathogen to the crop, in which contaminated flies successfully delivered GFP-labeled *E. coli* O157:H7 to leaves (Talley et al., 2009). Previous reports of flies as vectors for *E. coli* O157:H7 transmission has also been documented for apples (Janisiewicz et al., 1999).

Slugs have also been considered to be potential vectors. *E. coli* O157:H7 can survive on the surface of a slug for up to 14 days and within its feces for up to 3 weeks. In a field investigation, *E. coli* O157:H7 was isolated from 0.21% of field slugs from an Aberdeenshire sheep farm (Sproston et al., 2006).

2.2.3. *Transfer of* E. coli *O157:H7 from contaminated water and soil to the plant*

Screening for *E. coli* O157:H7 on leafy greens in actual production fields has shown the presence of avirulent *E. coli* on various leafy greens across the United States (Johnston et al., 2005; Mukherjee et al., 2006b), Canada (Arthur et al., 2007), and Europe (Oliveira et al., 2010), but has not revealed the presence of pathogenic *E. coli*. These studies indicated the presence of fecal material, suggesting the possibility for *E. coli* to follow a similar route to the field. Considering the intentional application of water and manure to fields, *E. coli* and coliform levels may not be the best indicator of contamination.

Contamination of leafy greens with *E. coli* O157:H7 has been accomplished experimentally through the use of contaminated manure-amended soils (Mootian et al., 2009; Islam et al., 2004; Franz et al., 2007) and water irrigation (Ibekwe et al. 2004; Mootian et al., 2009; Wachtel et al., 2002). Damage to the plant's leaf structure can also significantly increase *E. coli* O157:H7 persistence and survival in the field (Harapas et al., 2010; Barker-Reid et al., 2009), with potential for internalization of the pathogen (Solomon et al., 2002). Such conditions used for experimental research purposes are often exaggerated, using unrealistically high inoculation levels and may not reflect actual events as they occur in nature. Overall, internalization of *E. coli* O157:H7 into lettuce and spinach leaf tissue would require greater than

4.4 log CFU/leaf, and is therefore unlikely to occur in the field at low levels (Erickson et al., 2010).

2.2.4. *Washing/bagging*

The final steps of adding value to leafy greens, including shredding, washing, drying and bagging, have the potential to not only introduce contamination but also to spread incoming pathogens to otherwise clean product. Contamination can be introduced into the process by the incoming product, wash water (Hilborn et al., 1999), food handlers, and the equipment surfaces. A contaminated batch of lettuce can in turn contaminate the processing equipment, allowing the contamination to spread to subsequent product (Buchholz et al, 2009). Cross-contamination between leaves of fresh-cut lettuce may occur from contaminated water regardless of treatment with sanitized water (Lopez-Galvez et al., 2009). Furthermore, populations of *E. coli* O157:H7 can increase 4, 4.5, and 11-fold on lettuce leaves that are mechanically bruised, cut into large pieces, or shredded into multiple pieces, respectively, compared to only 2-fold increase on intact leaves under the same conditions (Brandl, 2008).

Once inside the bag, *E. coli* O157:H7 populations will decrease at proper refrigeration temperatures <5°C, while logarithmic growth will likely occur at higher temperatures >8°C (Luo et al. 2010; Abdul-Raouf et al., 1993; Francis and O'Beirne, 2001; Li et al., 2001; Koseki and Isobe, 2005). Such elevated temperatures may be reached during transport of the leafy greens to or from the market, and in storage at the market or home.

2.3. Stress and Sublethal Injury

Microorganisms encounter physical, chemical, and nutritional stressors throughout all phases of processing, challenging the organism's ability to survive and persist under diverse conditions. In the growth of leafy greens, bacteria may be stressed with temperature extremes, radiating energy from the sun, and a limited supply of nutrients in the field. Under processing conditions, the bacteria may be further exposed to agents such as chemical sanitizers, acidic environments, high pressure, and/or radiation. Under each agent and condition, the bacteria act to protect themselves against damage to their cellular barriers, metabolic processes, and reproductive functions.

Injured microorganisms are characterized by a greater sensitivity to chemical compounds used in selective environments, due to structural or metabolic damage (Brashears et al., 2001). Sublethal injury can be further explained as damage to an organism without complete kill, implying loss of function that may be either transient or permanent (Gilbert, 1984). Injury may occur from low levels of an applied stressor, with predictable and reproducible injury occurring rapidly, and is reversible under nonselective conditions (Camper and McFeters, 1979). Such attacks often leave the organism in a viable but not culturable state, void of detection using traditional methods (Hurst, 1977). The use of nonselective conditions is often used as a basis for quantifications of microbial injury, utilizing an organism's ability to proliferate on non-selective compared to a selective media (Wu et al., 2001). Often enrichment of the medium with added nutrients may further facilitate the recovery. Chlorine-injured cells of *E. coli* recover better in environments where ROS are neutralized (Tandon et al., 2007).

2.3.1. *Chemical sanitizers*

Sanitizers are agents that work to inactivate microorganisms through chemical interactions with the cell and the components therein. Such agents are used commercially, and vary in application depending on the primary mechanism of action, desired target organism, and physical properties of the material to which the sanitizer is applied. Based on these properties, sanitizers are commonly grouped into the following categories: alcohols, aldehydes, anilides, biguanides, diamidines, halogen-releasing agents, silver compounds, peroxygens, phenols, bis-phenols, halophenols, and quaternary ammonium compounds.

Chemical sanitizers have been used as the primary reduction strategy throughout the produce industry, especially in the treatment of leafy green vegetables, because of their ease of use and relatively low negative impact on product quality. Peroxygens and chlorine-based compounds (a halogen-releasing agent) are used in flume water during produce washing, while quaternary ammonium compounds are more commonly used to sanitize processing equipment surfaces between production runs.

Correlating the properties of sanitizers to the control of bacteria in flume water has proven to be problematic. During the processing of fresh produce, many variables act to inhibit optimum sanitizer function which leads to difficulties in reducing bacterial populations to acceptable levels. Considering the role of organic debris, bacterial defense strategies, and the structural composition of the product making up the organic load, there is a cumulative protective effect offered to the microorganism and a subsequent decrease in sanitizer efficacy.

2.3.2. *Oxidizing sanitizers*

Oxidants are a broad category of chemical sanitizers that function through the generation of reactive oxygen species (ROS) as charged particles that elicit deleterious effects on the composition of the bacterial cell. Such stress is known to damage proteins, membranes, and DNA (Dowds, 1994). The bulk of the research within this category, and the primary focus of this review has fallen within the subset of peroxygens, namely hydrogen peroxide and peroxyacetic acid; and the halogen-releasing agents, that of the chlorine-based compounds.

Using hydrogen peroxide as a model for oxidative stress, Imlay and Linn (1988) have attributed a major portion of oxidant toxicity towards *E. coli* to DNA damage mediated by a Fenton reaction that generates active forms of hydroxyl radicals, DNA-bound iron, and a constant source of reducing equivalents (Imlay and Linn, 1988). Cellular DNA was affected directly by a derivative of hydrogen peroxide, suggesting that the oxidant may be a ferryl radical as opposed to a hydroxyl radical (Imlay et al., 1988). Imlay and Linn (1987) have further characterized the killing of *E. coli* by H_2O_2 as a two-step mechanism: (1) DNA damage, requiring active metabolism during exposure; (2) uncharacterized damage, occurring in the absence of metabolism (Imlay and Linn, 1987). Increases in free-iron concentration have the effect of accelerating DNA oxidation (Keyer et al., 1995).

The action of oxidants has shown multiple stages of activity depending on the contact time and sanitizer concentration. Upon initial contact, sublethal injury is incurred by the cell, beginning with the cellular membrane and acting further on the physiological functions of the cell following uptake of the applied sanitizer. Lisle et al. (1999) quantified the progression of sublethal attack through a series of physiological indicators, suggesting that the site and extent of injury of bacterial cells can be determined following the order: viable plate counts > substrate responsiveness > membrane potential > respiratory activity > membrane integrity (Lisle et al., 1999). An increase in O_2- of more than two-fold above wild-type levels substantially diminished the activity of labile dehydratases, a four-fold or greater increase in O_2- measurably impaired growth, and a five-fold increase in O_2- sensitized the cells to DNA damage (Gort and Imlay, 1998).

2.3.3. *Peracetic acid-based compounds*

Peracetic acid, as it is commercially available, is prepared as a mixture of acetic acid, hydrogen peroxide, peracetic acid, and water according to the formula:

$$CH_3CO_2H + H_2O_2 \rightarrow CH_3CO_3H + H_2O$$

Where:

CH_3CO_2H = acetic acid

CH_3CO_3H = peracetic acid

H_2O_2 = hydrogen peroxide (Kitis, 2004)

These sanitizers, which function similar to hydrogen peroxide, act against DNA (Tutumi et al., 1974), oxidize essential enzymes (Baldry and Fraser, 1988), denature proteins and enzymes and increase cell wall permeability by disrupting sulfhydryl and sulfur bonds (Jolivet-Gougeon et al., 1996).

2.3.4. *Chlorine-based compounds*

Chlorine-based sanitizers have an extensive history compared to other forms of oxidizing agents and disinfectants. First discovered in 1774 by Carl Wilhelm Scheele, chlorine gas was found to be water soluble and capable of bleaching paper, vegetables and flowers (White, 1999). Some of the first recorded uses of chlorine as a disinfectant occurred in the early 1800's, when Guyton de Morveau in France and Cruikshank in England outlined its use as a common household cleaner by mixing together table salt, manganese, water, and sulfuric acid (Rideal, 1895). When added to water, chlorine gas almost completely hydrolyzes to form hypochlorous acid, the active form in common household bleach, which reversibly dissociates into hydrogen ions and hypochlorite ions (WHO, 1995).

The mechanism by which chlorine-based compounds inactivate bacterial cells is a topic of debate, one that has consisted of numerous research studies with inconclusive results. Chlorine-based compounds have been found to disrupt cell surface components (Ingols et al., 1953; Venkobachar et al., 1977; Haas and Engelbrecht, 1980; Virto et al., 2005), inhibit key cellular functions (Knox et al., 1948; Pereira et al., 1973; Barrette et al., 1989), and damage DNA (Patton et al., 1972; Hoyano et al., 1973).

Hypochlorous acid is generally considered to be a highly destructive, nonselective oxidant which strongly reacts with various subcellular compounds and affects metabolic processes, and therefore is unlikely itself to reach the DNA (Dukan and Touati, 1996). The progression of chlorine-based sanitizers to the interior components of the cell is therefore likely carried through by derivatives of chlorine generated through chemical reactions with the cell; hypochlorous acid formed by hydrolysis of the compound may be responsible for such reactivity. Marks et al. (1945) first described the binding of hypochlorous acid to nitrogen, and the production of the N-chloro compound. The reactivity of the N-chloro compound in this research was found to be dependent on the ratio of chlorine to nitrogen, and also on the nature of the nitrogen compound (Marks et al., 1945).

The primary focus of further reactive compounds is the generation of chloramines, the products of the reaction of hypochlorous acid or other chlorinating agents with primary and secondary amines (Thomas et al., 1986). Chloramines can effectively break both single- and double-stranded DNA in vitro and in vivo (Shih and Lederberg, 1976). Cho et al. (2010) determined that because of chloramines and other chlorine derivatives, chlorine, as a weaker oxidant, is actually a more effective bactericidal agent in comparison to stronger oxidants like chlorine dioxide. Chlorine action involves continuous penetration into the cell without significant surface damage or cell permeability changes as evidenced by limited protein release, and lipid peroxidation (Cho et al., 2010).

2.3.5. *Quaternary ammonium compounds*

In contrast to chlorine and peracetic acid, quaternary ammonium compounds (QACs) operate as surface-active agents or surfactants. Instead of using ROS to disrupt chemical bonds of the organism, these agents use a bipolar design consisting of two regions in their molecular structures, one a hydrocarbon, water-repellent (hydrophobic) group and the other a water-attracting (hydrophilic) group (McDonnell and Russell, 1999).

Research into the antimicrobial properties of QACs dates to the early 20th century, and has been captured in a detailed review by Rahn and Van Eseltine (1947). In general, QACs act against the cell membrane (Przestalski et al., 2000; Ioannou et al., 2007) through modifications to bacterial cell surface components.

Individual characteristics and composition of the bacterial cell surface are responsible for the efficacy of quaternary ammonium compounds, allowing the compounds to bind by chemisorption to the cell surface of bacteria because it is a negatively charged physiological pH (Neu, 1996). This property of QACs varies from one organism to the next depending on the genetic make-up of the individual species. Russel et al. (1987) found that higher levels of LPS, shown on rough strains of *E. coli*, exhibited greater inactivation in comparison to the smooth strains.

Another function provided by QACs, due to the bipolar nature of the compound, is to reduce bacterial attachment to food contact surfaces. QACs alter the hydrophobicity of the surface, resulting in a decrease of the contact angles (and an increase of the surface free energy), accompanied by a reduction in the number of adhered bacteria in comparison with the standard conditions (Sinde and Carballo, 2000). This has been the justification for using QACs in sanitation programs; in their application to clean surfaces of processing equipment as opposed to as an additive during production.

The ability to limit attachment has again varied between organisms. QACs were more effective in reducing attachment of *Salmonella spp.* than *L. monocytogenes* (Sinde and Carballo, 2000). Quaternary ammonium compounds have shown to inhibit and reduce the attachment of *Salmonella* to poultry tissues (Breen et al., 1995).

2.3.6. *Sanitizer limitations*

A primary limitation to the efficacy of chemical sanitizers in the processing of leafy greens has been the presence of organic matter and the inherent defense mechanisms of the plant. Organic matter may present itself in many forms in food processing systems, from noticeable residual soil or debris, to minute bacterial biofilms on the surface of equipment or food surfaces. Leafy greens in comparison to other produce may contain high levels of debris because of their growth very close to the ground. Greens also generally have high microbial populations on the leaf surface. In addition, the plant itself, composed of water, cellulose, vitamins, nutrients and phytochemicals, produces alternative sources to accept reactive oxygen species that are intended for microorganisms (Foyer and Halliwell, 1976; Gillham and Dodge, 1986; Luwe et al., 1993). The disruption of leaf cells during washing and cutting of leafy greens allows for high levels of these organic materials to be deposited into the processing water and onto equipment surfaces.

The concern with organic interference has been most apparent with chlorine-based sanitizers as opposed to peracetic acid or QACs. Small amounts of organic matter can drastically reduce chlorine efficacy. Trypticase Soy Broth at a concentration of 150 ppm in distilled water resulted in less leakage of UV-absorbing substances from cells of *E. coli, L. monocytogenes, B. subtilis* and *Y. enterocolitica* and no uptake of propidium iodide, an indicator of cytoplasmic membrane damage, compared to control cells in distilled water (Virto et al., 2005). The presence of food residues in the form of flour, whole-milk or egg yolk resulted in a decline in the sporicidal efficacy of sodium hypochlorite against *B. anthracis*, while the dose-response relationships for peroxyacetic acid and hydrogen peroxide showed little variation when challenged with the same residues (Hilgren et al., 2007).

2.4. Irradiation

X-ray irradiation as an inactivation strategy for fresh produce has been discussed in detail in a review paper by Moosekian et al (2012). A segment of this paper is included in this section.

Ionizing radiation is attracting renewed attention as a potential non-thermal microbial intervention strategy to ensure the safety of fresh fruits and vegetables. One of the newest strategies involves the development of machines that generate low-energy X-ray electromagnetic waves for in-line processing with these units likely to receive greater consumer acceptance than gamma irradiators that rely on a radioactive source.

Electromagnetic waves are inherent to the world in which we live, with human exposure coming from many diverse sources, ranging from the sun to cellular phones. Typical electromagnetic wavelengths/frequencies vary in size from 10^2 m (AM radio signals) to 10^{-10} m (X-rays). Based on the duality of matter as hypothesized by de Broglie in 1925 (Khan, 2003), electromagnetic waves also can be referred to as particle radiation. Depending on the wavelength/frequency, electromagnetic waves will yield different effects, with the particles or photons from shorter wavelengths able to more easily penetrate solid materials. Radiation - defined as the process by which electromagnetic waves or particles pass through a medium - is classified as either ionizing or nonionizing. If the electromagnetic wave energy is greater than the minimum required for ionization of atoms, it is categorized as ionizing radiation and if not it is nonionizing radiation. Ionizing radiation includes both direct (electrons, protons, alpha particles, heavy ions) and indirect radiation (X-ray, gamma rays) (Podgorsak, 2006).

Based on particle mass and speed, varying amounts of energy are transmitted to a food product or any other material upon impact. The amount of energy deposited in a unit mass ($J\ kg^{-1}$) is measured using a standard unit called a gray (Gy), which is named in honor of the British physicist Louis Harold Gray, the father of modern-day radiobiology. Typical ionizing radiation doses for treating food products range from 1 to 44 kGy. The dose required to reduce a microbial population by 90% (i.e., 1 log) is termed the D_{10} value (kGy) (Molins, 2001). Another convenient energy unit in atomic and nuclear physics is the electron volt (eV), which is the kinetic energy, equal to 1.602×10^{-19} J, needed to accelerate an electron across a one volt electric potential difference (Khan, 2003). The energy level for gamma rays is approximately 1×10^{6} eV or (1 MeV), with X-ray being in the range of a few keV to MeV.

Radioactive materials and particle accelerators are the two main sources for ionizing radiation. Gamma rays are a natural byproduct from the decay of radioisotopes (e.g., ^{60}Co or ^{137}Cs) and are emitted in all directions, whereas electron beams (E-beams) generated from particle accelerators can be aimed at specific targets. Consequently, each type of radiation has its inherent advantages and disadvantages in food, medical, and other types of applications.

2.4.1. *History of X-ray*

Ionizing irradiation - including gamma ray, E-beam, and X-ray - has long been recognized as a viable cold pasteurization strategy for reducing the levels of both pathogenic and spoilage microorganisms in a wide range of foods for the purpose of enhancing food safety and product shelf life. The introduction of X-rays as a source of ionizing irradiation dates back to the late nineteenth century, when German physicist W. C. Roentgen first observed the generation of radiation during his experiments with Hittorf-Crookes tubes, also known as modified cathode ray tubes. In his 1895 paper, *Eine neue Art von Strahlen* (A New Kind of Ray), Roentgen described how rays exiting the device generated transparencies of several objects, including paper, tinfoil, wood, rubber, and flesh. Roentgen noted that “if one holds a hand between the discharge apparatus and the screen, one sees the darker shadow of the bones within the slightly fainter shadow image of the hand itself" (Roentgen, 1895). Roentgen termed the unknown light rays that were emitted from the tube "X"-rays, forming the basis behind the function of medical X-rays.

Further understanding of X-rays and their properties as a source of ionizing irradiation developed in accordance with the discovery of radioactive elements. In 1896, A. H. Becquerel observed a form of radiation similar to that of Roentgen, stemming from elemental uranium, finding that when a photographic plate was exposed to salts of the element, a distinct impression was left on the plate (Strutt, 1904). Marie and Pierre Curie expanded upon the work of Becquerel with experimentation into understanding the source of energy and radioactivity of elements. Marie Curie further clarified the radiation emitted from radioactive materials, describing the similarities between Roentgen's rays and alpha, beta, and gamma rays, as penetrating rays that are unaffected by a magnetic field (Curie, 1961).

2.4.2. *X-rays as a form of ionizing irradiation*

X-rays, or Roentgen rays, appear next to gamma rays in the electromagnetic spectrum at frequencies of 10^{16} to 10^{19} Hz. The somewhat lower energy photons emitted by X-rays are formed from the interaction of a charged particle with matter, either from replacing displaced electrons from a low-lying orbit or through bremsstrahlung, also known as braking radiation (Newton, 1963). Machine sources of X-rays primarily use bremsstrahlung, where active photons emitted when high-velocity electrons strike a dense metal target, such as tungsten, tantalum, or gold, are directed toward the desired object. These high energy particles alone may also generate a lower level of ionization, the technology of which has been used in the development of high-energy E-beams.

Research into the ionizing effects of X-ray radiation did not begin until several years after its initial discovery, when researchers began to explore the power and effect of X-rays on various targets. At this time, researchers were still relatively unaware of the potential mechanism of action. Much of the work with X-rays during the early 1900s focused on food preservation and the control of insects or pests that negatively impact the quality of food and other consumer goods.

Through the early part of the twentieth century, it was generally concluded that the technology and efficiency of X-rays was simply too expensive for large-scale industrial use. In 1912, W. D. Hunter documented some of the first effects of Roentgen rays on insect control. In his review, he concluded that for the insects being tested with the technology of the time, there were no indications of any practical applications of X-rays for the destruction of insect pests (Hunter, 1912). A few years later, Morgan and Runner (1913) detailed the use of Roentgen rays in controlling the tobacco or cigarette beetle's infestation of tobacco products. Their work coincided with an attempt by the American Tobacco Company, under the assistance of Hunter, to commercialize X-rays for this particular purpose. The commercial design used two seven-inch X-ray tubes operating at 64,000 to 70,000 volts, with a current of 1.5 to 2.5 mA passing through the tubes. However, this treatment was ineffective in sterilizing tobacco because of variable X-ray penetration.

Using a subsequently modified Roentgen ray that allowed the operator to control both the intensity and penetrating power, higher doses applied at 600 mA (15 mA x 40 min) to infected tobacco yielded more promising results, showing signs of beetle egg infertility (Runner, 1916). The American Tobacco Company later implemented an in-line conveyor system for irradiating boxes of cigars in 1929, using a water-cooled X-ray at a maximum power of 30 mA at 200 kV.

However, the equipment still proved to be unsuitable for continuous use, as too much time and energy were wasted in machine maintenance and operation (Diehl, 1995).

In 1918, a U.S. patent was granted to D. C. Gillett for his design of an "Apparatus for Preserving Organic Materials by the Use of X-rays" (Gillett, 1918). Gillett's design was intended to "destroy utterly any destructive insects or other animal life that would tend to destroy perishable articles, or to sterilize these insects and prevent the further propagation of their species." In 1921, based on a design similar to Gillett's, the U.S. Department of Agriculture began research through the Zoological Division of the Bureau of Animal Industry on X-ray-initiated inactivation of *Trichinella*, the parasite in pork responsible for human trichinosis. The end results, however, were inconclusive and did not justify X-ray as a feasible means of trichinae destruction. X-rays appeared to injure the organism and/or disrupt reproduction, but the trichinae exhibited considerable variation in susceptibility (Schwartz, 1921).

The impact of X-rays and other forms of ionizing irradiation on matter had a monumental impact in the United States throughout the 1940s and 1950s. During the 1940s, interest focused on the application of irradiation toward preserving foods in an effort to extend the food supply. In 1953, President Eisenhower proposed the Atoms for Peace Program in a speech to the United Nations, describing and advocating the use of ionizing irradiation of food as a means to decrease world hunger by limiting the need for preservation and reducing pests. However, as the United States entered the Cold War, interest shifted toward the negative effects of ionizing irradiation on the nutritional properties of food and the side-effects of consuming irradiated foods.

Breakthroughs in the actual mechanism of X-rays and ionizing irradiation on cells and microorganisms took place toward the middle of the past century in conjunction with developments in DNA research. During that time, Bergonie and Tribondeau (1959) reported that

X-rays were more effective against rapidly growing cells, noting that they destroyed tumors as opposed to the surrounding tissue. Further information on the historical developments of X-rays and ionizing irradiation can be found in reviews by Josephson (1983) and Whitmore (1995), and greater detail pertaining to research done by the U.S. Army can be found in reviews by Josephson et al. (1978) and Brynjolfsson (1979).

2.4.3. *Mechanism of action*

When an atom is exposed to X-rays, energy transactions occur between the projected photons and the orbiting electrons. These interactions result in a net transfer of energy from X-rays to electrons in the absorbing material, raising the electron excitation level (Newton, 1963). Excitation, resulting from a low level of energy, moves an electron further out in its atomic orbit, thereby increasing the net energy. Ionization then occurs when the energy level sufficiently increases to produce highly reactive positive and negative ions by the removal of an orbiting electron (Wilkinson and Gould, 1998).

The extent to which ionization occurs in irradiated matter depends on the energy of the photon and the physical properties of the matter. Activity generated by the photons of X-rays may result in one of two energy absorption processes in the irradiated material: compton scattering or photoelectric absorption (Miller, 2005). When using low-energy photons, photoelectric absorption is generally seen with all of the photon's energy transferred to the electron, which then goes on to interact with other atoms. Compton scattering occurs at higher energy levels where photon interactions are confined to outer, loosely bound electrons, causing only a portion of the photon energy to be absorbed by the encountered electron (Pizzarello and Witcofski, 1975). Under these conditions, the initial photon and any excited or ionized particles may continue to react. It is estimated that an electron may produce 30,000 - 40,000 additional ionization processes and 45,000 - 80,000 excitations (Nawar, 1986).

The impact of charged particles on matter is classified according to the type of contact. Ionized or excited molecules may exert their effect by either direct contact of the photon with their target, i.e., the direct effect, or by the formation of cations created from target components such as water, i.e., the indirect effect (Podgorsak, 2006). Direct effects occur randomly and are dependent upon the electron density of the biological material. Water, a major component of foods and biological material, is the primary target for energy coming from the X-ray source. Reactions with photons and water lead to the generation of highly reactive free hydrogen and hydroxide radicals that are split from the hydrogen bond. Excited water molecules can further react to produce hydrogen and hydrogen peroxide, the only stable end products of water radiolysis (Miller, 2005).

The effects of ionizing irradiation can be seen throughout each component of an organism; however, the primary target remains the DNA. Approximately 20% of this attack is on DNA sugars and 80% on bases, with thymine being the most sensitive, followed by cytosine, adenine, and guanine (Moseley, 1990). Pollard (1966) attributed this observation to the relative mass of DNA compared with the organelles of the cell, stating that radiation sensitivity of organic substances is proportional to their molecular weight. He estimated that a dose of 0.1 kGy would damage 0.005% of the amino acids, 0.14% of the enzymes, and 2.8% of the DNA within a given cell. Within DNA, most strand breaks occur from the scission of the C-3′ phosphate ester bond, producing 5′-PO_4 and 3′-PO_4 termini in a 3:1 ratio (Johnston and Stevenson, 1990). Both single- and double-strand breaks can occur in DNA based on the random action of ionized particles with the latter occurring far less frequently.

2.4.4. *Dose measurements*

Radiation doses are measured using ionizing radiation sensitive materials that can be classified according to their accuracy and range. Based on accuracy of the measurement, the following four categories are now recognized: (*a*) primary standards (1%~2% uncertainty) maintained by national standards laboratories, (*b*) reference standards (~3% uncertainty) for calibrating radiation environments and routine dosimeters, (*c*) transfer standards for establishing traceability of an irradiation facility, and (*d*) routine standards (5%~10% uncertainty) for radiation process quality control, absorbed-dose monitoring, and mapping (ISO/ASTM, 2005). A water calorimeter (10^{-5}~10^{4} Gy) or ionization chamber is used for primary standards, an alanine dosimeter (10^{-1}~10^{4} Gy) is used for for reference standards, and a clear PMMA (polymethylmethacrylate) or radiochromic film (10^{2}~10^{5} Gy) is used for routine dosimeters (Molins, 2001). Of these, alanine-EPR (electron paramagnetic resonance) and radiochromic dosimeters are the most popular. The alanine-EPR dosimetry system uses an alanine dosimeter (film or pill type) and EPR spectroscopy to measure free radicals (ISO/ASTM 2004a; Maltar-Strmecki and Ravkin, 2004). In radiochromic dosimetry (film type), a spectrophotometer is used to measure radiation-induced color changes on a film as a series of absorption bands (ISO/ASTM, 2004b; Mehta and Parker, 2001). Alanine dosimeters are stable over long periods of time, whereas calibration curves must be continually established for radiochromic films.

2.4.5. *The need for alternative microbial reduction strategies*

Across the globe, interest in ionizing irradiation has increased steadily since the beginning of the millennium, with the market for irradiation equipment increasing from 19 billion to over 25 billion U.S. dollars. The United States alone claims roughly one quarter of this spending (Parker, 2005). Worldwide, various irradiation technologies are now being used in at least 55 countries to treat food products (IAEA, 2009). Renewed interest in ionizing irradiation has developed in response to continued outbreaks traced to fresh produce, including lettuce (Ethelberg et al., 2010; Irvine et al., 2009, Nygard et al., 2008; Sodha et al., 2001), spinach (Grant et al., 2008; Wendel et al., 2009), and raw nuts (Danyluk et al., 2007; Isaacs et al., 2005; Kirk et al., 2004) since these products are are adversely affected by thermal processing. From 1998 to 2007, a total of 1,999 outbreaks and 35,554 illnesses were associated with consuming meat, poultry, and seafood, with 684 outbreaks and 26,735 cases of illness from produce (CSPI, 2009b).

Microbial reduction strategies for fresh fruits and vegetables have remained largely ineffective because of current growing/harvest/processing practices and the nature of the material. Contamination of leafy greens and other produce, as discussed by Doyle and Erickson (2008), can occur in the field from irrigation water and animals as well as during processing from contaminated flume water. Because many produce items are traditionally consumed raw, to offer some degree of protection to the consumer, processing of fresh-cut produce typically includes one or more washing steps using chemical sanitizers. However, these means are largely ineffective, as evidenced from recent outbreaks, given that bacterial levels are typically reduced only 1 - 2 logs on the product (Sapers, 2001).

U.S. Food and Drug Administration's approval of irradiation began in 1963, with doses of up to 0.5 kGy being allowed for controlling insect infestation of wheat and flour (Table 1). This list has since expanded with 12 approved uses for irradiation, the most recent addition coming in response to the 2006 outbreaks that were traced to *Escherichia coli* O157:H7 on spinach and lettuce. In a report issued by the joint FAO/IAEA/WHO study group on the nutritional and safety impact of food irradiation, foods irradiated at doses below 10 kGy were deemed to be wholesome (WHO, 1997).

Table 1 Approved uses for food irradiation (USFDA, 2010)

Use	**Year**	**Dose**
Control of insects in wheat and flour	1963	≤ 0.5 kGy
Inhibiting spouting in potatoes	1964	≤ 0.15 kGy
Pork carcasses for *Trichinella spiralis*	1986	≤ 1 kGy
Culinary herbs, seeds, spices, vegetable seasonings	1986	≤ 30 kGy
Delay ripening of fruit and disinfecting fruits and vegetables of insects	1986	≤ 1.0 kGy
Fresh or frozen, uncooked poultry products	1990	≤ 3 kGy
Frozen, packaged meats used solely in NASA space flight programs	1995	Minimum dose 44 kGy
Refrigerated or frozen, uncooked meat products	1997	≤ 4.5 kGy (refrigerated); ≤ 7.0 kGy (frozen)
Fresh shell eggs for *Salmonella*	2000	≤ 3.0 kGy
Seeds for sprouting	2000	≤ 8.0 kGy
Fresh or frozen molluscan shellfish for *Vibrio* bacteria	2006	≤ 5.5 kGy
Iceberg lettuce and spinach	2008	≤ 4.0 kGy

2.4.6. *X-ray as a viable alternative*

Until very recently, food irradiation as a microbial reduction strategy focused almost exclusively on gamma rays and E-beams, with gamma rays identified as the only energy efficient means for cold pasteurization. This large body of literature on food irradiation has been reviewed elsewhere with respect to meat and poultry (O'Bryan et al., 2008), fish and seafood (Arvanitoyannis et al., 2009a; Venugopal et al., 1999), and fruits and vegetables (Arvanitoyannis et al., 2009b). It is only within the past decade that X-ray irradiation has garnered some attention as a viable microbial reduction strategy based on its now proven efficacy, minimal environmental impact, and potential for direct installation in commercial processing lines. Given the extensive shielding and other hazards associated with the radioactive sources for gamma radiation (e.g., ^{60}Co or ^{137}Cs), X-ray and E-beam are becoming more practical alternatives. In addition, these nonradionuclide machine-source irradiators can be turned on and off by a switch, allowing for more efficient commercial processing and greater operator control.

2.4.7. *Advances in X-ray technology*

Reinvention of X-ray machines with increased efficiency, combined with recent developments in legislation and engineering, is now allowing X-ray to actively compete with gamma irradiation and E-beam as a microbial reduction strategy for foods. In the generation of bremsstrahlung, one of the unfortunate outcomes is the inadequate conversion of energy from integrated photons to integrated electrons, which decreases process efficiency. This has been viewed by some as the primary limitation to X-ray use for commercial applications, but is also an area of debate. Initially, the approved maximum energy level permitted for X-rays was set at 5.0 MeV; however, at an October 16 - 18, 1995 meeting of the FAO/IAEC/WHO in Vienna, Austria, it was concluded that X-ray machines producing up to 7.5 MeV "can be used without any concern about induced radioactivity but would be a satisfactory, efficient and cost effective addition to other radiation sources available for food processing" (ICGFI, 1995). In light of this conclusion, FDA amended their food additive regulations in December 2004 by establishing a new maximum permitted energy level for X-rays of 7.5 MeV, provided that the X-rays are generated from machine sources that use tantalum or gold as the target material (Federal Resister, 2004).

The effective increase in energy from 5.0 to 7.5 MeV is not substantial, yet it is still important, as the emission efficiency increases proportionately with an increase in electron energy, as indicated in Table 2. Using Monte Carlo simulation, Meissner et al. (2000) found that bremsstrahlung yield - a measure of the emission efficiency - doubled when the energy level increased from 5.0 to 10.0 MeV. They also estimated a greater degree of penetration when an equivalent uniform dose was applied. Consequently, more powerful X-ray machines for food irradiation are now being designed based on these new rules. One manufacturer (L3 Communications Titan Pulse Sciences Division, San Leandro, CA) is preparing to develop an irradiator that incorporates both a 7.5 MeV electron linear accelerator capable of generating 100 kW of average power and a tantalum converter.

Table 2 Properties of X-ray at energy levels of 10.0, 7.5, and 5.0 MeV (Meissner et al., 2000)

Energy (MeV)	**Emission efficiency (%)**[a]	**Double-sided treatment (g $cm^{2\ -1}$)**[b]	**Dose uniformity ratio (D_{max}/D_{min})**[c]
10	16.2	43	1.54
7.5	13.3	38	1.54
5.0	8.23	34	1.54

[a]Efficiency of the bremsstrahlung yield (X-ray generation) in the forward direction assuming normal electron incidence on to tantalum converter (conversion efficiency)

[b]Aerial density (g $cm^{2\ -1}$) of stacked polyethylene plates (density 0.96 g $cm^{3\ -1}$, dimension 49 × 80 × 40 cm height)

[c]Max to min dose ratio for the stacked polyethylene plates

In 2009, these L3 devices were used as an 18 kW E-beam linear accelerator and a 15 kW, 10 MeV X-ray linear accelerator, and then compared with a hydrostatic pressure treatment for inactivation of *E. coli* O157:H7 in ground beef inoculated at 10^3 CFU g^{-1} (Schilling et al., 2009). Using X-ray and E-beam doses of 2 kGy, the *E. coli* O157:H7 population decreased below the limit of detection, whereas hydrostatic pressure (300 mPa) did not completely eliminate the pathogen. Furthermore, sensory panelists found the irradiated and nonirradiated control samples to be comparable and overall more acceptable than ground beef treated with hydrostatic pressure. No potentially hazardous volatile compounds were detected in irradiated or hydrostatic pressure-treated samples.

Further technological advancements in X-ray technology have focused on improved efficiency. Rad Source Technologies (Suwanee, GA) led these developments with their RS 2000 X-ray design. This machine uses a patented Rad Source RAD+ Chamber and a point source X-ray tube (Rad Source Technologies, 2008) to deliver a uniform irradiation dose over a large area. Additional energy is provided from a reflector of low Z (atomic number), high-density material that is positioned within the chamber to reflect radiation back to the product (Gueorguiev, 2002).

The RS 2000 X-ray irradiator has been tested at several institutions across the United States, with the bulk of this work having been done at Mississippi State University. Using this irradiator at a maximum dose of 2.0 kGy (dose rate of 1 kGy per 50 min at 145 kV and 19 mA), Robertson et al. (2006) were able to reduce initial *Listeria monocytogenes* populations of 4.4 log CFU g^{-1} in vacuum-packaged smoked mullet to undetectable levels with no recovery seen when the fillets were held for 90 or 17 days at 3°C or 10°C, respectively. Sensory panelists were also unable to detect any differences between the treated and untreated samples (Robertson et al., 2006). However, the 100 min exposure time to achieve these results was cited as being very problematic for the industry.

In 2009, Collins et al. (2009) expanded on this research by evaluating the same system and operating conditions in treating fresh channel catfish fillets for the reduction of *L. monocytogenes*, mesophilic aerobic bacteria, psychrotrophic bacteria, and total coliforms. Their findings were similar to those of Robertson et al. (2006), with 40%, 27%, 0%, and 7% of samples yielding *L. monocytogenes* after receiving irradiation doses of 0, 0.5, 1.0, and 1.5 kGy, respectively. They also reported the development of an off aroma in unirradiated control samples during storage at 5°C over 17 days, which was not detected on the irradiated fillets, with these changes attributed to fewer inherent bacteria and spoilage organisms on the treated fillets.

In other developments, Rad Source Technologies received a U.S. patent **(**number 7,346,147) for 4pi X-ray emitters (Kirk and Gorzen, 2008). The manufacturer claims that this device delivers higher dose rates, comparable to gamma irradiators, through the use of an extended anode design in which X-rays are generated from a cylindrical rather than a point source, using all of the photons produced (Rad Source Technologies, 2008). Their design results in both the creation of X-rays proceeding through the anode along its length and X-rays that are reflected back through the length of the anode and throughout the circumference of the cylindrical anode (Kirk and Gorzen, 2008).

This 4pi technology has been used by Rad Source (RS) Technology in the construction of their RS 2400 and RS 2500 irradiators. Specifications for the RS 2400 X-ray irradiator in Table 3 illustrate how high dose rates can be achieved under low-energy conditions (< 1 MeV). However, the operating capabilities of the cabinet remain a limitation to the design, allowing only small batches of product to be treated within an exposure chamber measuring 91.4 × 60.0 × 63.5 cm.

Table 3 Characteristics of the RS 2400 Irradiator (Mehta and Parker, 2011)

Characteristic	**Value**
Maximum tube voltage	150 kV
Maximum tube current	45 mA
Maximum power	6.75 kW
X-ray converter	12 ìm gold
Dose rate (to water) in center of rice-filled canister	14.1 Gy min^{-1}
Dose energy ratio at this location	0.0374 Gy $(kW\ s)^{-1}$

Efficacy of this improved RS 2400 irradiator (dose rate of 1.0 kGy per 16 min at 145 kV and 45 mA) has been demonstrated through a series of reports on the treatment of shellfish. In 2009, whole live and half-shell oysters were irradiated to inactivate *Vibrio parahaemolyticus* (Mahmoud and Burrage, 2009) and later *Vibrio vulnificus* (Mahmoud, 2009a). In this work, *V. parahaemolyticus* populations decreased more than 6 logs to levels below the limit of detection using X-ray irradiation doses of 0.75, 2.0, and 5.0 kGy for pure-culture, half-shell, and whole-shell oysters, respectively. Under the same conditions, *V. vulnificus* was more susceptible, with reductions of greater than 6 logs seen after exposing the pure-culture, half-shell, and whole-shell oysters to doses of 0.75, 1.0, and 3.0 kGy, respectively. In addition, the shelf life of whole oysters could be extended using a dose of 5.0 kGy, as evidenced by oyster survival at the high dose, and inherent microorganisms were reduced to levels below the limit of detection. Mahmoud (2009b) reported equally effective results using the same system on ready-to-eat shrimp, demonstrating more than a 6 log CFU reduction for *E. coli* O157: H7, *Salmonella* *enterica*, *Shigella flexneri*, and *V. parahaemolyticus* using doses of 2.0, 4.0, 3.0, and 3.0 kGy X-ray, respectively. Lower X-ray doses of 0.75 kGy also significantly reduced the initial microflora in ready-to-eat shrimp samples from 3.8 ± 0.2 to < 1.0 log CFU g^{-1}.

Mahmoud later went on to demonstrate the efficacy of this same X-ray irradiator for a series of produce items, including spinach (Mahmoud et al., 2010), iceberg lettuce (Mahmoud, 2010a), and Roma tomatoes (Mahmoud, 2010b). In these experiments, results indicated a greater than 5 log reduction for *E. coli* O157:H7, *L. monocytogenes, S. enterica,* and *S. flexneri* at 2.0, 1.0, and1.5 kGy for iceberg lettuce, spinach, and Roma tomatoes, respectively. In addition, treatment at these dose levels significantly decreased inherent microflora, which in turn enhanced product shelf life during refrigerated storage. Results obtained on leafy greens were similar to those reported for both gamma (Niemmira et al., 2002; Niemira, 2008) and E-beam (Gomes et al., 2008) radiation (Table 4).

Table 4 Microbial efficacy (D_{10} value) of X-ray irradiation on various products

Products	**Microorganisms**	**Inoculation method**	**D_{10} value (kGy)**	**Reference**	**X-ray irradiator**
Oysters (whole live and half shell)	*Vibrio parahaemolyticus*	Immersion	2.0 log reduction kGy^{-1} (whole shell)[a]	(Mahmnoud and Burrage, 2009)	150 kV 6.75 kW^{-1} (RS 2400, Rad Source Technologies Inc., Alphretta, GA)
			4.9 log reduction kGy^{-1} (half shell)[a]		
Soy broth (TSB)	*Enterobacter sakazakii*	Mix	0.41 ± 0.1	(Mahmoud, 2009b)	
Skim milk			0.54 ± 0.04		
Low-fat milk (1%)			0.65 ± 0.02		

Table 4 (cont'd)

Low-fat milk (2%)			0.71 ± 0.3		
Whole-fat milk (3.5%)			0.74 ± 0.03		
Spinach (leaves)	*Escherichia coli* O157:H7	Spot	1.1	(Mahmoud et al., 2010)	
	Listeria monocytogenes		1.0		
	Salmonella enterica		1.2		
	Shigella flexneri		0.96		
Iceberg lettuce (shredded)	*E. coli* O157:H7	Spot	4.4 log reduction kGy^{-1} [(a)]	(Mahmoud, 2010a)	
	L. monocytogenes		4.1 log reduction kGy^{-1} [(a)]		
	S. enterica		4.8 log reduction kGy^{-1} [(a)]		
	S. flexneri		4.4 log reduction kGy^{-1} [(a)]		
Roma tomatoes (whole)	*E. coli* O157:H7	Spot	0.39 ± 0.5	(Mahmoud, 2010b)	
	L. monocytogenes		0.66 ± 0.1		
	S. enterica		0.56 ± 0.1		
	S. flexneri		0.98 ± 0.3		
Shrimp (frozen, cooked, peeled ready to eat)	*E. coli* O157:H7	Immersion	1.1 ± 0.01	(Mahmoud, 2009a)	
	S. enterica		1.3 ± 0.03		
	S. flexneri		1.2 ± 0.01		
Mullet (vacuum-packaged smoked)	*L. monocytogenes*	Immersion	1.6 log reduction kGy^{-1} [(a)]	(Robertson et al., 2006)	145 kV 2.76 kW^{-1} (RS 2,000, Rad Source Tech, Boca Raton, FL)

Table 4 (cont'd)

Iceberg lettuce (leaves)	*E. coli* O157:H7	Immersion	0.040 ± 0.001	(Jeong et al., 2010b)	70 kV 4 kW^{-1} (Rayfresh Foods Inc., Ann Arbor, MI)
		spot	0.078 ± 0.008		

[a] D_{10} values were not reported.

The RS 2400 irradiator was also evaluated for inactivation of *Enterobacter sakazakii* in milk (Mahmoud, 2009c). X-ray doses of 5.0 and 6.0 kGy reduced ($P < 0.05$) *E. sakazakii* populations to < 1 log CFU ml^{-1} in skim milk and milk containing ≥ 1% fat, respectively. However, this study did not investigate the possible negative impact of X-ray doses on product quality and rancidity, which is an important consideration in high-fat products.

Rayfresh Foods (Ann Arbor, MI) also developed a patent-pending process termed The Rainbow Process, which is based on low-energy (< 1 MeV) X-rays (Rayfresh Foods, 2009). This technology has been evaluated at Michigan State University over the past five years using a wide range of products, including lettuce, spinach, parsley, asparagus, blueberries, almonds, walnuts, chestnuts, and ground beef. Research to date on this system has been conducted using a pilot-scale irradiator (maximum power 4 kW, operating energy 70 kV) comparable in design to the RS 2400 irradiator. Rayfresh Foods currently is focused on development of a commercial scale irradiator for in-line processing.

Experiments assessing the efficacy of this new technology against *E. coli* O157:H7 in ground beef yielded a D_{10} value of 0.100 kGy (Jeong et al., 2007), which was significantly lower than previously published D_{10} values for gamma or E-beam irradiation (~0.270 kGy)

(Thayer and Boyd, 1993). This system also has proven to be highly effective against *E. coli* O157:H7 on leafy green vegetables. Treatment of iceberg lettuce yielded a D_{10} value of 0.040 kGy (Jeong et al., 2010b), which is 3.4 times lower than the previously reported value of 0.136 kGy using gamma radiation (Niemira et al., 2002) (Table 4). When ten stacked leaves were irradiated from both sides, a dose of 0.2 kGy was achieved at the center of the stack, with a surface dose of 1 kGy corresponding to a 5-log reduction for *E. coli* O157:H7 at the center of the stack. Lower D_{10} values were also observed when spinach (0.035 kGy), parsley leaves (0.0522 kGy), and parsley stems (0.067 kGy) were surface inoculated with *E. coli* O157:H7 (Moosekian et al., 2010a).

Efficacy against *Salmonella* on tree nuts (almonds and walnuts) has also been demonstrated (Jeong et al., 2010a). Nuts were inoculated with *Salmonella* Enteritidis PT30 or *Salmonella* Tennessee, and conditioned to different water activities (0.23 - 0.84). The efficacy was significantly ($P < 0.05$) affected by both nut type and water activity, with greater inactivation on the surface of almonds than on walnuts, for equivalent doses, and maximum resistance at a water activity ~0.6. Also, irradiation of uninoculated samples to a dose that would achieve a 5-log reduction (1.1 and 2.4 kGy for almonds and walnuts, respectively) resulted in no perceivable sensory changes in almond quality, as determined by a consumer panel, but did result in a decrease in acceptability for the walnuts, indicating the importance of product-specific data in evaluating the technology.

The Palletron system is another proposed commercial design for implementing X-ray technology in food-processing facilities. This irradiator increases dose uniformity through a rotational pallet system comprising a radiation source, adjustable collimator, turntable, and control system (Kotler and Borsa, 2003), with the goal of reducing the dose uniformity ratio

(DUR) or the ratio between the maximum and minimum dose absorbed by different areas of the food or within a food container (Grandison, 2006). According to Lazurik et al. (2007), application of X-ray beams from multiple angles and orientations increases both dose uniformity and efficiency. When applied from four sides, beam efficiency was more than 60%, which allowed large objects to be processed at a DUR < 1 (Lazurik et al., 2001). Monte Carlo simulation studies with the Palletron further demonstrated that it is possible to reach a DUR $>$ 1.5 for all densities up to 0.8 g cm$^{3\ -1}$ while preserving a high treatment capacity (Stichelbaut et al., 2004a).

2.4.8. *X-ray irradiation process control and validation*

Irradiation doses need to be uniformly delivered to the product to avoid an overdose because the energy from X-ray (unlike E-beam) is attenuated exponentially along the projection axis of the material. Therefore, processing models are needed to predict both X-ray dose distribution in the food matrix and microbial inactivation efficacy. Because of the complexities in generating ionizing radiation, transport, and interaction with matter, construction of a purely analytical model is clearly challenging. Unlike mono-energetic gamma rays and E-beam, X-ray's multi-energetic energy spectrum results in angular dependence of the radiation, energy dependence of X-ray absorption, and product particle scattering (Miller, 2003).

For food irradiation, the dose estimation methods and modeling techniques resemble the radiation treatment plans for medical patients (Ay and Zaidi, 2005; Borsa et al., 2002; Reynaert et al., 2007). Given recent computer advances, simulations of particle transport and interactions are now being used to estimate dose distributions within the intended target. A typical stochastic process suited for this type of simulation is the Monte Carlo method, in which the history of each particle (trajectory, interactions) is traced in detail (Cantone and Hoeschen, 2011). A detailed comparison among several Monte Carlo particle transport codes, including MCNPX, GEANT4, FLUKA, MARS, and PHITS, is available (Los Alamos National Laboratory, 2011). Typically, the composition of the food matrix is mapped using computer tomography images (CT scan), after which a three-dimensional image of the object is reconstructed with embedded density/composition information (Borsa et al., 2002). This material composition model is then coupled with Monte Carlo particle transport codes to predict the radiation dose received at a specific location (kGy), with this dose then converted into a process lethality based on the D_{10} value for the target organism.

Some readily available Monte Carlo codes include MCNPX (McKinney, 2011) (license needed from the Radiation Safety Information Computation Center, Oak Ridge National Laboratory, Oak Ridge, TN) and GEANT4 (Agostinelli et al., 2003) (free and downloadable from http://geant4.web.cern.ch/geant4/index.shtml). The main difference is that GEANT4 can handle electromagnetic field problems, which enables the modeling of accelerated electrons in an electromagnetic field.

Using these Monte Carlo codes, Stichelbaut et al. (2004a; 2004b) successfully modeled the Rhodotron® TT300 (X-ray from 5, 7, and 10 MeV) and Palletron™ using the GEANT Monte Carlo simulation toolkit. For complex foods, including chicken carcass/broccoli (Kim et al., 2007; Kim et al., 2006), apple (Brescia et al., 2003; Kim et al., 2006), an apple surrogate (Rivadeneira et al., 2007), and bagged spinach (Gomes et al., 2008), this modeling procedure was successful using machine source irradiation. Even though Monte Carlo simulation provides an accurate dose-distribution map of the product, this is not an ordinary task. Therefore, Miller (2003) developed a faster and more convenient analytical/empirical model for X-ray irradiation that can be validated using integrated TIGER series Monte Carlo codes. After model validation, an economic analysis is then needed to assess industry feasibility. Based on available economic models, all three available irradiation technologies, gamma, E-beam, and X-ray, have their own benefits and hindrances that must be specifically addressed by each user (Kunstadt, 2001; Sadat, 2004).

2.4.9. *Consumer acceptance*

The efficacy and safety of food irradiation have been recognized for more than a century, especially in recent decades, by the scientific community as well as numerous health organizations and governmental agencies. Given this consensus, food irradiation is now approved in more than 50 countries, 30 of which are irradiating multiple commodities (Mostafavi et al., 2010). However, some hurdles to further expansion of food irradiation remain because of negative consumer perception of irradiated foods, industry costs associated with adopting the technology; and the labeling requirement (e.g., Radura symbol) by regulatory agencies.

Despite increased efficacy and continued research on the safety of irradiated foods, some consumers remain skeptical (Lyndhurst, 2009). Therefore, the issues are how much negative perception can be tolerated and can consumer perceptions of food irradiation be improved through education? Consumer acceptance, perception, and attitude are influenced by many factors including socio-demographic/economic status, risk-benefit perceptions, knowledge, trust in the source of information, and labeling (Rollins et al., 2011). In the United States (Bruhn, 2001), Europe (Rollin et al., 2011), and other countries, including Brazil (Behrens et al., 2009; Martins et al., 2008), Chile (Junqueira-Goncalves et al., 2011), South Korea (Byun et al., 2009), Argentina (Curzio and Croci, 1998), Africa (Mostafavi et al., 2010), Turkey (Gunes and Tekin, 2006), Egypt (El-Fouly et al., 2002), consumer attitudes and negative perceptions (or fear) of food irradiation improved after consumer education. Commodity-specific consumer acceptance of various irradiated products, including ground beef (Lorenzen and Heymann, 2003; Spaulding et al., 2006; Wheeler et al., 1999), fruits (Deliza et al., 2010; Martins et al., 2008), pork (Fox et al., 2002; Wolfe et al., 2005), fish (Aworh et al., 2002), meat (Eustice and Bruhn, 2010), onions (Curzio and Croci, 1998), apple cider (Yulianti et al., 2004), and turkey meat (Lee et al., 2003), also has been assessed. Although most of this work focused on consumer acceptance, adoptive behavior of irradiation by retailers was also assessed (Jaenicke et al., 2006). A recent trend has been seen toward improved consumer acceptance of certain health-promoting food additives, such as antioxidants, which can also reduce radiation byproducts and increase product value (Over et al., 2010; Yan et al., 2006). One approach to improving consumer acceptance involved the public display and advertisement of irradiated foods (Furuta, 2004). After consumer acceptance improves to the level of other technologies, food irradiation is likely to become far more widely adopted by the food industry.

2.5. Cross-protection

2.5.1. *Hurdle approach for pathogen control*

Combining irradiation with other treatments, including chemical preservatives and growth inhibitors in a hurdle approach, has been proposed as an additional option for enhancing product safety and quality. Thayer et al. (2006) found that irradiation and chlorination acted synergistically in the inactivation of *Salmonella, E. coli* O157:H7, and *L. monocytogenes* on fresh produce. In a separate report, Foley et al. (2004) determined that although water, chlorine

(200 ppm), and irradiation (1.05 kGy) significantly reduced levels of *E. coli* O157:H7 on cilantro, combined use of irradiation with a wash treatment was superior to irradiation alone.

2.5.2. *Development of cross-protection*

Cross-protection can be described as an innate means of protection from destructive conditions or treatments (Sykes, 1963). This infers that microorganisms have the ability to adapt to adverse situations for their temporary survival. Cross-resistance has the potential to occur when different antimicrobial agents attack the same target, initiate a common pathway to cell death, or share a common route of access to their respective targets (Chapman, 2003).

Treatment with sublethal levels of stress has been found to spur cross-protection by inducing proteins which protect the cells against high levels of the same stress and often against additional, unrelated stressors (Browne and Dowds, 2001). When utilizing multiple barriers at sublethal levels to achieve bacterial inactivation, there is the concern for bacterial adaptation to the applied stressor and the possibility that a hurdle approach may be creating more harm than good.

Cross-protection has been investigated across many distinct categories of stressors; however, oxidizing sanitizers have been a primary focus of scrutiny in this area, considering their inefficacy in processing and the subsequent demand for a combined approach to bacterial inactivation. Research has demonstrated that sanitizer damage can infer protection against further oxidative damage. Jenkins et al. (1988) found that starvation or prior adaptation of *E. coli* (K-12) to heat, hydrogen peroxide, or ethanol was protective against further H_2O_2 oxidative damage. Zook et al. (2001) later observed that *E. coli* O157:H7 sublethally injured by low levels of peroxyacetic acid sanitizer induced peroxidative adaptation, but not thermal resistance.

The occurrence of cross-protection from oxidizing agents has primarily been seen when using low levels of the primary stressor. This is often attributed to the theory that at a sublethal dose the cellular components react with the oxidant, while at higher concentrations the ability for these components to scavenge the oxidants is overwhelmed. In 1983, Demple and Halbrook reported that prior treatment with peroxide doubled the survival rate for *E. coli* (AB1157) that was further exposed to gamma irradiation, with the survival greatest for radiation doses <10 krad (0.1 kGy) (Demple and Halbrook, 1983). Dukan and Touati (1996) found that pretreatment with a low concentration of HOCl protected cells from H_2O_2; however, at higher concentrations this was not seen (Dukan and Touati, 1996).

Cross-protection may also develop in nature from repeated exposure to environmental stress and expression of inherent systems to survive. Superoxide dismutase (SOD) acts to reduce the affects of oxidation and ROS (Keyer et al., 1995). Induction of microbial defense systems in response to oxygen radicals from exposure to peroxides results in the synthesis of proteins such as catalases, superoxide dismutase, and alkyl hydroperoxidases (Chapman, 2003). Exposure of *E. coli* to hydrogen peroxide also resulted in a 10-fold increase in catalase levels and enhanced ROS scavenging (Imlay and Linn, 1987).

Resistance can be achieved by mutation, acquisition of new genetic information by horizontal gene transfer, expression of previously silent genes, growth in a biofilm, and other poorly defined phenotypic alterations which can give rise to a transiently resistant phenotype (Chapman, 2003). Lisle et al. (1998) found that *E. coli* O157:H7 chlorine resistance progressively increased through the starvation period, demonstrating that the organism adapts to starvation conditions by developing a chlorine resistant phenotype.

Some clades of *E. coli* O157:H7 have demonstrated greater resistance to chlorine treatment than others. Wang et al. (2009) found that strains in clade 8, including the strain implicated in the 2006 spinach outbreak, showed significantly ($P < 0.05$) higher resistance to chlorine than strains from other clades of *E. coli* O157:H7.

Gene analysis has shown that the response of bacteria, in regards to gene activation, is similar regardless of the stressor. Imlay and Linn (1988) found that exposing *E. coli* to H_20_2 also resulted in the induction of functions under control of the OxyR regulon, which enhances the scavenging of active oxygen species. Similarly, Dukan and Touati (1996) found resistance to HOCl to be largely mediated by genes involved in resistance to hydrogen peroxide. In their research, rpoS was the gene responsible for protection in stationary phase, while in exponential phase, induction of the OxyR regulon protected against HOCl exposure (Dukan and Touati, 1996).

Stressors have been found to activate similar sets of genes in the bacterial defense response. In investigating the changes in gene expression after treatment of *E. coli* cultures with mitomycin C, over 1000 genes were induced during the damage response including those involved in the SOS response as well as other stress response pathways, such as those of oxidative stress and osmotic protection (Khil and Camerini-Otero, 2002). In a study of the transcriptional changes in *Pseudomonas aeruginosa* following exposure to sodium hypochlorite, peracetic acid and hydrogen peroxide, 40 common genes were upregulated and 23 common genes were downregulated, suggesting similar response mechanisms to oxidative stress (Small et al., 2007). More recently, Wang et al. (2009) demonstrated a similar response to chlorinated water. Here it was found that over 380 genes were expressed in response to low levels of chlorine or hydrogen peroxide, in particular several regulatory genes responsive to oxidative stress such as the OxyR regulons and genes encoding putative oxidoreductases.

2.6. Summary

Contamination of leafy greens may occur at any stage throughout processing due to the extensive use of poor irrigation practices and the uncontrollable interactions that the growing plants will have with wildlife in the field. Increased demand for leafy greens, including conveniently packaged fresh-cut leafy greens, has amplified these concerns by allowing the spread of pathogens throughout entire lots of food during processing. Chemical sanitizers, the current microbial control strategy, have their limitations for use in produce processing in that the high organic load allow for the target organisms to receive lower than optimum contact time with the sanitizer. The result of sanitizer treatment of leafy greens has been minimal inactivation and sublethal injury of the target pathogens.

Ionizing irradiation, including X-ray, has received greater attention in the inactivation of pathogens on produce following the approval by the FDA allowing the use of up to 4 kGy on fresh and bagged iceberg lettuce and spinach. X-ray irradiation has proven to be a viable pathogen reduction strategy for several food commodities including, iceberg lettuce, roma tomatoes, oysters, shrimp, mullet, and milk; however, information is still lacking on the efficacy of X-rays for pathogen reduction on baby spinach. Of concern however, is that X-ray irradiation has a similar inactivation mechanism to that of chemical sanitizers, with the primary target being cellular DNA. Considering the continual use of chemical sanitizers in produce processing, there is a legitimate concern for cross-protection if and when X-ray technology is implemented into the current processing conditions.

3. CHAPTER 2

INACTIVATION OF *ESCHERICHIA COLI* O157:H7 ON BABY SPINACH USING LOW-ENERGY X-RAY IRRADIATION

3.1. Abstract

Low-energy X-ray irradiation was assessed as a means of eliminating *Escherichia coli* O157:H7 on baby spinach. Round-cut baby spinach leaves (2.54 cm diameter) were dip-inoculated in a 3-strain cocktail of *E. coli* O157:H7, and then irradiated at four different dose levels up to 0.176 kGy using a prototype low-energy (70 kV) X-ray irradiator. Radiochromic film dosimeters were then used to map the X-ray dose rate distribution on the surface and at the mid-plane region of 225-g bags of baby spinach. D_{10} values for round-cut leaf pieces were used to estimate inactivation of *E. coli* O157:H7 in commercial-sized bags of baby spinach. Inoculated bags were irradiated for a total duration of up to 480 s per treatment to simulate 120 s of processing in a four tube commercial scale irradiator to determine actual reduction values within the bag. The D_{10} value for *E. coli* O157:H7 on baby spinach was 0.033 ± 0.001 kGy. Dose rate distribution in the bags of baby spinach ranged from 1.93 ± 0.75 Gy/s at the mid-plane to 7.18 ± 3.85 Gy/s on the bag's surface. The predicted minimum log reduction for *E. coli* O157:H7 in bagged baby spinach using a simulated exposure time of 120 s was 4.10 log CFU, while the maximum dose received by the surface was 1.32 kGy. The observed reduction value, determined as an average throughout the bag, was 3.99 log CFU. Based on these findings, low-energy X-ray irradiation appears to be a promising means to inactivate *E. coli* O157:H7 on baby spinach, with a 4 – log reduction achievable without exceeding the maximum allowable dose of 4 kGy at the surface of the bag.

3.2. Introduction

Increased interest in convenience, healthy eating, and unprocessed, raw fruits and vegetables has greatly escalated the market for fresh-cut, pre-washed bagged salad greens in the United States (Mintel, 2008). However, meeting this demand through expansion in farming and mechanization of production practices has not come without costs. The industry has been plagued with product recalls and occasional outbreaks. From 1998 to 2007, the Center for Science in the Public Interest (CSPI) reported that three pathogens - *Salmonella*, *E. coli* O157:H7, and Norovirus - combined for a total of 265 outbreaks and 9,001 illnesses from consumption of lettuce and leafy green-based salads (CSPI, 2009b). Baby spinach moved to the forefront of consumer concerns following an *E. coli* O157:H7 outbreak in September 2006 that resulted in 205 confirmed cases in 28 states and 3 deaths (CDC, 2006).

Considering the numerous and still undefined contamination routes for baby spinach (CFERT, 2007), pathogen control has been targeted at value-added processing. Chemical sanitizers, including chlorine- and peracetic acid-based sanitizers, used in commercial flume tanks are the only current means for decreasing microbial contamination. However, these methods have proven to be marginally effective, generally reducing pathogens < 2 logs (Sapers, 2001; Beuchat et al., 2004; Keskinen et al., 2009; Lopez-Galvez et al., 2009).

In response, the FDA has allowed the use of ionizing irradiation at levels up to 4 kGy for bagged spinach (FDA, 2008). Ionizing irradiation is commonly produced using either radioactive sources, such as ^{60}Co or ^{137}Cs to generate gamma rays, or through machine sources in the form of electron beams (E-beam) and X-rays, the latter of which has been gaining attention given the hazards and negative public perception associated with radioactive sources. E-beam has proven effective for spinach. Gomes et al. (2008) established a D_{10} value of 0.186

kGy for *E. coli* O157:H7 on spinach using E-beam (10 MeV) as a form of ionizing irradiation (Gomes et al., 2008). A dose of 0.70 kGy was also shown to decrease *E. coli* O157:H7 in bagged spinach by 4 logs (Neal et al., 2008).

Using a newly developed low-energy X-ray irradiator (Rayfresh Technologies, Ann Arbor, MI), we obtained a D_{10} value of 0.040 kGy for *E. coli* O157:H7 on the surface of iceberg lettuce (Jeong et al., 2012) which is 3.4 times lower than the previously reported value of 0.136 kGy using gamma radiation (Niemira et al., 2002). Low-energy X-ray technology can be incorporated into existing processing lines due to the low shielding requirement and small machine footprint. X-rays can also be used to treat packaged products, thereby minimizing the risk of post-treatment contamination. Consequently, the objectives of this study were to (1) establish the D_{10} value for *E. coli* O157:H7 on baby spinach using low-energy X-ray irradiation, and (2) determine the time required to inactivate *E. coli* O157:H7 in commercial bags of baby spinach using simulated processing conditions.

3.3. Materials and Methods

3.3.1. *Bacterial strains*

Three *E. coli* O157:H7 outbreak strains - K3995 from a 2006 spinach outbreak, K4830 from a 2006 bagged lettuce outbreak, and K4492 from a different 2006 bagged lettuce outbreak were obtained from Dr. Michael Doyle at the Center for Food Safety, University of Georgia, Griffin, GA and stored at -80°C in trypticase soy broth containing 0.6% (w/v) yeast extract (TSBYE) (Becton Dickinson, Sparks, MD) and 10% (v/v) glycerol. Each strain was subjected to two consecutive 37ºC/24 h transfers – the first in 10 ml of TSBYE, and the second in 15 ml of TSBYE, and then combined to obtain 45 ml of a 3-strain cocktail containing ~10^9 CFU/ ml as determined by optical density at 600 nm using a Genesys 20 ThermoSpectronic spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA) and plating in duplicate on trypticase soy agar containing 0.6% (w/v) yeast extract (TSAYE) (Becton Dickinson, Sparks, MD).

3.3.2. *X-ray irradiator*

A pilot-scale, custom designed, low-energy X-ray food irradiator (Rayfresh Foods Inc., Ann Arbor, MI) housed in the biosafety level 2 pilot plant at Michigan State University was used to irradiate the samples. This irradiator, which contained a 53 × 53 × 58 cm treatment chamber shielded by 25 mm-thick lead, generated 70 kV with a 4 kW maximum capacity. X-rays were filtered using a thin beryllium window (30 mm in diameter and 0.127 mm in thickness). The typical dose rate from this irradiator was 17 Gy/s in air at 10 cm from the source, as calibrated using an ion chamber dosimeter.

3.3.3. D_{10} *value determination for* E. coli *O157:H7 on baby spinach*

Baby spinach was purchased from a local supermarket and then stored at 4°C for ≤ 24 h before use. Baby spinach leaves were round-cut (2.54 cm diameter) in a biosafety hood using a sterilized cork borer to allow for uniform X-ray dose distribution across the leaf, and acting to simulate actual tissue damage incurred during leafy green processing. The 3-strain *E. coli* O157:H7 cocktail was diluted 1:1 in sterile, pre-chilled (4°C) 0.1% phosphate buffer solution (PBS), after which triplicate sets of 20 round-cut spinach samples were submerged in the inoculum for 3 min, and then partially spin-dried for 1 min in a biosafety hood using a salad spinner (Oxo Intl., New York, NY). Two additional samples served as uninoculated controls.

Each sample was aseptically inserted into a 4 oz sterile Whirl-Pak® bag (NASCO, Fort Atkinson, WI) using sterile tongs and a sterile spatula. Trapped air in the bag was purged by hand to prevent the sample from moving inside the bag during subsequent handling. All bagged samples were then held 24 h at 4°C until irradiated.

Round-cut baby spinach samples were irradiated at room temperature (~20°C) on one side inside the Whirl-pak® bag, after which the bag was flipped to achieve X-ray doses of 0, 0.050, 0.101, 0.126, 0.151, or 0.176 kGy per side (maximum treatment time = 7 s per side). Irradiation dose was measured using radiochromic film dosimeters (GAF3001DS; maximum dose of 3 kGy; GEX Corporation, Centennial, CO) which were read 24 h after exposure at 500 or 550 nm using a Spectronic Genesys 20® spectrophotometer (Thermo Electron Corporation, Madison, WI).

For quantification of *E. coli* O157:H7, 15 ml of PBS was added to each bagged sample. After homogenizing in a stomacher for 3 min, the samples were serially diluted in PBS or filtered using 0.45 μm membranes and then plated in duplicate on Sorbitol MacConkey Agar containing 0.005% (w/v) cefixime and 0.25% (w/v) tellurite (CT-SMAC, Becton Dickinson). Preliminary tests showed no significant difference in recovery for treated samples plated on CT-SMAC and CT-SMAC overlayed with TSAYE, indicating negligible sublethal injury as a result of X-ray exposure. All *E. coli* O157:H7 colonies were counted after 18 – 24 h of incubation at 37 ± 2ºC, with these counts converted to log CFU/cm^2. Log reductions were then calculated by subtracting the log survivor counts from those of the unirradiated controls. Negative control samples were also spread-plated onto CT-SMAC, with any suspect colonies further evaluated using Reveal® test strips for *E. coli* O157:H7 (Neogen, Lansing, MI).

3.3.4. *Determination of dose rate and dose in bags of baby spinach*

Dose rate (Gy/s) at the mid-plane region of the bag was determined by inserting a cardboard section (236 cm^2) - mounted with nine radiochromic film dosimeters positioned 7.6 cm apart (figure 1) - into the center of an uninoculated bag. The bag was then irradiated for 60 s upon on a miniature conveyor belt system within the X-ray irradiation chamber, programmed to oscillate at 8 – 10 cm/s. To evaluate the dose rate (Gy/s) at the surface of the bag the cardboard section was irradiated directly for 30 s as it oscillated upon the conveyor belt within the X-ray chamber. Dosimeters were read 24 h after exposure as previously described. Dose distribution mapping was performed using the values generated by the dosimeters at each position to illustrate the variations throughout the bag.

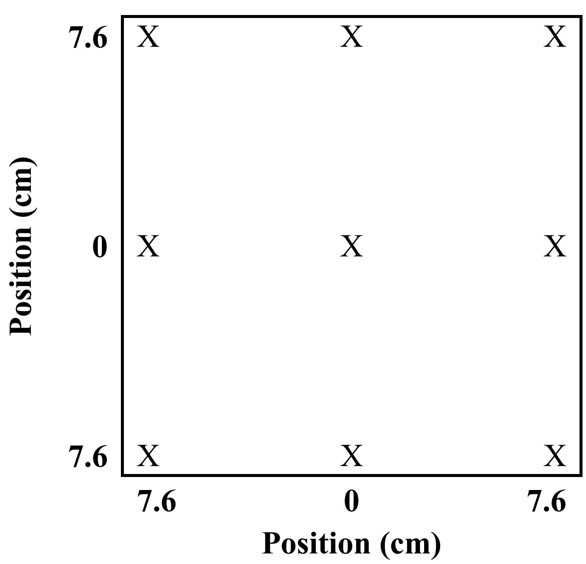

Figure 1. Diagram illustrating the positioning of radiochromic film dosimeters on a cardboard segment.

3.3.5. *Reduction of* E. coli *O157:H7 in bags of baby spinach*

Bags of baby spinach (225 g) were purchased locally on the day of inoculation. Spinach bags were inoculated with 2.25 ml of inoculum by inserting a sterile syringe into one end of the bag to achieve a final population of 10^7 CFU/g. After covering the syringe holes with tape, the bags were gently agitated by hand for 1 min to evenly distribute the inoculum. A prior trial using Glo-germ® was done to demonstrate the efficacy of this syringe inoculation method. A visual inspection using a black light showed even distribution of the Glo-germ® across the entire bag using these procedures. Following agitation, the bags were held for 24 h at 4°C.

Commercial bags of baby spinach were irradiated using a miniature conveyor belt system within the X-ray irradiation chamber, programmed to oscillate at 8 – 10 cm/s. Triplicate sets of bags were irradiated twice on each side of the bag (4 exposures) for a total duration of 0, 120, 240, 360, and 480 s per treatment to simulate 0, 30, 60, 90, and 120 s of processing in a four tube commercial scale irradiator.

After irradiation, the bags were again agitated for 1 min to account for the uneven dose distribution across the bag. Subsequently, two 25 g samples were aseptically taken from each bag using sterile tongs and placed into 10 oz Whirl-pak® bags. *E. coli* O157:H7 populations were determined by homogenizing samples in 100 ml of sterile PBS in a stomacher for 3 min, and then plating appropriate serial dilutions on CT-SMAC. Survivors were counted after 24 h of incubation at 37°C.

3.3.6. *Statistical analysis*

The X-ray radiation D_{10} value for the 3-strain *E. coli* O157:H7 cocktail on round-cut baby spinach was calculated as the absolute value of the reciprocal slope for the linear regression of log reduction vs. dose. The maximum dose (kGy) received by the bag was determined as the maximum average dose rate seen at the surface of the bag throughout the maximum exposure time using the equation:

Max. Dose = (Max. dose rate surface) x t

Where: t = time

Log reduction values for *E. coli* O157:H7 in bags of baby spinach were predicted as the dose rate exposure over time at the mid-plane region and at the surface of the bag using the equations:

R_{Min} = (Dose Rate mid-plane x t)/Dv

R_{Max} = (Dose Rate surface x t)/Dv

Where: R_{Min} = Predicted minimum reduction value

R_{Max} = Predicted maximum reduction value

t = time

Dv = Calculated D_{10} value for *E. coli* O157:H7 on baby spinach

3.4. Results and Discussion

3.4.1. *Uninoculated controls*

None of the uninoculated baby spinach leaves had quantifiable numbers of *E. coli* O157:H7 as determined by spread-plating on CT-SMAC and subsequent testing of suspect colonies using Reveal® test strips specific for *E. coli* O157:H7.

3.4.2. D_{10} *value for* E. coli *O157:H7 on baby spinach*

Unirradiated, dip-inoculated baby spinach contained an *E. coli* O157:H7 population of ~6.00 log CFU/cm^2, with a maximum reduction of ~4.91 log CFU/cm^2 seen after irradiation. The D_{10} value for baby spinach was 0.033 ± 0.001 kGy (Figure 2), which is comparable to our previously reported value of 0.040 ± 0.001 kGy for iceberg lettuce (Jeong et al., 2010b), 5.3 times lower than the previously reported value of 0.186 kGy using E-beam irradiation (Gomes et al., 2008), and 33 times lower than the value of 1.1 kGy reported by Mahmoud using X-ray irradiation (Mahmoud et al., 2010).

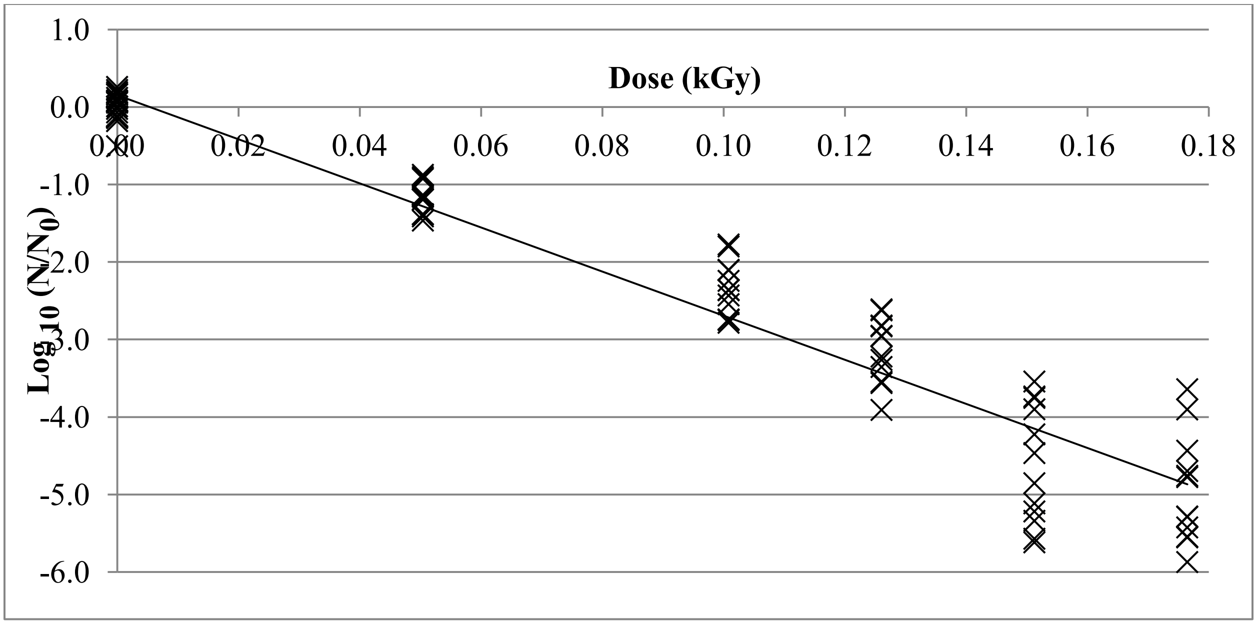

Figure 2. Reduction of *E. coli* O157:H7 on baby spinach using X-ray irradiation (95% confidence limit).

The observed differences in D_{10} values between inoculated and irradiated leafy green varieties is consistent with the observations of Niemira et al. (2002), who found that *E. coli* O157:H7 was significantly more sensitive to gamma radiation on red leaf (D_{10} value = 0.119 ± 0.004) and green leaf lettuce (D_{10} value = 0.123 ± 0.003) than on iceberg (D_{10} value = 0.136 ± 0.004) or Boston lettuce (D_{10} value = 0.140 ± 0.003). Mahmoud also observed similar differences in reduction of *E. coli* O157:H7 on lettuce as compared to spinach using X-ray irradiation (Mahmoud, 2010a; Mahmoud et al., 2010). The reason for the sensitivity differences between leaf varieties was not explored in these experiments. These slight variations in D_{10} values for different leafy greens will likely impact the exposure times needed for commercial processing of bagged salad greens with a standardized process needed to ensure equivalency between leaf varieties.

3.4.3. *Determination of dose rate and dose*

Average dose rates across the mid-plane region and surface of the bags containing baby spinach were 1.93 ± 0.75 and 7.18 ± 3.85 Gy/s, respectively (Figures 3 and 4).

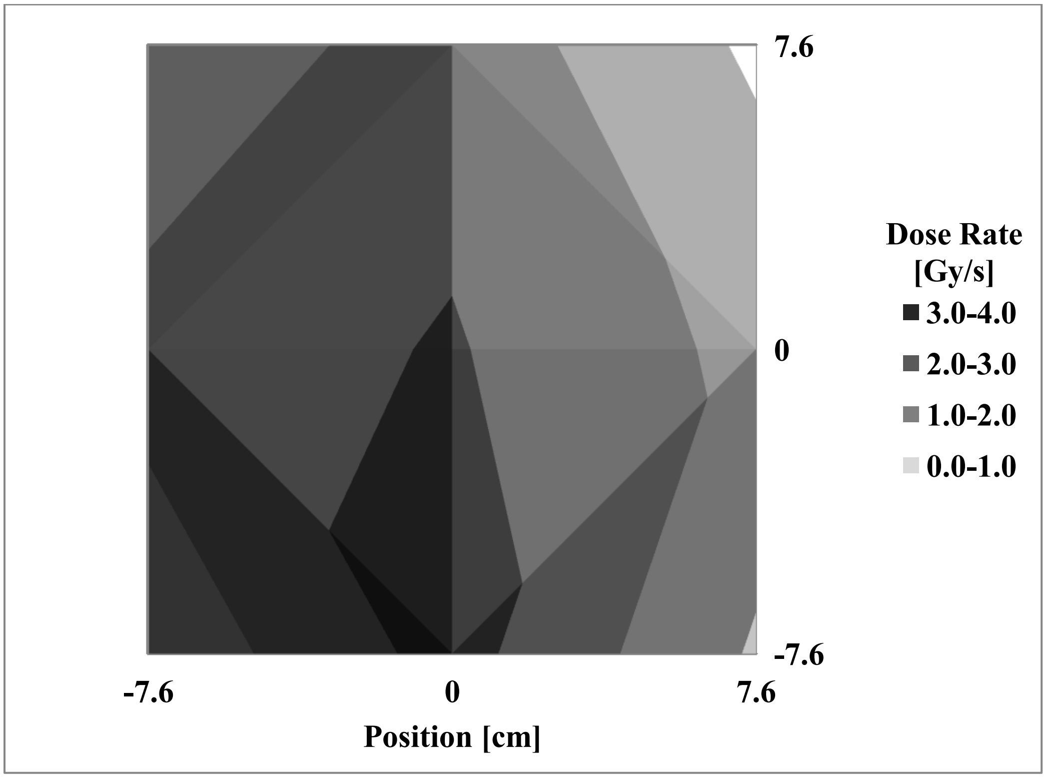

Figure 3. Dose rate distribution at the mid-plane region of a 225 g bag of baby spinach.

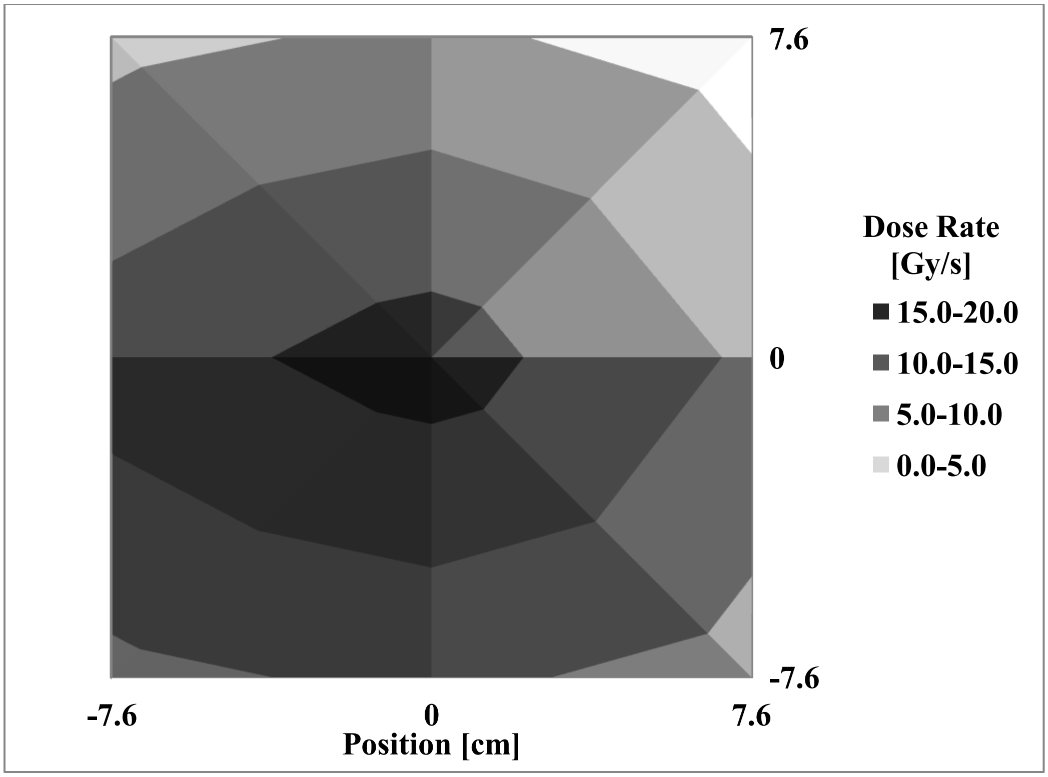

Figure 4. Dose rate distribution at the surface of a 225 g bag of baby spinach.

Differences in D_{10} values seen between X-ray technologies may be associated with differences in dose rate. Rad Source technology's RS 2400 generates X-rays at a rate of 0.235 Gy/s (Mehta and Parker, 2011), which is 5 times lower than the minimum dose rate of 1.18 Gy/s established within a bag of baby spinach using the Rayfresh technology. Longer exposure times at lower dose rate may allow time for the target organism to repair, with a higher dose needed to achieve inactivation.

E-beam dose rates are generally much higher than those for X-rays, at a rate of $10^3 – 10^6$ Gy/sec, while a typical ^{60}Co irradiator generates 1 – 100 Gy/min (Moreira, 2006). After conversion to X-rays the dose rate of electrons generated by E-beams is significantly reduced, while the penetrating power of the resulting photon exceeds that of the electrons alone.

Differences in efficacy between the sources of ionizing irradiation may therefore be explained by the beam characteristics and energy levels of the technology, specifically the relationship between linear energy transfer (LET) and relative biological effectiveness (RBE) of the targeted cell. Barendsen (1968) quantified this relationship and reported that the RBE needed to kill mammalian cells increases with LET up to about 100 keV per μm and then decreases. Consequently, low LET sources produce a more mutations compared to high LET sources (Stapleton et al., 1952; Mortimer et al., 1965).

Varying levels of LET also contribute to the bystander effect (BE) on neighboring cells. BE accounts for indirect actions of ionizing irradiation on intracellular communication, providing another route for cell death (Prise and O'Sullivan, 2009). The cellular response to bystander signals is considered to be non-linear and may involve greater involvement from the development of reactive oxygen species (Kundrat and Friedland, 2012).

This concept of further reactivity indirectly supports the results for lower dose reduction values achieved using X-ray irradiation in comparison to other sources. However, direct comparisons between sources of ionizing irradiation using similar methods need to be performed

3.4.4. *Reduction of* E. coli *O157:H7 in commercial bags of baby spinach*

The predicted log reduction for *E.* coli O157:H7 in a bag of baby spinach ranged from 4.10 to 38.5 log CFU using a simulated exposure time of 120 s. However, the observed log reduction at 120 s, calculated as the average log reduction in a bag, was 3.99 log CFU (Table 5).

Table 5. Predicted and observed average reductions for *E. coli* O157:H7 in bagged baby spinach

		$Log_{10}(N/N_0)$		
Simulated Time (s)	**Max. Dose Received at Surface (kGy)**	**Min. Predicted Mid-plane**	**Max. Predicted Surface**	**Tested Avg.**
0	0.00	0.00	0.00	0.00
30	0.331	-1.03	-9.63	-1.63
60	0.662	-2.05	-19.30	-2.82
90	0.993	-3.08	-28.90	-3.81
120	1.32	-4.10	-38.50	-3.99

The predicted mid-plane dose rate appears to provide a better indication of actual *E. coli* O157:H7 reduction in the bag of spinach, with the observed average reductions more closely resembling the predicted minimum value at the mid-plane region of the bag. The observed *E. coli* O157:H7 reductions were expected to lie between the predicted values, considering that higher dose levels at the bag surface would balance the overall average. Some limitations of the predicted values should be considered in this regard. Dosimeters, while able to measure the dose received, do not fully account for the dose received by the target organism. Dosimetry also does not factor the recovery of injured cells following X-ray treatment. The predicted reductions also assumed that *E. coli* O157:H7 inactivation follows first-order kinetics.

These results clearly demonstrate that low-energy X-ray can be an effective strategy for inactivating *E. coli* O157:H7 in commercial bags of baby spinach, achieving approximately a 4 log reduction within the bag. The maximum dose of 1.32 kGy received by the bag under these conditions was also significantly lower than the limit of 4.0 kGy set by the FDA. In addition, there were no obvious visual changes in the product quality at these dose levels; however, sensory evaluation will need to be performed under these conditions to fully evaluate product acceptability.

These small-scale experiments cannot express the full potential of this technology, since many modifications were made to simulate actual processing conditions. Full scale X-ray irradiators will likely contain multiple X-ray tubes, expanding the exposure range of the instrument and shortening exposure duration.

4. CHAPTER 3

INACTIVATION OF SANITIZER-INJURED *ESCHERICHIA COLI* O157:H7 ON BABY SPINACH USING X-RAY IRRADIATION

4.1. Abstract

This study assessed the potential of sanitizer-induced cell injury to enhance resistance of *E. coli* O157:H7 to X-ray irradiation on baby spinach. A 3-strain *E. coli* O157:H7 cocktail was exposed to two different sanitizers used in flume washing systems - 5.2 ppm peroxyacetic acid-based (PABS, Tsunami® 100) and 22 ppm chlorine-based (CBS, XY-12®) sanitizer, and 18 ppm quaternary ammonium compound-based sanitizer used in equipment sanitation (QACBS, Whisper™) - to obtain 90 to 99% injury. Pre-irradiated, round-cut (2.54 cm diameter) baby spinach leaves were immersed for 5 min in the injured cocktail and irradiated in Whirl-pak® bags at doses of up to 0.063 kGy using a low-energy X-ray irradiator (Rayfresh Foods, Ann Arbor, MI). Healthy and injured survivors were respectively quantified by plating appropriate dilutions on SMAC overlayed with TSAYE and SMAC. *E. coli* O157:H7 injury on inoculated spinach decreased from 90-99 to 66, 63, and 1% for PABS-, CBS-, and QACBS-treated cells, respectively. D_{10} values for PABS-, QACBS-, and CBS-injured *E. coli* O157:H7 on baby spinach were 0.0136 ± 0.000, 0.0223 ± 0.001, and 0.0242 ± 0.001 kGy, respectively. Prior exposure to PABS significantly ($P < 0.05$) enhanced *E. coli* O157:H7 susceptibility to X-ray irradiation, while exposure to CBS significantly ($P < 0.05$) reduced susceptibility. Results suggest that PABS may be preferred for irradiated baby spinach.

4.2. Introduction

Consumption of baby spinach and other leafy greens continues to be a topical issue among nutritionists and food safety experts. However, concerns have been raised regarding the increasing number of leafy green-associated outbreaks due to *Escherichia coli* O157:H7. During 1998 to 2007, *E. coli* O157:H7 was implicated in 13 mixed green salad and12 lettuce outbreaks, resulting in a total of 652 total illnesses (CSPI, 2009b). Leafy green concerns climaxed in 2006 with three prominent *E. coli* O157:H7 outbreaks: the first in September associated with baby spinach resulting in 205 confirmed cases in 11 states, with 31 cases of hemolytic uremic syndrome (HUS) and 3 deaths (CFERT, 2007), followed by two in December traced to shredded lettuce that included a total of 152 confirmed cases in 5 northeastern states, and 10 cases of HUS (U.S. FDA, 2007a; U.S. FDA, 2007b). These outbreaks confirm that currently used chemical sanitizers are not sufficiently effective, with bacterial populations decreasing only 1 to 2 logs on leafy greens during commercial processing (Sapers, 2001; Beuchat et al., 2004; Keskinen et al., 2009; Lopez-Galvez et al., 2009). Consequently, alternative microbial reduction strategies including irradiation need to be explored.

In 2008, the US Food and Drug Administration issued a rule allowing the use of irradiation at doses up to 4 kGy for loose and bagged iceberg lettuce and spinach (FDA, 2008). Previous work focused on gamma rays and E-beams as irradiation sources, with X-ray technology, until very recently, considered being unfeasible economically. Using gamma radiation, *E. coli* O157:H7 internalized in spinach and on the surface of lettuce yielded D_{10} values of 0.27 and 0.14 kGy, respectively (Niemira, 2002; Niemira, 2007). Similar *E. coli* O157:H7 inactivation values have been observed using E-beam, with a D_{10} value of 0.20 kGy for baby spinach (Neal, 2008).

Given several recent technological advances, X-ray irradiation has reemerged as a viable non-thermal pathogen reduction strategy for a wide range of foods, including ground beef (Jeong et al., 2007; Schilling, 2009), almonds (Jeong et al., 2008; Jeong and Marks, 2010), milk (Mahmoud, 2009b) various types of seafood (Mahmoud, 2009a; Robertson et al., 2006; Mahmoud and Burrage, 2009; Mahmoud, 2009c) and fresh produce (Mahmoud, 2010a; Mahmoud, 2010b; Jeong et al., 2010; Mahmoud et al., 2010). X-rays inactivate microorganims primarily through single- or double-strand DNA breaks (Pollard, 1966), which can occur both directly through high energy photons and indirectly through the excitation and ionization of electrons within a food (Podgoršak 2006). In our most recent work (Jeong et al., 2010), *E. coli* O157:H7 populations decreased 5 logs when fresh-cut iceberg lettuce was exposed to an X-ray irradiation dose of 0.20 kGy. Further studies demonstrated that X-ray doses of 0.18 and 0.26 kGy decreased *E. coli* O157:H7 populations 5 logs on baby spinach and flat-leaf parsley, respectively (Moosekian et al., 2010). Regardless as to whether or not X-ray irradiation can eventually be commercialized as a final kill step in leafy green processing, prior washing of the product in sanitizer-treated flume water will still remain an integral step in the process.

Several previous reports have raised concerns regarding enhanced protection of *E. coli* following sublethal exposure to chemical sanitizers. Zook et al. (2001) reported that *E. coli* O157:H7 cultures treated with peroxyacetic acid exhibited substantially increased tolerance to further peroxidative stress, while Dukan and Touati (1996) found that pre-treating *E. coli* O157:H7 with hydrochloric acid conferred resistance to hydrogen peroxide. Similarly, prior exposure to an oxidizing agent may also increase resistance to ionizing irradiation. Demple and Halbrook (1983) reported that prior treatment with peroxide doubled the survival rate of *E. coli*

K-12 that was further exposed to gamma irradiation, with enhanced survival greatest at radiation doses <0.1 kGy.

Inactivation of bacterial cells by chlorine and peroxyacetic acid is well documented but still poorly understood with their oxidative ability directed towards the cell membrane (Baldry and Fraser, 1988; Venkobachar et al., 1977), functional processes of the cell (Barrette et al., 1989; Benarde et al., 1967; Bloomfield 1996; Winter et al., 2008), and potentially DNA. Quaternary ammonium compounds (QACs) also target the bacterial cell membrane (Hotchkiss, 1946; Ioannou et al., 2007; Przestalski et al., 2000).

Given similarities in the inactivation mechanisms for many chemical sanitizers and ionizing irradiation, the potential for enhanced microbial resistance from sublethal sanitizer stress should be addressed before implementing low-energy X-ray technology as a microbial reduction strategy in the processing of leafy greens. Hence, in this study three commonly used commercial sanitizers - one chlorine- and one peroxyacetic acid-based sanitizer commonly used in flume washing systems and one quaternary ammonium-based sanitizer used in post-production clean-up - were investigated for their potential to enhance the resistance of *E. coli* O157:H7 on baby spinach to X-ray irradiation.

4.3. Materials and Methods

4.3.1. *Bacterial strains*

Three *E. coli* O157:H7 strains - K3995 (2006 spinach outbreak), K4830 (2006 lettuce outbreak A), and K4492 (2006 lettuce outbreak B) were obtained from Dr. Michael Doyle at the Center for Food Safety, University of Georgia, Griffin and maintained at -80°C in trypticase soy broth containing 0.6% (w/v) yeast extract (TSBYE; Becton Dickinson, Sparks, MD) and 10%

(v/v) glycerol (Mallinckrodt Baker, Phillipsburg, NJ). Each strain was transferred from the frozen stock cultures and grown in TSBYE for 24 h at 37°C. Following a second transfer in 200 ml of TSBYE, the strains were pelleted by centrifugation for 15 min at 2200 x *g*, resuspended in phosphate buffer solution (PBS), combined in equal volumes and adjusted to 1 L with PBS to obtain a 3-strain cocktail containing ~ 9.7 log CFU/ml *E. coli* O157:H7 as determined by plating on trypticase soy agar containing 0.6% (w/v) yeast extract (TSAYE; Becton Dickinson).

4.3.2. *Peroxyacetic acid-based sanitizer (PABS) injury*

Five 250 ml Erlenmeyer flasks, each containing 200 ml of the *E. coli* O157:H7 cocktail, were agitated at 200 rpm on a Gyrotory Shaker (Model G2; New Brunswick Scientific Co., Edison, NJ) during exposure to 5.2 ppm of PABS (Tsunami® 100, Ecolab, St. Paul, MN). After 2 min, the reaction was stopped by adding 1 ml of 38.5x neutralizing buffer (Difco™ neutralizing buffer, Becton Dickinson) to obtain ~90% injury. The injured cocktail was then pelleted by centrifugation at 2200 x *g* for 15 min and resuspended in PBS to obtain a population of ~8.0 log CFU/ml.

4.3.3. *Quaternary ammonium compound-based sanitizer (QACBS) injury*

Four 2.8 l Fernbach flasks, each containing 250 ml of the *E. coli* O157:H7 cocktail, were agitated at 200 rpm on a Gyrotory Shaker during exposure to 18 ppm QACBS (Whisper™, Ecolab). After 2 min, the reaction was stopped by adding 1 ml of 38.5x neutralizing buffer to obtain ~86% injury. The injured cocktail was then pelleted by centrifugation and resuspended in PBS to obtain a population of ~8.4 log CFU/ml.

4.3.4. *Chlorine-based sanitizer (CBS) injury*

Four 2.8 l Fernbach flasks, each containing 250 ml of the *E. coli* O157:H7 cocktail, were agitated at 200 rpm on a Gyrotory Shaker during exposure to 22 ppm CBS (XY-12®, Ecolab). After 4 min, the inoculum was transferred to a sterile 400 ml beaker containing 10 ml of 77x neutralizing buffer to halt the reaction and obtain ~99% injury. The injured cocktail was then pelleted by centrifugation and resuspended in PBS to obtain a population of ~8.5 log CFU/ml.

4.3.5. *Quantification of injury*

Initial cell injury was determined by plating each *E. coli* O157:H7 suspension on TSAYE and Sorbitol MacConkey Agar (SMAC; Becton Dickinson). After 48 h of incubation at 37°C, percent injury was determined following the equation:

$$\% \text{ Injury} = [(N_{\text{TSAYE}} - N_{\text{SMAC}}) / N_{\text{TSAYE}}] \times 100$$

Where: N_{TSAYE} = CFU/ml recorded from TSAYE plates

N_{SMAC} = CFU/ml recorded from SMAC plates

4.3.6. *X-ray irradiator*

A pilot-scale, custom designed, low-energy X-ray food irradiator (Rayfresh Foods Inc., Ann Arbor, MI) housed in the biosafety level 2 pilot plant at Michigan State University was used to irradiate the samples. This irradiator, which contained a 53 × 53 × 58 cm treatment chamber shielded by 25 mm-thick lead, generated 70 kV with a 4 kW maximum capacity. X-rays were filtered using a thin beryllium window (30 mm diameter, 0.127 mm thick). The typical dose rate from this irradiator was 17 Gy/s in air at 10 cm from the source, as calibrated using an ion chamber dosimeter.

4.3.7. *Baby spinach preparation*

Leaves of baby spinach, originating from California and purchased in 5 oz clamshell containers from a local supermarket on the day of use, were round-cut (2.54 cm diameter) using a sterile cork borer and placed in a Whirl-pak® bag. Prior to inoculation, all leaves were irradiated for 10 s on each side for a combined dose of 0.167 kGy to reduce background microflora confirmed using radiochromic film dosimeters **(**GAF3001DS, GEX Corporation, Centennial, CO). The spinach samples were aseptically removed from the bag, immersed in the injured *E. coli* O157:H7 cocktail for 5 min, spin-dried in a salad spinner (Oxo Intl., New York) for 1 min in a biosafety cabinet, placed in a clean Whirl-pak® bag and irradiated within 30 min of inoculation.

4.3.8. *X-ray irradiation*

Fifteen round-cut leaves (three replicates at each dose) were irradiated on each side for combined doses of 0.012, 0.028, 0.043, 0.054 and 0.063 kGy. The irradiation dose was again confirmed using radiochromic film dosimeters.

4.3.9. *Enumeration*

Following irradiation, spinach samples were diluted in 20 ml of PBS and homogenized in a Seward Stomacher 400 circulator for 3 min at 260 rpm. *E. coli* O157:H7 survivors were enumerated by plating appropriate dilutions on SMAC and SMAC overlayed with approximately

10 ml TSA-YE less than one hour prior to plating for recovery of healthy and healthy plus sublethally injured cells, respectively. Survivors were counted after 48 h of incubation at 37°C.

4.3.10. *Statistical analysis*

All sanitizer experiments were performed in triplicate. A Wilcoxon signed-rank test was used as a non-parametric alternative to the Student's t-test to analyze the injury results (α = 0.05), since there was an abnormal distribution of the matched population. D_{10} values were determined from the absolute value of the inverse slope of the linear regression for the log reduction values, using Microsoft Office Excel 2007. D_{10} values between treatments were then compared by Analysis of CoVariance (α = 0.05), using Minitab® 14 statistical software.

4.4. Results and Discussion

Combined use of multiple microbial reduction strategies, including X-ray irradiation as one such strategy, during leafy green processing will likely remain the most effective approach for maintaining both end product quality and safety. However, some concern is warranted regarding the efficacy of ionizing irradiation for leafy greens given the sublethal concentrations of sanitizers currently used in the industry. This study aimed to address this concern, with the particular focus on evaluating the efficacy of X-ray irradiation against cells that have been sublethally injured by chemical sanitizers and have become attached to baby spinach.

Prior treatment of *E. coli* O157:H7 cell suspensions reduced the initial levels present on the leaf following inoculation from 8.27 cfu/g to approximately 4.84, 5.09, and 5.79 cfu/g, for PABS-, CBS-, and QACBS-treated inoculum, respectively. All cell populations exposed to X-ray irradiation decreased linearly regardless of prior sanitizer exposure (Figure 5).

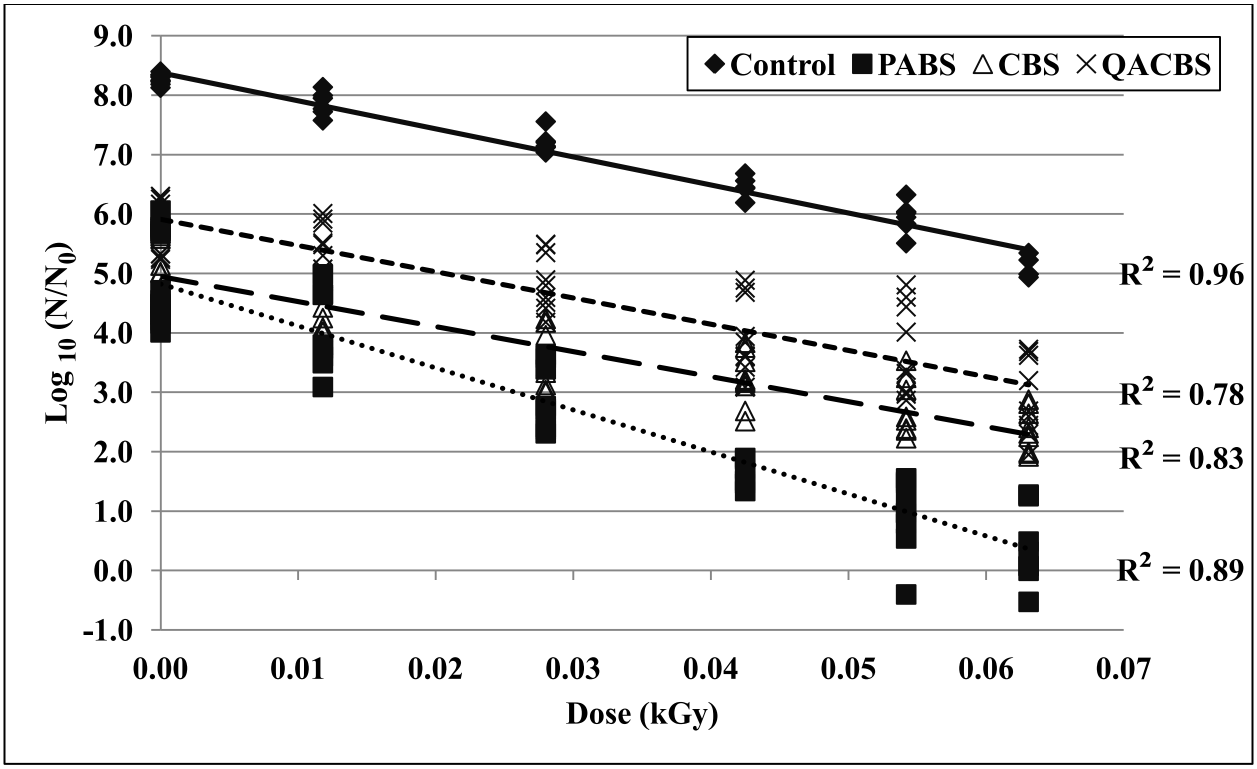

Figure 5. X-ray reduction of Control, PABS-, CBS-, and QACBS-treated cells of *E. coli* O157:H7.

Significant ($P < 0.05$) repair from sublethal injury was observed for each of the three sanitizer-treated *E. coli* O157:H7 cocktails after inoculation onto baby spinach leaves. The extent of injury, however, varied for the different sanitizer treatments, with QAC-treated cells undergoing virtually complete recovery from an initial injury level of 88%. In contrast, PAB- and CB-treated cells exhibited less repair with percent injury decreasing from 89 to 66% and 95 to 63%, respectively (Table 6).

Table 6 *E. coli* O157:H7 cell injury following sanitizer treatment

Sanitizer Treatment	**Inoculum Injury (%)***	**30 min Post-inoculation Injury (%)***
Peroxyacetic acid based	89 ± 4^{a}	66 ± 11^{c}
Quaternary ammonium compound based	88 ± 4^{a}	1 ± 10^{b}
Chlorine based	95 ± 3^{a}	63 ± 4^{c}
None (Control)	10 ± 10^{b}	8 ± 6^{b}

*Letters indicate significant difference between treatments ($P < 0.05$).

Recovery of *E. coli* O157:H7 on baby spinach 30 minutes after exposure to CB- and PAB-based sanitizer injury offers insight into the similarities in the mechanism of action of these two sanitizers and the cellular response to stress. This is to be expected, as both sanitizers have some of the same targets for inactivation. The action of oxidants has shown multiple stages of activity depending on the contact time and sanitizer concentration. Upon initial contact, sublethal injury is incurred by the cell, beginning with the cellular membrane and acting further on the physiological functions of the cell following sanitizer uptake. Lisle et al. (1999) quantified the progression of sublethal attack from chlorine through a series of physiological indicators, suggesting that the site and extent of injury of bacterial cells can be determined following the order: viable plate counts > substrate responsiveness > membrane potential > respiratory activity > membrane integrity.

Depending on the type of prior sanitizer exposure, sublethally injured cells of *E. coli* O157:H7 exhibited different degrees of X-ray resistance on baby spinach leaves. D_{10} values for PABS-, QACBS-, and CBS-injured *E. coli* O157:H7 on baby spinach were 0.014, 0.022, and 0.024 kGy, respectively (Table 7). Prior exposure to PABS significantly ($P < 0.05$) decreased *E.*

coli O157:H7 resistance to X-ray irradiation, while exposure to CBS significantly ($P < 0.05$) increased resistance. Significant cell injury after irradiation was not seen ($P > 0.05$) for cells pretreated with either the PABS or QACBS; however, CBS-treated cells demonstrated an initial decline in percent injury at low doses of irradiation with a significant ($P < 0.05$) increase in injury seen at higher doses (Figure 6). Graphical comparisons illustrating the recovery of cells between each sanitizer can be found in Appendix E. These results can be attributed to the physiological state of the organism before X-ray exposure and the mechanism of sanitizer action as it interacts with the spinach leaf.

Table 7 D_{10} values for sanitizer-treated inoculum

Sanitizer Exposure	**D_{10} values (kGy)***
None (Control)	0.021 ± 0.001 [a]
PABS	0.014 ± 0.000 [b]
CBS	0.023 ± 0.001 [c]
QACBS	0.022 ± 0.001 [ac]

*Letters indicate a significant difference between treatments ($P < 0.05$).

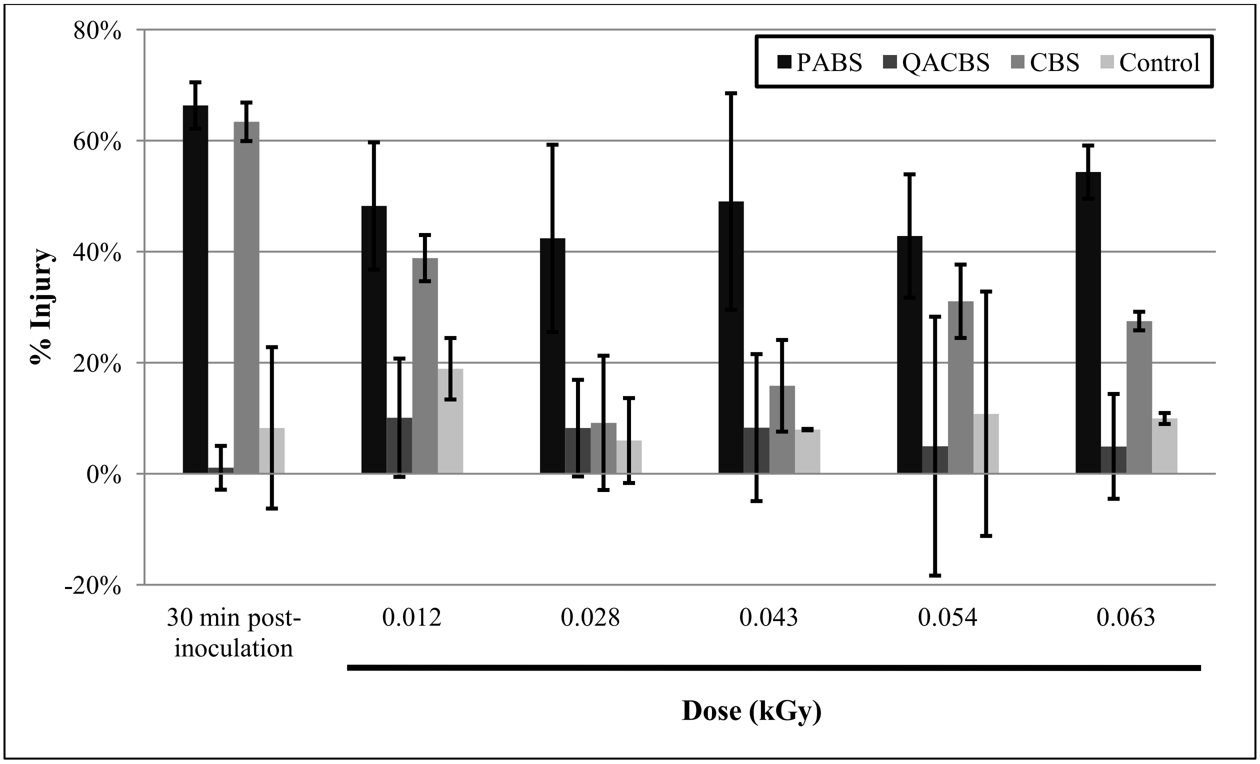

Figure 6. *E. coli* O157:H7 cell injury following X-ray irradiation.

The mechanism of action for oxidizing chemical sanitizers may again help explain the differences in D_{10} values seen between PABS- and CBS-treated cultures. Commercial chlorine- and peroxyacetic acid-based sanitizers exhibit different rates of oxidation. Strong oxidizing agents such as peroxyacetic acid primarily target cell surface components, while weaker oxidizing agents such as chlorine diffuse through the protective cell barrier to react with inner cellular components (Cho et al., 2010; Kitis, 2003).

Dukan and Touati's research (1996) demonstrated how the further reactivity of chlorine compounds impacts the efficacy of chlorine-based sanitizers. In their experiments, hypochlorous acid (HOCl) reacted with many cellular components and was unlikely to reach the DNA; however, mutants in recombinational repair genes exhibited increased sensitivity to HOCl, indicating DNA damage. This result was attributed to hypochlorous acid derivatives including chloramines, which are less reactive and thus more diffusible and efficient at breaching cellular barriers. Chloramines are formed when HOCI or other chlorinating agents react with primary and secondary amines (Thomas, 1986), which make up the structure of bacterial cells. The concept of further reactivity offers a mechanistic separation between chlorine- and peroxyacetic acid-based sanitizers, suggesting that chlorine compounds can penetrate to the core of the cell. Consequently, chlorine and ionizing irradiation attack similar targets.

The effect of X-ray irradiation on QAC-injured cells of *E. coli* O157:H7 could not be assessed due to almost total repair on the spinach leaves prior to X-ray exposure. This may be explained by the additional role of QACs as a surfactant, which binds by chemisorption to the cell surface to influence the zeta potential of the cell (Neu, 1996). On equipment surfaces, QACs provide a barrier to prevent bacterial attachment by decreasing the contact angle, and increasing surface free energy, which in turn reduces bacterial adherence (Sinde and Carballo, 2000). Pretreatment with QAC was also shown to completely inhibit *S.* Typhimurium attachment to chicken skin (Breen et al., 1995). Consequently, pre-treating *E. coli* O157:H7 with a QAC-based sanitizer may have consequently decreased the organism's ability to attach to the leaves of baby spinach, preferentially allowing for the attachment of healthy, unaffected cells.

Properties inherent to baby spinach may also have lead to the repair of sublethally injured cells of *E. coli* O157:H7 or increased attachment to the leaf surface during contact with the leaves prior to X-ray exposure. In this study, sanitizer-injured cells of *E. coli* O157:H7 had two extended 30 min periods of contact with the spinach – the first after inoculation to allow for attachment to the spinach surface and transport to the X-ray irradiator, and the second after irradiation as the spinach leaves were crushed to fully homogenize the samples. A high concentration of naturally present vitamins and antioxidants including ascorbate in spinach leaves aide in neutralizing reactive oxygen species (Foyer and Halliwell, 1976). Ascorbate fluctuates between the reduced ascorbate and the oxidized form, dehydroascorbate to prevent oxidative leaf damage from environmental ozone (Luwe et al. 1993) and hydrogen peroxide (Nakano and Asada, 1981).

Overall, these findings suggest that peroxyacetic acid-based sanitizers may be preferred for X-ray irradiated baby spinach, because exposure to a chlorine-based sanitizer resulted in cross-protection to X-ray irradiation. Hence, combined use of PAB sanitizers with X-ray irradiation can allow for shortened X-ray exposure times, with fewer negative impacts on spinach quality from high levels of sanitizer and/or irradiation alone. While these results do indicate that sublethal injury of *E. coli* O157:H7 due to prior treatment with a chlorine-based sanitizer may reduce the efficacy of X-ray irradiation compared to those cells that did not receive prior sanitizer exposure, this research does not suggest that combined treatment of a CBS and X-ray will not be more effective. To this end, it is recommended for those processers using chlorine in the wash water to carefully monitor levels of sanitizer, and to frequently clean equipment and reduce the build-up of product in an effort to minimize the presence of sublethally injured cells during production.

5. CHAPTER 4

SUGGESTIONS FOR FUTURE WORK WITH LOW-ENERGY X-RAY IRRADIATION

Several questions and concerns need to be addressed prior to full implementation of low-energy X-ray irradiation for leafy green processing. As with the implementation of any new technology, one must consider both the cost and impact on the product.

Consumers eat spinach and other leafy greens for the crisp texture and high nutrient quality. Additional processing using ionizing irradiation, heat, or chemical treatments, may potentially disrupt the quality attributes of the product. This thesis focused on the ability of low-energy X-ray irradiation to reduce levels of *E. coli* O157:H7 on spinach, while past work using the Rayfresh irradiator was concerned with pathogen reduction levels on iceberg lettuce. In this research, other attributes, such as sensory or quality changes, were not considered. In other published research, gamma ray and E-beam irradiation doses of up to 3.85 kGy did not adversely affect the nutritional value or texture of leafy greens (Denise et al., 2004; Gomes et al., 2008; Han et al., 2004; Likui et al., 2006; Niemira and Fan, 2006; Prakash et al., 2000). X-ray's impact on nutritional composition should likewise be fully evaluated considering the treatment times and conditions used for a 5-log reduction in commercial bags of baby spinach and other leafy greens.

Results of this thesis have also suggested that the efficacy of X-ray irradiation may be commodity-specific. X-ray dose levels to reduce *E. coli* O157:H7 differed for spinach, lettuce, and parsley, with similar findings seen using gamma ray irradiation (Niemira et al., 2002). Previous studies also reported dose differences between pathogens (Mahmoud, 2010a; Mahmoud et al., 2010). Such variations highlight the need to further explore additional leafy greens and potential pathogens, and identify target levels for industry.

Scale-up of the data for commercial processing must also be considered. D_{10} values are valuable tools for demonstrating the efficacy of X-rays at their most basic level, but not under actual processing conditions. This topic was touched upon in Chapter 2, with the irradiation of bagged spinach; however, it should be expanded to include industry scale equipment as well as consideration for distribution of the product within the bag.

Lastly, this research has highlighted the need for expanding the understanding of the interaction between chemical sanitizers, ionizing irradiation and components of the bacterial cell. Prior treatment of the cell with peracetic acid-based sanitizers in conjunction with X-ray enhanced inactivation of *E. coli* O157:H7, while prior exposure to chlorine-based sanitizers reduced the efficacy of X-ray irradiation. Genetic analysis has suggested similar routes of bacterial inactivation by oxidizing agents (Dukan and Touati, 1996; Khil and Camerini-Otero, 2002; Wang et al., 2009); however, the details of inactivation are still not clearly understood, and minor differences in the genetic response of the cell may have significant implications. Exploring the reaction of the cell following exposure to chemical sanitizers and ionizing irradiation will be useful in determining the route of inactivation and the ideal sanitizer to be used when processing leafy greens with X-ray.

Given the limitations of this thesis, future research objectives using low-energy X-ray irradiation should focus on expanding our current understanding of the technology, with special consideration given to the economics and commercialization of the technology, the impact of X-rays on the sensory attributes of the leaf, the reduction of spoilage microorganisms, and the mechanisms of bacterial inactivation.

5.1. Determine the Cost Associated with X-ray Irradiation

Cost of any food safety intervention is critically important to a business. An in-depth cost analysis must be done to determine whether produce companies are capable of absorbing the added cost of implementing the technology with the potential for a contamination response. Implementing X-ray technology into an existing processing line will include a modification of the production process, retraining of employees, and the upfront cost of the technology itself. In addition, there will be the maintenance of the equipment, including specially designed parts and experienced technicians. On the other hand, total retail sales for bagged spinach declined $201.9 million during the first 68 weeks after the FDA announced the 2006 outbreak of *E. coli* O157:H7 (Arnade et al., 2009). The cost of this outbreak included untold millions in lost product, recalls, and lawsuits. The final considerations will be the shelf-life extension of the product and consumers' view of irradiation.

5.2. Application to Commercial Processing

The D_{10} values generated in this thesis must be translated to real-life processing conditions and quantities of product. Key concepts to evaluate will therefore include dose-mapping of irradiated packages, and applying the D_{10} values determined in this thesis to that dose information. Tests will then need to be performed to evaluate whether the calculated doses are in fact effective and practical for commercial processing.

5.3. Sensory Analysis

When attempting to sell a product, two of the most important factors include the taste and the willingness to purchase. Consumer acceptance is therefore another key component in the implementation of X-ray into the processing of leafy greens. A sensory study should be conducted to evaluate consumer response to irradiation of spinach, lettuce, and parsley, using doses determined to be lethal to *E. coli* O157:H7. This study should also aim to address consumer interest in purchasing X-ray irradiated produce in addition to their taste perceptions of the treated product.

5.4. Enhanced Growth from Elimination of Background Microflora

In addition to cost and customer considerations, the implementation of X-ray irradiation must be fully integrated with the safety of the technology as the primary focus. This thesis focused solely on the reduction of pathogens specifically applied to the surface of the leaf, but had no regard to the safety of the treated product, including how it may fair in the household and after opening. A risk analysis should be conducted to evaluate the potential for consumer mishandling and to assess whether the dose applied to reduce pathogen levels does not also allow for other, unseen concerns.

Ionizing irradiation is a very effective strategy for reducing microorganisms on produce, including background and spoilage microflora. In the absence of competitive or spoilage organisms, opportunistic organisms or pathogens, such as *Listeria monocytogenes*, could grow uncontrolled on the surface of irradiated foods. Given the extended shelf-life of irradiated foods, growth of these pathogens in comparison to product spoilage should be investigated. Appendix C outlines the beginning of this process.

5.5. Sanitizer Mechanisms for Microbial Inactivation

Developing the optimal sanitizer to be used in combination with X-ray irradiation requires a greater understanding of the role that sanitizers play in bacterial inactivation and how the sanitizer alters the target organism prior to X-ray exposure. Individual chemical sanitizers should be investigated for their role in eliciting a genetic response by the bacterial cell, based on exposure time and sanitizer concentration. These results can be built to track the genetic markers of cell exposure, provide an indication of inactivation, and help to identify key components of the sanitizer that make the cell vulnerable to X-ray irradiation.

6. APPENDIX A

INACTIVATION OF *ESCHERICHIA COLI* O157:H7 ON FLAT LEAF PARSLEY USING X-RAY IRRADIATION

6.1. Purpose

This study was conducted to determine the dose of X-ray irradiation to create a 1-log decrease in the population (D_{10} value) of *Escherichia coli* O157:H7 on the surface of flat-leaf parsley. The D_{10} value can vary between different organisms, across different food commodities, and between technologies. This research has been a part of a greater goal to apply X-ray irradiation to the safe production of bagged, cut leafy green vegetables. The first step to understanding how X-ray interacts with flat-leaf parsley is to determine the D_{10} value for the destruction of *E. coli* O157:H7. This value then needs to be scalable to larger quantities of product.

6.2. Materials and Methods

6.2.1. *Strain preparation*

Three *E. coli* O157:H7 strains - K3995 (2006 spinach outbreak), K4830 (2006 lettuce outbreak A), and K4492 (2006 lettuce outbreak B) preserved at -80°C were grown in trypticase soy broth containing 0.6% (w/v) yeast extract (TSBYE) for 24 h at 37°C. Each individual strain was then transferred to 20 ml TSBYE in centrifuge tubes. Following overnight growth at 37°C, the strains were pelleted by centrifugation for 15 min at 4500 rpm, resuspended in phosphate buffer solution (PBS), and combined in equal volume.

6.2.2. *Sample preparation*

Flat-leaf parsley leaves, purchased from the local supermarket on the day of experimentation, were aseptically removed from the stem and digitally imaged with these pixilated images factored into a computer algorithm to determine the leaf surface area. Parsley stems were aseptically removed from the leaf using sterile scissors.

6.2.3. *Inoculation*

Prepared samples of parsley leaves and parsley stems were dip-inoculated in the 3-strain cocktail of *E. coli* O157:H7 for 3 minutes, and spun dry in a salad spinner for 1 minute. Leaves of parsley were placed directly within a 4 oz Whirl-pak^{TM} bag, while the parsley stems were cut into 2 cm segments, of which 3 segments were arranged side-by-side in the corner of the Whirl-pak bag. All samples were then held 24 h at 4°C.

6.2.4. *X-ray irradiation*

Three replicates of 15 samples each were irradiated on each side of the leaf or stem to achieve five combined surface doses of up to 0.205 kGy for flat-leaf parsley leaves and stems using a prototype low-energy X-ray irradiator (Rayfresh Foods, Ann Arbor, MI). Irradiation dose was confirmed using radiochromic film dosimeters (GAF3001DS, GEX Corporation, Centennial, CO).

6.2.5. *Enumeration*

Numbers of *E. coli* O157:H7 in homogenized samples were determined by dilution or filtering and plating on Sorbitol MacConkey Agar with cefixime and tellurite (CT-SMAC). The highly selective media was used to prevent the growth of background bacteria present on the spinach and parsley, after determining that there had been no evidence of injury using this medium in previous work. Survivors were counted after 24 h incubation at 37°C.

6.2.6. *Statistics*

D_{10} values were determined from the absolute value of the inverse slope of the linear regression for the log reduction values.

6.3. Results and Discussion

D_{10} values for flat-leaf parsley and parsley stems were 0.052, and 0.067 kGy, respectively (Figure 7).

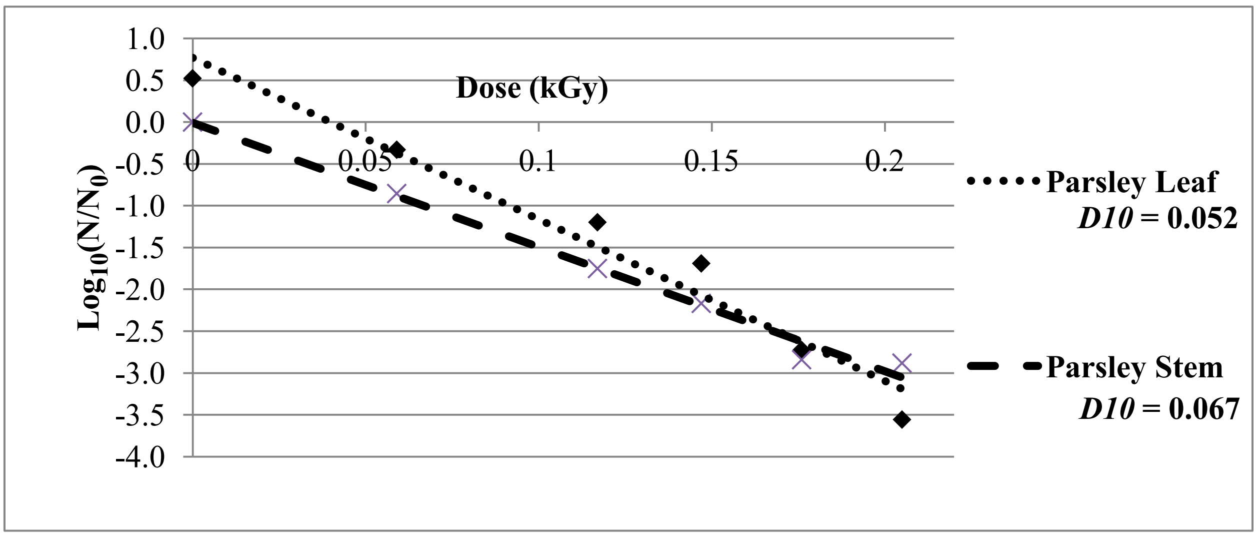

Figure 7. D_{10} values for *E. coli* O157:H7 on the leaves and stems of flat-leaf parsley.

This research suggests that X-ray irradiation needs to be tailored to the commodity being treated, and consideration must be made to the additional, inedible portions of product, specifically the stems of flat-leaf parsley if it is intended to be packaged along with the leaves.

6.4. Raw Data

Table 8 Reduction of *E. coli* O157:H7 on the surface of flat-leaf parsley leaves

Date	7/17/2009	3/3/2009	3/18/2009		
Initial Conc. (CFU)	2.00*10^9	2.30*10^9	2.30*10^9		
Control Avg. (log CFU)	5.8873	5.70230	5.7285		
Dose [kGy]	#1	#2	#3	Log Reduction	Std. Dev.
0	0.192148479	0.042946	-0.031394	0.522827	-0.51197
	-0.045469482	-0.85274	-0.718641		
	-0.210850396	-0.84689	-1.368647		
	-0.089897273	-0.88273	-0.856825		
	-0.01641547	-1.05127	-1.10573		
0.059	1.253038016	0.004884		-0.33065	-0.58839
	1.001787521	-0.09041	-0.24191		
	0.807514275	-0.01728	-0.072445		
0.117	1.955636981	0.977047	0.7001719	-1.19797	-0.70846
	2.155460313	0.770461	0.3316571		
	2.21183976	0.768892	0.9106054		
0.147	2.869958769	1.537592	1.5149451	-1.69053	-0.76468
	1.879187759	1.620674	1.4655402		
	2.788211004	0.423361	1.1153441		
0.176	3.41366791	2.082121	1.9234307	-2.72652	-0.6457
	3.584788866	2.710039	2.5167755		
	3.453787258	2.823199	2.0308585		
0.205	3.997871985	3.163645	3.4548041	-3.55734	-0.39977
	3.952483914	3.816698	2.9917274		
	4.043642486	3.251514	3.3437066		

Table 9 Reduction of *E. coli* O157:H7 on the surface of flat-leaf parsley stems

Date	7/17/2009	3/3/2009	3/18/2009		
Initial Conc. (CFU)	2.00*10^9	2.30*10^9	2.30*10^9		
Control Avg. (log CFU)	5.4549	4.73197	4.9776244		
Dose [kGy]	#1	#2	#3	Log Reduction	Std. Dev.
0	-0.2830	0.16524	-0.372139	-2E-16	-0.29346
	0.3580	-0.40236	0.2907648		
	-0.0750	0.23712	0.0813738		
0.059	1.2525	0.67364	1.797929	-0.85466	-0.61352
	0.3966	0.91906	0.0883227		
0.117	1.5656	1.32225	2.3244119	-1.75168	-0.36889
	1.9897	1.48431	1.8238095		
0.147	2.0724	1.97705	1.2689911	-2.16833	-0.69018
	2.1512	2.13675	3.4035931		
0.176	1.5976	1.92749	3.2086165	-2.84173	-0.87522
	3.2134	3.26399	3.8393217		
0.205	3.4743	2.50796	2.6711994	-2.88251	-0.43539
	2.4917	3.37979	2.7701247		

7. APPENDIX B

INACTIVATION OF *ESCHERICHIA COLI* O157:H7 IN BAGGED ICEBERG LETTUCE USING X-RAY IRRADIATION

7.1. Purpose

The purpose of this research is to determine the time of X-ray irradiation required to result in the inactivation of *Escherichia coli* O157:H7 in commercial bags of iceberg lettuce. The results of this research can be used to determine processing conditions considering the implementation of X-ray irradiation within a commercial processing line.

7.2. Materials and Methods

7.2.1. *Strain preparation*

Three *E. coli* O157:H7 strains - K3995 (2006 spinach outbreak), K4830 (2006 lettuce outbreak A), and K4492 (2006 lettuce outbreak B) preserved at -80°C were grown in trypticase soy broth containing 0.6% (w/v) yeast extract TSBYE for 24 h at 37°C. Each individual strain was then transferred to 20 ml TSBYE in centrifuge tubes. Following overnight growth at 37°C, the strains were pelleted by centrifugation for 15 min at 4500 rpm, resuspended in phosphate buffer solution (PBS), and combined in equal volume.

7.2.2. *Sample preparation*

Bags of iceberg lettuce/romaine blend (312 g) were purchased from the local supermarket at the day of inoculation. Lettuce was inoculated with 3.15 ml of prepared inoculum to achieve a final concentration of 10^7 CFU/g using a sterile 2 in syringe. Syringe holes were then covered with tape, and the bags were agitated gently by hand for approximately 1 min. Following agitation, the bags were held for 24 h at 4°C.

7.2.3. *X-ray irradiation*

Samples bags were placed onto a conveyor system within the prototype low-energy X-ray irradiator (Rayfresh Foods, Ann Arbor, MI) to simulate processing conditions as outlined in section 3.3.4.

7.2.4. *Enumeration*

Following irradiation, 2 – 25 g samples were aseptically taken from each bag and placed within a 10 oz Whirl-pak™ bag. Numbers of *E. coli* O157:H7 in homogenized samples were determined by dilution or filtering and plating on Sorbitol MacConkey Agar with cefixime and tellurite (CT-SMAC). Survivors were counted after 24 h incubation at 37°C.

7.3. Results

7.3.1. *Dose determination*

Table 10 Dose rate distribution at the mid-plane region in bags of iceberg lettuce

Product	Position	Absorbance	Exp Time (s)	Dose [Gy]
Lettuce	0	0.241	60	54.1
	1	0.281	60	73.3
	2	0.23	60	48.9
	3	0.241	60	54.1
	4	0.257	60	61.8
	5	0.211	60	39.9
	6	0.247	60	57
	7	0.232	60	49.8
	8	0.199	60	34.2

7.4. Raw Data

Table 11 Log reduction values for *E. coli* O157:H7 in bags of iceberg lettuce on 10/19/2010

Treatment	Sample		Plate Counts (CFU)		Plate Dilution	Bag Dilution	CFU/25 g	Avg. CFU/25 g	Weight (g)	Avg. CFU/Bag	Avg. log CFU/Bag	Log Reduction
Control	1	A	104	126	10000	100	1.15E+08					
		B	160	166	10000	100	1.63E+08					
Control	2	A	136	130	10000	100	1.33E+08					
		B	84	84	10000	100	8.40E+07					
Control	3	A	136	118	10000	100	1.27E+08					
		B	18	24	100000	100	2.10E+08	1.39E+08	312	1.73E+09	9.24	0.00
1-log	4	A	105	95	1000	100	1.00E+07					
		B	167	198	1000	100	1.83E+07					
1-log	5	A	40	55	1000	100	4.75E+06					
		B	161	144	100	100	1.53E+06					
1-log	6	A	26	23	1000	100	2.45E+06					
		B	37	34	1000	100	3.55E+06	6.75E+06	312	8.43E+07	7.93	1.31
3-log	7	A	12	13	10	100	1.25E+04					
		B	31	38	10	100	3.45E+04					
3-log	8	A	48	41	100	100	4.45E+05					
		B	30	40	1000	100	3.50E+06					
3-log	9	A	110	80	100	100	9.50E+05					
		B	135	143	100	100	1.39E+06	1.06E+06	312	1.32E+07	7.12	2.12
5-log	10	A	190	191	100	100	1.91E+06					
		B	86	94	10	100	9.00E+04					
5-log	11	A	92	97	10	100	9.45E+04					
		B	38	43	100	100	4.05E+05					
5-log	12	A	9	15	10	100	1.20E+04					
		B	1	1	10	100	1.00E+03	4.18E+05	312	5.22E+06	6.72	2.52

Table 12 Log reduction values for *E. coli* O157:H7 in bags of iceberg lettuce on 12/7/2010

Treatment	Sample		Plate Counts (CFU)		Plate Dilution	Bag Dilution	CFU/25 g	Avg. CFU/25 g	Weight (g)	Avg. CFU/Bag	Avg. log CFU/Bag	Log Reduction
Control	1	A	183	195	10000	100	1.89E+08					
		B	359	322	10000	100	3.41E+08					
Control	2	A	98	97	10000	100	9.75E+07					
		B	482	580	10000	100	5.31E+08					
Control	3	A	348	326	10000	100	3.37E+08					
		B	448	406	10000	100	4.27E+08	3.20E+08	312	4.00E+09	9.60	0.00
1-log	4	A	108	107	1000	100	1.08E+07					
		B	55	41	1000	100	4.80E+06					
1-log	5	A	162	150	1000	100	1.56E+07					
		B	680	384	1000	100	5.32E+07					
1-log	6	A	332	328	1000	100	3.30E+07					
		B	1500	1500	1000	100	1.50E+08	4.46E+07	312	5.56E+08	8.75	0.86
3-log	7	A	0	0	10	100	0.00E+00					
		B	4	1	10	100	2.50E+03					
3-log	8	A	53	72	100	100	6.25E+05					
		B	540	452	100	100	4.96E+06					
3-log	9	A	2	2	10	100	2.00E+03					
		B	0	0	10	100	0.00E+00	9.32E+05	312	1.16E+07	7.07	2.54
5-log	10	A	0	0	0.1	100	0.00E+00					
		B	4	1	0.1	100	2.50E+01					
5-log	11	A	86	70	1	100	7.80E+03					
		B	35	27	1	100	3.10E+03					
5-log	12	A	0	2	0.1	100	1.00E+01					
		B	0	0	0.1	100	0.00E+00	1.82E+03	312	2.27E+04	4.36	5.24

8. APPENDIX C

EVALUATING *LISTERIA MONOCYTOGENES* GROWTH ON IRRADIATED SPINACH

8.1. Purpose

In the absence of competitive or spoilage organisms, opportunistic organisms or pathogens such as *Listeria monocytogenes* could grow uncontrolled on the surface of irradiated foods. This concern should be addressed when implementing X-ray irradiation as a pathogen control strategy for packaged leafy green vegetables. X-ray has demonstrated 5-log reduction of several pathogens on a variety of produce, as well as a decrease in the levels of inherent microbes and an overall extension in the shelf-life of the produce.

8.2. Materials and Methods

8.2.1. *Sample preparation*

Spinach samples were round-cut (2.54 cm diameter) using a sterile cork borer, and divided into 4 groups based on the following treatments:

1. Irradiated/non-inoculated
2. Irradiated/inoculated
3. Non-irradiated/inoculated
4. Non-irradiated/non-inoculated

Samples that received a pre-irradiation treatment were transferred to Whirl-pak bags and irradiated for 10 seconds on each side of the leaf, for a combined dose of 0.167 kGy as confirmed using radiochromatic film dosimeters.

8.2.2. *Culture preparation*

Four strains of *Listeria monocytogenes*, preserved at -80°C, were grown in trypticase soy broth containing 0.6% (w/v) yeast extract (TSBYE) for 24 h at 37°C. Each individual strain was then transferred to 9 ml TSBYE. Following overnight growth at 37°C, individual strains were combined and adjusted to an optical density of 0.967 and an initial concentration of 1.6 x 10^8 CFU.

8.2.3. *Inoculation*

The prepared inoculum was serially diluted through 6 dilutions in sterile phosphate buffer solution (PBS), and 50µl was then spot inoculated onto treatments 2 and 3. Inoculated samples were then placed into Whirl-pak bags and held 12 days at 4°C.

8.2.4. *Enumeration*

At 2 day intervals, two samples of each treatment were removed from refrigeration, homogenized in 10 ml of PBS, and appropriate dilutions were plated in duplicate to either trypticase soy agar containing 0.6% (w/v) yeast extract (TSAYE) (non-inoculated spinach) for enumeration of total aerobic plate count (APC), or Modified Oxford Agar (MOX) (inoculated spinach) for enumeration of *L. monocytogenes*. Colonies were counted following incubation for 24 – 48 hr at 37°C.

8.3. Results

Initial background levels of bacteria decreased from 5.33 to 5.01 log CFU/cm^2 spinach. Following 12 days storage at 4°C, total aerobic growth increased to 6.12 and 5.73 CFU/ cm^2 on irradiated and non-irradiated spinach, respectively. *L. monocytogenes* grew better on pre-irradiated spinach than on untreated spinach, with levels reaching 4.13 and 2.75 CFU/cm^2 on pre-irradiated and non-irradiated spinach, respectively following 12 days at 4°C.

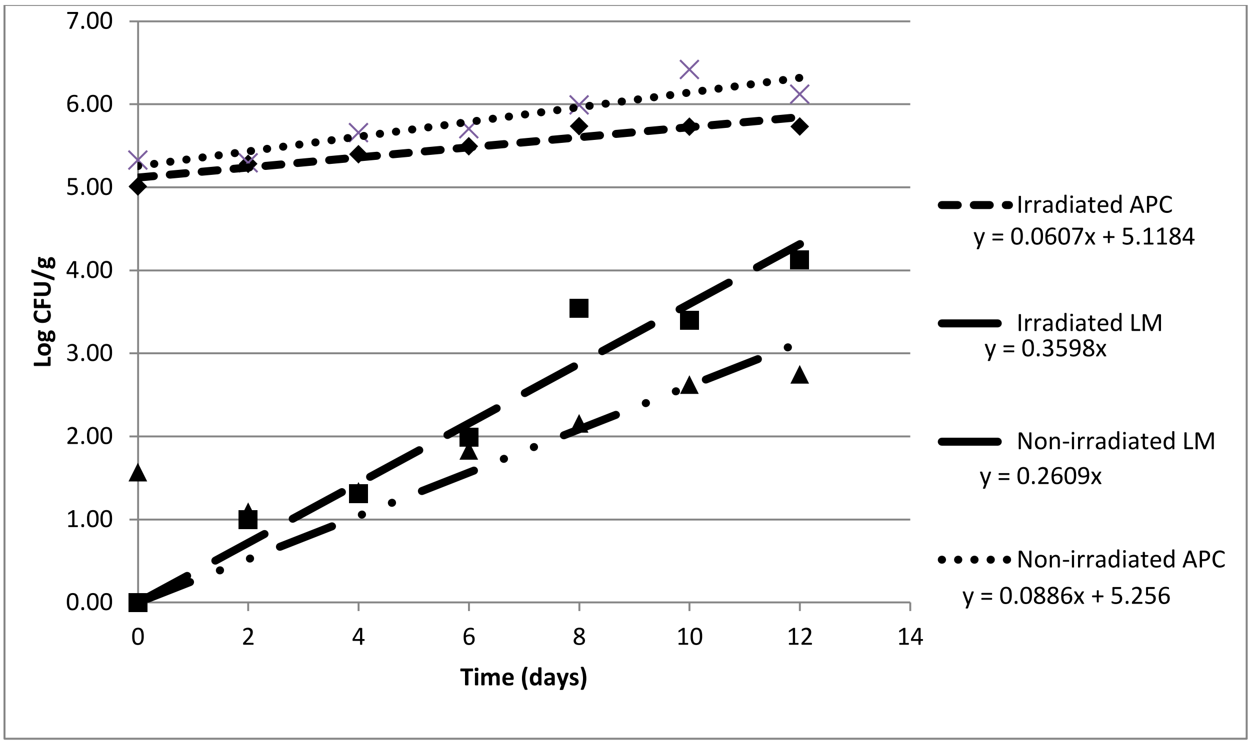

Figure 8. Growth of total aerobic bacteria and *L. monocytogenes* on irradiated and non-irradiated baby spinach.

8.4. Interpretation/Limitations

L. monocytogenes can grow to a higher level on X-ray irradiated spinach compared to non-irradiated spinach. Further work with this study should include a comparison with higher initial irradiation levels, extended storage times, and also at a variety of storage temperatures.

8.5. Raw Data

Table 13 Growth of total aerobic bacteria and *L. monocytogenes* on irradiated and non-irradiated baby spinach

Treatment	Time (day)	TSAYE Count (CFU)				Avg. (CFU)	Dilution (ml)	Surface area (cm^2)	CFU/cm^2	Log CFU/cm^2
Irradiated; non-inoculated	0	193000	215000	3200	2500	103425	10	10.12	102198.6	5.01
	2	183000	189000	198000	205000	193750	10	10.12	191452.6	5.28
	4	280000	280000	210000	240000	252500	10	10.12	249505.9	5.40
	6	420000	330000	210000	300000	315000	10	10.12	311264.8	5.49
	8	870000	570000	380000	380000	550000	10	10.12	543478.3	5.74
	10	580000	570000	540000	480000	542500	10	10.12	536067.2	5.73
	12	720000	580000	400000	480000	545000	10	10.12	538537.5	5.73
Irradiated; inoculated		**MOX Count (CFU)**								
	0	0	0	0	0	0	10	10.12	0	0.00
	2	0	10	10	20	10	10	10.12	9.881423	0.99
	4	14	23	26	19	20.5	10	10.12	20.25692	1.31
	6	120	96	100	80	99	10	10.12	97.82609	1.99
	8	1150	770	11240	930	3522.5	10	10.12	3480.731	3.54
	10	4500	2000	1680	1890	2517.5	10	10.12	2487.648	3.40
	12	21000	22000	5800	5300	13525	10	10.12	13364.62	4.13
Non-irradiated; inoculated		**MOX Count (CFU)**								
	0	30	80	30	10	37.5	10	10.12	37.05534	1.57
	2	10	0	0	40	12.5	10	10.12	12.35178	1.09
	4	16	11	30	31	22	10	10.12	21.73913	1.34
	6	108	95	36	35	68.5	10	10.12	67.68775	1.83
	8	180	140	160	100	145	10	10.12	143.2806	2.16
	10	350	400	580	370	425	10	10.12	419.9605	2.62
	12	750	800	340	370	565	10	10.12	558.3004	2.75

Table 13 (cont'd)

Non-irradiated; non-inoculated		TSAYE Count (CFU)								
	0	200000	140000	180000	340000	215000	10	10.12	212450.6	5.33
	2	228000	216000	193000	160000	199250	10	10.12	196887.4	5.29
	4	320000	370000	690000	460000	460000	10	10.12	454545.5	5.66
	6	710000	560000	370000	410000	512500	10	10.12	506422.9	5.70
	8	910000	900000	1110000	1050000	992500	10	10.12	980731.2	5.99
	10	4200000	3900000	1320000	1180000	2650000	10	10.12	2618577	6.42
	12	1900000	2300000	630000	520000	1337500	10	10.12	1321640	6.12

9. APPENDIX D

INACTIVATION OF BIOFILM-ASSOCIATED *ESCHERICHIA COLI* O157:H7 ON LABORATORY GROWN BABY SPINACH

9.1. Purpose

Contamination of leafy greens is thought to occur in the field by any number of interactions that the plant has with the environment, with the primary source thought to be by means of irrigation water. Contamination of irrigation can occur through close contact with wildlife and manure, and has shown the capability of transfer to growing plants.

Biofilms have also demonstrated the ability to resist means of disinfection and irradiation. The plant leaf surface has been shown to be a highly unfavorable environment for the growth of bacteria. Therefore in an attempt to encourage survival, bacteria form biofilms. It is likely that in the field environment, this biofilm will consist of many other bacteria common to the field. Considering that *E. coli* in general are relatively poor biofilm formers, many others may aid in the development of the film. In previous research there has been no consideration for the natural microflora present on the leaf or the potential benefits of advanced biofilms in the protection of pathogens.

This study aims to determine the persistence of *E. coli* O157:H7 on the surface of baby spinach within an established biofilm, and to further assess whether a natural biofilm provides a means of protection from exposure to X-ray irradiation.

9.2. Design/Objectives

- Collect and characterize the microflora of field-grown spinach.
- Determine biofilm-forming ability of chosen bacteria strains and compatibility with *E. coli* O157:H7.
- Establish laboratory growing conditions suitable for spinach
- Grow spinach with inoculation procedures replicating contaminated irrigation practices.

- Evaluate the growth/survival of *E. coli* O157:H7, pseudomonad, lactic acid bacteria, and total aerobic bacteria throughout the growth period.
- Evaluate the efficacy of X-ray irradiation on the reduction of *E. coli* O157:H7.

9.3. Materials and Methods

9.3.1. *Bacterial collection and characterization*

9.3.1.1. Field sampling

Baby spinach was aseptically collected from two different growth conditions at the Michigan State University Student Organic Farm (Holt, MI) - inside of a greenhouse and from an outdoor plot, on August 8th, with outdoor conditions approximately 70°F, cloudy, with a light rain. Organic spinach was collected in order to determine naturally occurring microorganisms that may reside on the surface of the plants and that have not been exposed to common processing conditions such as sanitizer treatment and employee handling.

9.3.1.2. Enumeration and isolation

Two 25 g samples from each location were placed in Whirl-pak bags to which 100 ml phosphate buffer solution (PBS) was added and homogenized for 60 s at 260 rpm. Appropriately diluted samples were placed on Trypticase Soy Agar with 0.6% yeast extract (TSAYE) and incubated at 37°C for 24 hr. Plates were evaluated for total growth, and then individual colonies of differing morphologies were selected for isolation and identification.

9.3.1.3. Biofilm analysis

Biofilm analysis was conducted on organisms isolated from field-grown spinach, GFP-labeled *E. coli* O157:H7, and laboratory Pseudomonad strains in order to determine a suitable cocktail for inoculation. Pseudomonas were selected because of their known ability to form a biofilms. Testing procedures were based on a modification of the assay described by Stepanovic *et al*. (2000). All strains ere grown in TSBYE at 37°C for 18 h, and then streaked onto TSAYE to obtain confluent growth after incubation at 37°C for 18 h. Cells were then harvested from the plate by flooding the agar surface with 10 ml of 0.1% sterile PBS. Cell concentrations were then estimated using MacFarland Turbidity Standards, and adjusted to a final O.D. of 1.0 ± 0.5. Cultures were then serially diluted to a final concentration of 10^2 CFU/ml in TSBYE. 200 µl of each diluted cell suspension was then transferred in triplicate to wells of a 96-well untreated polystyrene microtiter tissue culture plate (BD Falcon Microtest Flat Bottom; Becton Dickinson, Franklin Lakes, NJ), with sterile TSBYE serving as a negative control. Plates were incubated for 4 days at 22 ± 2°C. Following incubation, microtiter plate wells were emptied, rinsed three times with 0.85% physiological saline, while being gently shaken to remove unattached cells, and allowed to air-dry. Remaining cells were fixed to the well by the addition of 200 µl 99% methanol (Fisher Chemicals, Fair Lawn, NJ) with the methanol decanted 15 min later. After allowing the plates to air dry, the microtiter wells were stained using 200 µl of 2% crystal violet (Remel, Lenexa, KS) for 5 min. After decanting the crystal violet, the wells were rinsed five times with deionized water and air-dried. The remaining dye was resolubilized in 160 µl of 33% (v/v) glacial acetic acid (Sigma Chemical Company, St. Louis, MO) and optical densities were read at 570 nm using a Synergy HT Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT).

9.3.2. *Spinach growth and inoculation*

9.3.2.1. Planting instructions

Soil (Scott's Premium Potting Soil) (0.07% total nitrogen, 0.01% phosphate, 0.03% soluble potash) was appropriately portioned into each of 32 cells in a 2 in flat (Jiffy seed starter) and pressed in lightly. Distilled water was then applied to moisten the soil. Three spinach seeds (Spinach double choice hybrid, W. Atlee Burpee and Co., Warminster, PA) were added per cell and a covered with a light layer of soil (1-2 cm). After the plants were 1 – 2 in tall, the plants were thinned to 1 plant per cell.

9.3.2.2. Growth conditions

Plants were grown under a grow lamp (Sun System® 10) equipped with a 400 W metal halide bulb (Econo Gro®). The grow system provided an 11.5 hr light cycle, and the temperature was consistent at 25 – 28 °C with approximately 70% relative humidity.

9.3.2.3. Inoculation

Individual colonies of 4-environmental isolate (EI) bacterial strains (determined to be better biofilm formers), 5-Pseudomonad (Ps) strains, and 4-avirulent GFP-labeled *Escherichia coli* O157:H7 (EC) strains are selected from TSAYE and transferred to 9 ml TSBYE and grown for 48 h at 25 °C. Following incubation, EI and Ps strains were combined in a 250 ml centrifuge container and centrifuged at 4500 rpm for 15 min. The supernatant is discarded and the culture is resuspended in 100 ml DI water. (Initial concentration determined by spread plating following appropriate dilutions.) EC strains were combined in TSBYE. Spray inoculum consisted of 100 ml EI mixture in DI water, 5 ml EC in TSBYE, 100 µl fertilizer, and 400 ml DI water. 300 ml was then evenly applied to the sheet of spinach within a biosafety cabinet. Spinach inoculation was conducted daily over a three week span following three weeks of growth.

9.3.2.4. Spinach sampling

At 3-day intervals for 3 wks, three spinach leaves were aseptically cut from mature spinach plants prior to watering and daily inoculation. Each leaf was weighed, transferred to a 2 oz Whirl-pak bag, and hand-homogenized in 5 ml PBS. Appropriate dilutions were plated onto TSAYE, TSAYE w/ampicillin, MRS, and PIA for enumeration of total aerobic bacteria, GFP-labeled *E. coli*, lactic acid bacteria, and Pseudomonas, respectively. Plates were incubated for 24 h at 37°C.

9.3.3. *X-ray efficacy*

Tests were conducted to compare the efficacy of X-ray on *E. coli* O157:H7 on the surface of spinach and ingrained within a natural biofilm on the leaf.

9.3.3.1. X-ray on surface-inoculated *E. coli* O157:H7

Spinach was grown following the procedures outlined above, with the *E. coli* O157:H7 omitted from the routine inoculum. Four strains of GFP-labeled E. coli O157:H7, preserved at -80°C, were grown in TSBYE for 24 h at 37°C. Each individual strain was then transferred to 20 ml TSBYE in centrifuge tubes. Following overnight growth at 37°C, the strains were pelleted by centrifugation for 15 min at 4500 rpm, resuspended in PBS, and combined in equal volume.

Following plant maturity, spinach leaves were round-cut using a size 10 sterile cork-borer, dipped inoculated in a 1:10 dilution of the prepared culture for 5 min, and spun dry for 1 min in a salad spinner. Spinach rounds were X-ray irradiated in triplicate at 5 dose levels of 1 – 5 seconds. Experiment was done in duplicate from two successive batches of grown spinach.

9.3.3.2. X-ray on biofilm-incorporated *E. coli* O157:H7

This portion of the experiment was not conducted. Levels of *E. coli* O157:H7 did not reach high enough levels on the growing spinach to be irradiated and demonstrate logarithmic inactivation.

9.4. Results

9.4.1. *Bacterial collection and characterization*

9.4.1.1. Enumeration and isolation

Total aerobic growth was 5.52 and 5.76 log CFU/g on spinach taken from the inside of the greenhouse and outside of the greenhouse, respectively.

Table 14 Characterization of organisms isolated from field-sampled spinach

Isolate	Location	Colony Morphology	Gram Stain	Organism Description
1	Outside	Yellow, circular, raised, sticky, medium sized	neg	short rod, ~1-2 µm
2	Outside	Yellow, circular, raised, fragile, medium sized	neg	short rod, ~1-2 µm
3	Outside	white, circular, cratered, medium	pos	rod, ~4-5 µm
4	Greenhouse	yellow w/white clearing, raised, large	neg	rod, ~1 µm
5	Outside	Yellow, divot in center, circular, med-large	pos	diplococci, 2-3 µm
6	Greenhouse	Pale yellow, irregular shape, flat, large	pos	rod, ~5-6 µm

Table 14 (cont'd)

7	Greenhouse	pale white, slightly raised, rough edges, medium sized	pos	rod, ~10 μm, chained
8	Outside	glossy white, raised, circular, large size	pos	rod, ~10-15 μm
9	Greenhouse	irregular shaped, large	pos	rod, ~6 μm
10	Greenhouse	white, circular, cratered, large	pos	rod, ~10-12 μm
11	Greenhouse	white, convex, circular, very small	pos	cocci, ~1 μm diameter
12	Outside	pale white, flat, circular, rough edges, large size	pos	rod, ~10-12 μm
13	Outside	cream colored, very irregular shaped, raised, mucoid	pos	rod, ~4-5 μm

9.4.1.2. Biofilm analysis

Of the organisms tested, 4 of the environmental isolates (strains 1, 2, 4, and 11) were found to be acceptable biofilm formers, having an absorbance >0.10 in the microtiter well assay. All of the pseudomad strains tested had acceptable biofilm-forming ability as did one of the GFP-labeled *E. coli* O157:H7.

Table 15 Biofilm-forming ability of EI, Ps, and EC

	Strain	Absorbance
Env. Isolates	1	0.13
	2	0.57
	3	0.02
	4	0.26
	5	0.01
	6	0.03
	7	0.01
	8	0.01
	9	0.00
	10	0.01
	11	0.26
	12	0.00

Table 15 (cont'd)

	13	0.00
GFP- E.C.	ATCC 43888	0.20
	CV2b7	0.01
	6980-2	0.08
	6982-2	0.04
Ps. Strains	20	0.17
	21	0.15
	22	0.28
	23	0.24
	24	0.19

9.4.2. *Persistence of E. coli O157:H7 during spinach growth*

Levels of *E. coli* O157:H7 remained consistent during inoculation of spinach plants, ranging from 3.07 – 3.43 log CFU/g. At the last time point, following 6 days without inoculation, levels dropped to approximately 1 CFU/g.

Table 16 Growth of Total Aerobic Bacteria, *E. coli* O157:H7, Pseudomonas, and Lactic Acid Bacteria over the growth period

Day	**Total Aerobic Growth (log CFU/g)**	***E. coli* O157:H7 (log CFU/g)**	**Pseudomonad (log CFU/g)**	**Lactic acid bacteria (log CFU/g)**
0	2.58	0.00	0.00	1.92
3	5.48	3.26	3.09	5.44
7	5.69	3.21	3.60	5.40
10	5.61	3.07	3.43	5.40
14	5.57	3.29	3.34	5.31
17*	5.53	3.43	4.08	5.07
21	4.37	1.06	1.16	3.32

*Day 17 was the last day of inoculation

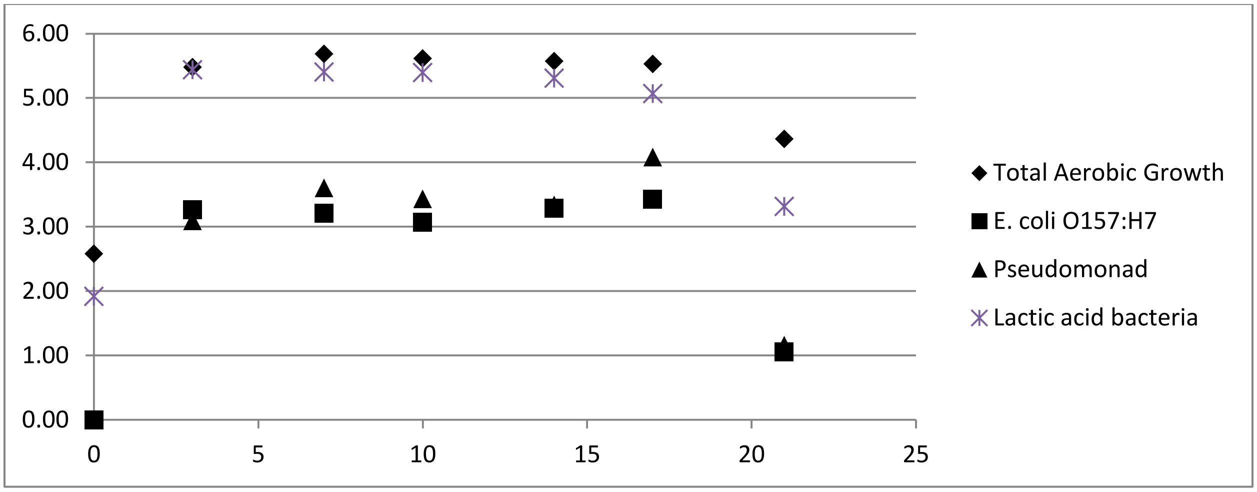

Figure 9. Growth of total aerobic bacteria, *E. coli* O157:H7, Pseudomonad, and lactic acid bacteria over the growth period.

9.4.3. *X-ray efficacy on surface-inoculated vs. biofilm-incorporated cells of* E. coli *O157:H7.*

Table 17 X-ray inactivation of surface inoculated laboratory grown spinach- Batch 1

Sample	Dose Time (s)	Plate Count A (CFU)	Plate Count B (CFU)	Average Plate Count (CFU)	Amount Plated	Initial Dilution	Sur Area (cm^2)	Log CFU/cm^2	Avg Log CFU/cm^2	Log Reduction
1	0	2	0	1	1.00	15	3.08	0.0000	0.00	**0.00**
2	0	0	0	0	1.00	15	3.08	0.0000		
3	0	0	0	0	1.00	15	3.08	0.0000		
4	0	10200	10700	10450	1.00	15	3.08	4.7071	4.68	**0.00**
5	0	10900	8300	9600	1.00	15	3.08	4.6702		
6	0	9700	7000	8350	1.00	15	3.08	4.6096		
7	0	12900	10000	11450	1.00	15	3.08	4.7467		
8	1	5500	4800	5150	1.00	15	3.08	4.3997	4.38	**0.30**
9	1	6700	4200	5450	1.00	15	3.08	4.4243		
10	1	4600	4000	4300	1.00	15	3.08	4.3214		
11	2	1700	1610	1655	1.00	15	3.08	3.9067	4.08	**0.60**
12	2	2390	2170	2280	1.00	15	3.08	4.0459		
13	2	4000	4000	4000	1.00	15	3.08	4.2900		
14	3	810	820	815	1.00	15	3.08	3.5991	3.68	**1.01**
15	3	1050	930	990	1.00	15	3.08	3.6836		
16	3	1130	1150	1140	1.00	15	3.08	3.7448		
17	4	550	590	570	1.00	15	3.08	3.4438	3.47	**1.21**
18	4	650	530	590	1.00	15	3.08	3.4588		
19	4	700	660	680	1.00	15	3.08	3.5204		
20	5	370	280	325	1.00	15	3.08	3.1998	3.28	**1.41**
21	5	510	460	485	1.00	15	3.08	3.3737		
22	5	470	270	370	1.00	15	3.08	3.2561		

Table 18 X-ray inactivation of surface inoculated laboratory grown spinach- Batch 2

Sample	Dose Time (s)	Plate Count A (CFU)	Plate Count B (CFU)	Average Plate Count (CFU)	Amount Plated	Initial Dilution	Sur Area (cm^2)	Log CFU/cm^2	Avg Log CFU/cm^2	Log Reduction
1	0	0	0	0	1.00	15	3.08	0.0000	0.00	**0.00**
2	0	0	0	0	1.00	15	3.08	0.0000		
3	0	0	0	0	1.00	15	3.08	0.0000		
4	0	6100	5500	5800	1.00	15	3.08	4.4514	4.45	**0.00**
5	0	5700	5500	5600	1.00	15	3.08	4.4361		
6	0	6100	6400	6250	1.00	15	3.08	4.4838		
7	0	6100	5300	5700	1.00	15	3.08	4.4438		
8	1	3000	2100	2550	1.00	15	3.08	4.0945	4.47	**-0.02**
9	1	3900	3200	3550	1.00	15	3.08	4.2382		
10	1	44500	5200	24850	1.00	15	3.08	5.0833		
11	2	1330	1680	1505	1.00	15	3.08	3.8655	3.90	**0.56**
12	2	1890	1380	1635	1.00	15	3.08	3.9015		
13	2	1740	1710	1725	1.00	15	3.08	3.9247		
14	3	1350	1720	1535	1.00	15	3.08	3.8740	3.82	**0.63**
15	3	1940	1370	1655	1.00	15	3.08	3.9067		
16	3	950	1030	990	1.00	15	3.08	3.6836		
17	4	920	750	835	1.00	15	3.08	3.6096	3.59	**0.87**
18	4	840	870	855	1.00	15	3.08	3.6199		
19	4	720	660	690	1.00	15	3.08	3.5268		
20	5	540	520	530	1.00	15	3.08	3.4122	3.44	**1.02**
21	5	590	440	515	1.00	15	3.08	3.3997		
22	5	590	700	645	1.00	15	3.08	3.4975		

10. APPENDIX E

COMPARISON OF SELECTIVE AND NON-SELECTIVE PLATING FOR THE DETERMINATION OF D_{10} VALUES FOR SANITIZER-TREATED AND X-RAY IRRADIATED *ESCHERICHIA COLI* O157:H7 ON BABY SPINACH

10.1. Purpose

A direct comparison between selective and non-selecting plating of X-ray irradiated and sanitizer-injured *Escherichia coli* O157:H7 illustrates the impact of the treatment on the injury of the cell, as well as the affect of the medium on the determination of dose-reduction values.

10.2. Materials and Methods

Methods for this comparison are detailed in section 3.2.

10.3. Results

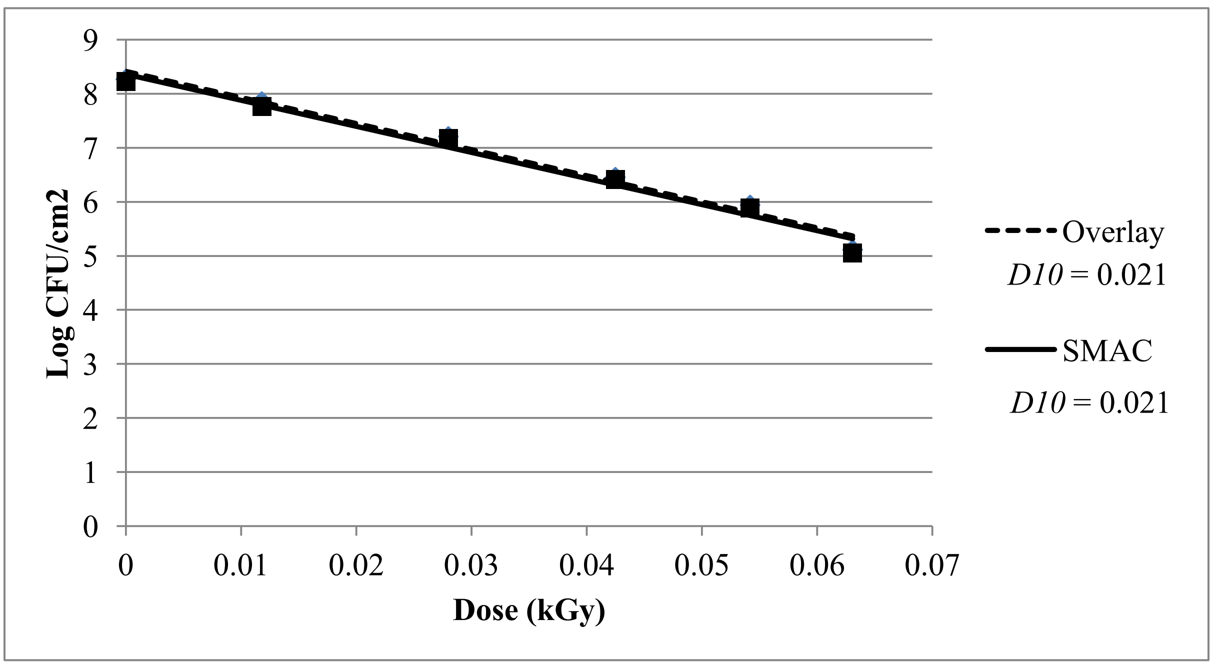

Figure 10. D_{10} values for selective and non-selective control samples of baby spinach.

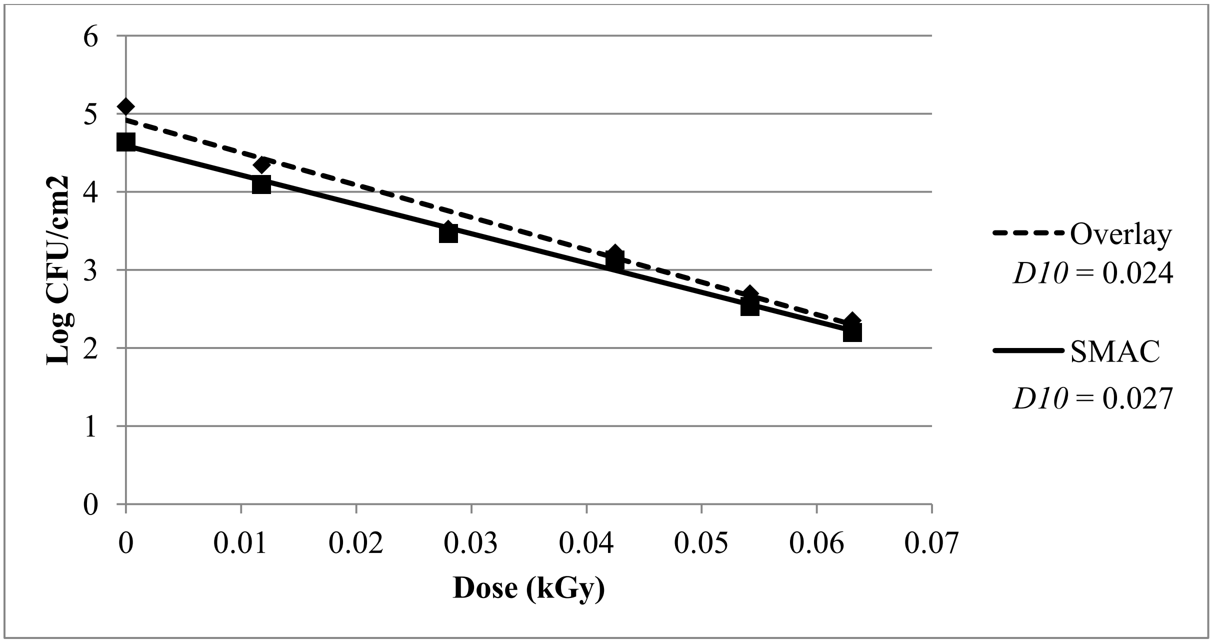

Figure 11. D_{10} values for selective and non-selective XY-12-treated samples of baby spinach.

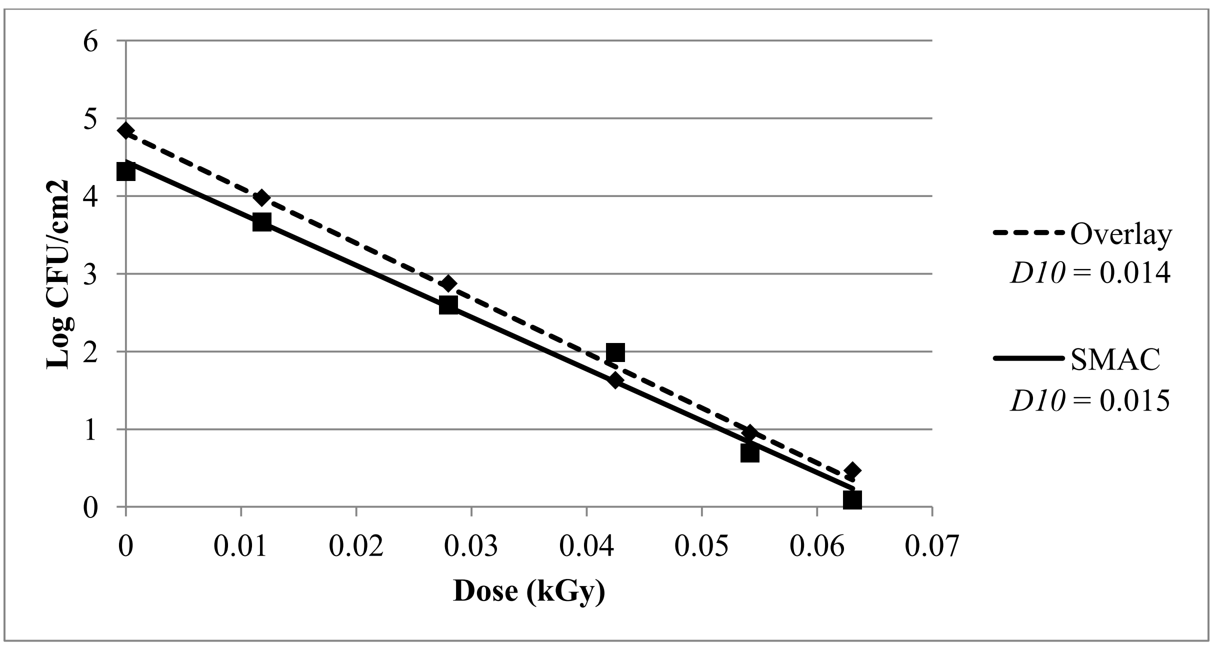

Figure 12. D_{10} values for selective and non-selective Tsunami-treated samples of baby spinach.

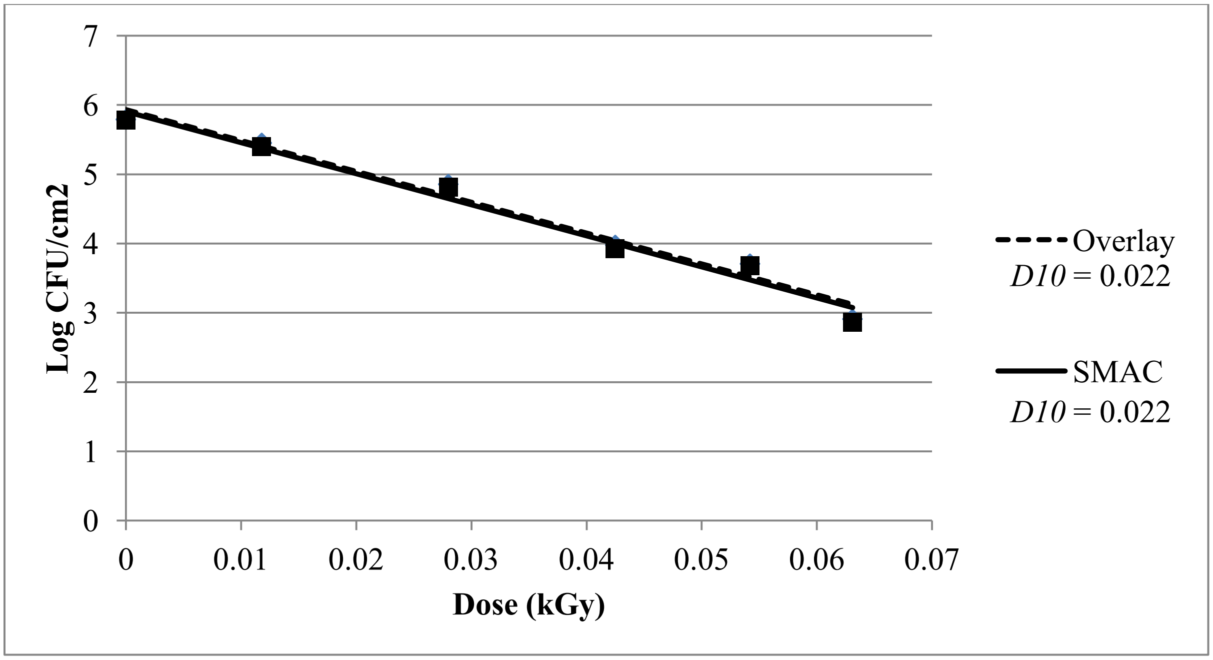

Figure 13. D_{10} values for selective and non-selective QUAT-treated samples of baby spinach.

10.4. Conclusion

Results of the selective vs. non-selective plating show that the plating medium is an important consideration in assessing the recovery of injured microorganisms and in accurately reporting dose-reduction values. These results clearly illustrate that there is no clear indication of injury obtained from X-ray treatment alone (figure 10) or from sanitizer exposure using a quaternary ammonium compound-based sanitizer (figure 13), while treatment with a chlorine-based (figure 11) and peracetic acid-based (figure 12) sanitizer did show signs of injury from the sanitizer treatment.

By evaluating the data with the injured microorganisms in mind through the use of a selective medium with a non-selective overlay, the D_{10} value is significantly reduced compared to the D_{10} value of the selective medium alone. Without a selective medium, the D_{10} value may be over-reported.

11. REFERENCES

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