1-1-2003

Starvation-induced cross-protection of Escherichia coli O157:H7 against electron-beam radiation or ultraviolet radiation in physiological saline and in apple juice

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Starvation-induced cross-protection of *Escherichia coli* O157:H7 against electron-beam radiation or ultraviolet radiation in physiological saline and in apple juice

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
Aubrey Mendonca (Major Professor)
Bonita Glatz
Mark Gleason

Iowa State University
Ames, Iowa
2003

Graduate College

Iowa State University

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 is a foodborne pathogen of major concern to the food industry. Although this organism was first isolated in the United States in 1975 (Griffin and Tauxe, 1991), it was not until 1982 that it was fully recognized as a foodborne pathogen (Wells et al., 1983). *E. coli* O157:H7 infections are commonly caused by eating undercooked ground beef containing the pathogen but cases of illness involving contaminated drinking water ground beef, raw milk and other food products have been reported (CDC, 1993; Smith, 1997; Padhye and Doyle, 1992). This pathogen has a low infectious dose and is fairly resistant to moderate acidity in foods (Doyle et al., 2001). For example, it has been reported to survive in mayonnaise (pH 3.6 to 3.9) (Zhao and Doyle, 1994) and non-pasteurized apple cider (pH 3.6 to 4.0) for extended periods (Zhao et al., 1993). Several outbreaks associated with fruit juices, yogurt, fermented dry salami, and non-pasteurized apple cider have highlighted the health risk posed by this organism in low-pH food products (Leyer et al., 1995).

The destruction of human enteric pathogens in low-pH foods such as fruit juices can be achieved by heat pasteurization. In fact, the vast majority of fruit juices sold in the United States are pasteurized using heat (Splittstoesser et al., 1996). Although apple juice is usually pasteurized to destroy undesirable organisms, apple cider producers have depended on the cider's natural acidity, refrigeration, and addition of chemical preservatives for ensuring the microbial safety of this product (Miller and Kaspar, 1994; Zhao et al., 1993). Heat pasteurization causes loss of essential oils and certain heat-labile vitamins, and alters the flavor of the juice. Customers value non-heated fruit juices for their desirable flavor and aroma; however, these products have been implicated

in foodborne disease outbreaks involving *Salmonella* and *E. coli* O157:H7. In response to foodborne disease outbreaks implicating fruit juices, the Food and Drug Administration (FDA) required processors of non-heat pasteurized fruit juices to adapt their process to give 5-log (99.999%) reductions of human pathogens in the final product.

Technologies such as high hydrostatic pressure, pulsed electric fields, and those that involve ionizing or non-ionizing radiation seem to have good potential for eliminating human pathogens in juices without heating the product to cause undesirable changes (Wright et al., 2000). Most of the research conducted on irradiation of fruit juices has focused on reduction of spoilage microorganisms such as yeast and molds. More recently, studies have been conducted on the use of radiation to destroy pathogens in juices (Buchanan et al., 1998). Results of recently published research indicated that ionizing radiation (gamma or electron beam) or non-ionizing radiation such as ultraviolet light may offer an alternative way of achieving 5-log reduction in pathogen populations in fruit juices (Buchanan et al., 1998; Wright et al., 2000). Radiation resistance (D-values) for pathogens such as *Salmonella* and *E. coli O157:H7* in fruit juices have been reported. However, food processors should be cautious in fully adopting these D-values. The data are likely to underestimate the radiation resistance of pathogens unless the most resistant state of the pathogens was tested in the studies. For example, Buchanan et al. (1998) demonstrated that prior acid adaptation of three strains of *E. coli* O157:H7 increased their radiation resistance in apple juice by 54 to 67%.

In the natural environment and often in the food processing environment, foodborne pathogens are exposed to a variety of stressful conditions including drying, starvation, acid or alkaline pH, osmotic shock, and heat shock. Stressed foodborne pathogens are a major problem to the food industry because these organisms may become stress-adapted and acquire increased resistance to food preservation processes (Rowe and

Kirk, 1999). This problem is further exacerbated when pathogens endure nutrient starvation because this type of stress elicits a strong increase in microbial resistance to various physical or chemical challenges (Rowe and Kirk, 2000). The main objective of this study was to evaluate the effects of starvation in *E. coli* O157:H7 on the resistance of this pathogen against ionizing (electron-beam) and non-ionizing (ultraviolet) radiation. Results of this study should assist food processors in designing radiation processes with an extra margin of safety fro inactivating vegetative foodborne pathogens.

Thesis Organization

This thesis consists of two papers to be submitted to the Journal of Food Protection. Each paper has its own abstract, introduction, materials and methods, results, discussion, and references cited. The general introduction is in front of the first paper and the general conclusions follow the second paper. References cited in the general introduction may be found after the literature review.

Literature Review

E. coli is a member of the family Enterobacteriaceae, a group of Gram-negative, rod-shaped, facultative anaerobic microflora that are commonly found in the intestinal tracts of humans and warm-blooded animals (Doyle et al., 2001). Although most *E. coli* strains are harmless commensals, some strains are pathogenic and cause diarrheal disease. *E. coli* strains that cause diarrheal illness are categorized into specific groups based on virulence properties, mechanisms of pathogenicity, clinical syndromes, and distinct O:H serogoups. These categories include: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAggEC), and enterohemorrhagic *E. coli* (EHEC) (Doyle et al.,

2001).

Pathogenic *E. coli*

The EPEC cause diarrhea. The general characteristics of EPEC do not include the production of heat-labile enterotoxin, heat-stable enterotoxin, or *Shigella*-like invasiveness (Doyle et al., 2001). However, they induce the attaching and effacing (AE) lesions in cells to which they bind. These organisms cause symptoms by invading the epithelial cells and destroying the microvilli (Donnenberg et al., 1989). Humans are an important reservoir. The symptoms caused by EPEC include severe diarrhea, vomiting, fever, and abdominal pain. Ill adults usually have watery diarrhea with large amounts of mucus lasting up to three days (Bell, 1998).

The ETEC are a major causative agent of infantile diarrhea in developing countries. They are also the agents most frequently responsible for traveler's diarrhea with symptoms including watery diarrhea, vomiting, chills, headache, and rarely, fever. The ETEC colonize the proximal small intestine by fimbrial colonization factors (e.g., CFA/I and CFA/II) and ETEC symptoms are induced by the action of heat-stable (ST) and/or heat-labile (LT) enterotoxins. These toxins cause fluid accumulation and a diarrheal response (Doyle et al., 2001). Humans are the principal reservoir of ETEC that cause human illness.

The EIEC causes nonbloody diarrhea and dysentery, which are similar to symptoms caused by *Shigella* spp.. These organisms invade and multiply within colonic epithelial cells; however, they do not produce Shiga toxin (Salyers and Whitt, 1994). Humans are a major reservoir. The colon is the major site of bacterial localization, where EIEC invade and proliferate in epithelial cells, and cause cell death (Doyle et al., 2001).

The DAEC isolates have been associated with mild diarrhea without blood or

fecal leukocytes and identified by a characteristic diffuse-adherent pattern of attachment to epithelial cells. These bacteria cover the cell surface uniformly. Primarily this strains cause diarrhea in young children who are older than infants. However, the reason for this age-related infection is not known. The DAEC usually do not prduce heat-labile or heat-stable toxins or elevated levels of shiga toxins, nor do they possess EPEC adherence factor plasmids or invade epithelial cells (Doyle et al., 2001).

The EAggEC are known for persistent diarrhea in infants and children in several countries (Nataro and Kaper, 1998). These *E. coli* strains have a uniquely different manner of adhering to the mucosal surface. The EAggEC adhere as small aggregates rather than uniformly. Then they produce a ST-like toxin and a hemolysin (Salyers and Whitt, 1994). However, more epidemiologic information on EAggEC as an agent of diarrheal disease is needed.

Enterohemorrhagic *E. coli* and Serotype O157:H7

Characteristics

Highly virulent strains of enterohemorrhagic *Escherichia coli* O157:H7 were first identified in the United States in 1982, when they were associated with food-related diarrheal outbreaks (Doyle, 1991). Most *E. coli* O157:H7 strains have several different characteristics from most other *E. coli*. For example, *E. coli* O157:H7 cannot grow well at temperatures above 44.5 °C, ferment sorbitol within 24 h, or produce ß- glucuronidase (measured as ability to hydrolyze 4-methy-umbelliferyl-D-glucuronide (MUG)). Also this pathogen possesses an attaching and effacing (*eae*) gene and uncommon 5,000 to 8,000-molecular-weight outer membrane protein (OMP). Unlike most foodborne pathogens, *E. coli* O157:H7 is uniquely tolerant to acidic environments (Doyle et al., 2001). When high levels of *E. coli* O157:H7 were added to certain food products, they

survived in mayonnaise (pH 3.6 to 3.9) for 5 to 7 weeks at 5 °C and for 1 to 3 weeks at 20 °C (Zhao and Doyle, 1994). They also survived in apple cider (pH 3.6 to 4.0) for 10 to 31 days or 2 to 3 days at 8 °C or 25 °C, respectively (Zhao et al., 1993). In addition, *E. coli* O157:H7 can develop increased resistance to antibiotics but no unusual resistance to heat (Doyle et al., 2001).

Disease

E. coli O157:H7 is a known cause of food poisoning that can result in severe complications such as hemorrhagic colitis, hemolytic uremic syndrome (HUS), or thrombotic thrombocytopenic purpura (TTP) (Doyle, 1991). Particularly, Shiga-like toxins produced by enterohemorrhagic strains of *E. coli* during infections can cause death in young children and the elderly (Griffin and Tauxe, 1991).

Symptoms of hemorrhagic colitis begin with a sudden onset of severe abdominal pain followed within 1 to 2 days by watery diarrhea which progressively becomes bloody diarrhea that lasts for 4 to 10 days. Typically there is little of no fever. The HUS is a leading cause of acute renal failure in children and usually begins with the production of bloody diarrhea. Patients usually have a triad of features: acute nephropathy (kidney disorder), microangiopathic hemolytic anemia (intravascular coagulation of erythrocytes that results in mechanical damage of erythrocytes as they go through abnormally narrow channels), and thrombocytopenia (low circulating platelets). Persons with HUS often require dialysis and blood transfusions, and may develop central nervous system disease characterized by frequent seizures and coma. The TTP affects adults and has very similar clinical and pathogenic characteristics to HUS. Distinct neurological abnormalities are caused by blood clots in the brain which can result in death. Fortunately, TTP is a rare syndrome of *E. coli* O157:H7 infection (Doyle, 1991; Doyle et al., 2001).

Outbreaks

Recent outbreaks of *E. coli* O157:H7 foodborne disease have been associated with consumption of contaminated high-acid foods, including apple cider and fermented dry salami. An early outbreak of hemorrhagic uremic syndrome associated with apple juice or cider occurred in Canada before the recognition of *E. coli* O157:H7 as a foodborne pathogen; however, the responsible agent was not identified, possibly due to delays between sampling and analysis (Steele et al., 1982). Outbreaks of hemorrhagic uremic syndrome in Massachusetts in 1991 (Besser et al., 1993) and Connecticut in 1996 (CDC, 1997) were attributed to *E. coli* O157:H7 in apple cider. In a 1996 outbreak in the Pacific Northwest, Odwalla brand unpasteurized apple juice and juice mixtures contaminated with *E. coli* O157:H7 were implicated. This prompted a nationwide recall of Odwalla products (CDC, 1996). Since apple cider or juice is known as a novel vehicle for outbreaks of *E. coli* O157:H7 infections, much attention has been focused on this problem (Wright et al., 2000). Since more than 90% of the apples used in cider are collected from the ground (dropped apples), they are likely to be contaminated with microorganisms from the soil or from fecal material of animals. Apples are sometimes pressed without washing and the cider might not be pasteurized, or contain preservatives. Prior to outbreaks involving apple cider this product was considered safe from pathogens because of its high acidity and low pH (<4.0). Inoculation studies revealed that *E. coli* O157:H7 is acid-tolerant and able to survive in apple cider for 20 days at 8°C (Zhao et al., 1993). Since the infectious dose of *E. coli* O157:H7 is low, ingestion of even a small number of surviving *E. coli* O157:H7 could cause an illness. Severe symptoms combined with its apparent low infectious dose (<100 cells) qualify *E. coli* O157:H7 to be among the most serious of known foodborne pathogens (Doyle et al., 2001).

Food Irradiation.

The National Advisory Committee on Microbiological Criteria for Foods (1997) recommended that production of fruit juices should include treatments capable of producing a cumulative 5-log-unit reduction in the levels of *E. coli* O157:H7 (Buchanan et al., 1998). In the apple juice industry, the target level of enterohemorrhagic *E. coli* inactivation has been already achieved by thermal pasteurization; however, the high cost of thermal pasteurization equipment and unique flavor changes from the mild heat processing of pasteurization make this process unattractive and unfeasible for both producers and consumers of fresh apple juice (Buchanan et al., 1998; Duffy et al., 2000). Recently, research in food microbiology has focused on nonthermal processing alternatives for juice and ready-to-eat foods. Treatment with low-dose electron beam irradiation at refrigerated temperatures should not alter the organoleptic characteristics of the product and could be an attractive alternative technology for reducing enterohemorrhagic *E. coli* (Buchanan et al., 1998). Treatment with UV light, already approved by FDA for pathogen reduction in water, is also a promising, lower-cost alternative to pasteurization (Anonymous, 1999).

Ionizing radiation.

Research on the application of ionizing radiation to food began in the early 1950s. Radiation sources approved for food use are gamma rays (produced by the radioisotopes cobalt-60 or cesium-137), machine-generated X-rays (with a maximum energy of 5 million electron volts, MeV), and electrons (with a maximum energy of 10 MeV). Depending on the dose of radiation energy applied, foods may be pasteurized to reduce or eliminate pathogens, or they may be treated to eliminate all microorganisms, except for some viruses (Crawford and Ruff, 1996; IFT, 1983). Low (up to 1 kGy) to medium

doses (1-10 kGy) kill insects and larvae in wheat and wheat flour and destroy pathogenic bacteria and parasites (Olson, 1998). Low to medium doses also prevent sprouting from potatoes and other foods and slow the ripening and spoilage of fruit and vegetables. Higher doses (10-50 kGy) sterilize foods for a variety of uses such as for astronauts during space flight and for immune-compromised patients who must have pathogen-free food (Olson, 1998).

Electron beams possess enough energy to ionize molecules in their paths and can inactivate foodborne microorganisms without increasing the temperature of the irradiated food. The principal effect of radiation on foodborne microorganisms is cellular destruction by disruption of the genetic material in the cells (Olson, 1998). Even though an extremely small percentage of chemical bonds are broken down during the irradiation, the effect can be dramatic. For example, if the bonds in the deoxyribose nucleic acid (DNA) are broken, the cell loses its ability to replicate. Therefore, a relatively small change in the DNA of a bacterial cell can destroy the cell (Olson, 1998). The cellular destruction caused by disruption of the genetic material in a living cell is the principal effect of radiation on foodborne microorganisms (Murano, 1995).

When molecules absorb ionizing energy, they become reactive and form ions or free radicals that react to form stable radiolytic products (Woods and Pikaev, 1994). However, ionizing radiation at approved doses for foods cannot make food radioactive. The radiolytic products that form when food is irradiated are generally the same as those that are formed when food is cooked (Crawford and Ruff, 1996).

Factors affecting microbial inactivation by irradiation

Inactivation of foodborne microorganisms by ionizing radiation is affected by several factors. These factors include irradiation dose, numbers and types of

microorganisms, food composition and preservation methods, temperature, and atmospheric gas composition (Mendonca, 2002).

Irradiation dose

Usually higher doses of ionizing radiation result in greater damage to microorganisms. However, due to the lower rate of oxidizing reactions that produce free radicals and toxic oxygen derivatives makes microbial destruction at a given irradiation dose less under anaerobic or dry conditions (Mendonca, 2002).

Numbers and types of microorganisms

As observed with heating and other food preservation processes, the numbers of microorganisms affects the efficacy of irradiation. For example, high populations of microorganisms can reduce the antimicrobial effectiveness of a given dose of radiation. With regard to types of microorganisms, viruses have the highest radiation resistance followed by bacterial spores. Bacterial spores are more resistant than vegetative cells of bacteria, which are more resistant than yeast and molds (Satin, 1993). Generally, more complex life forms are more sensitive to irradiation than simpler life forms. Among bacteria, gram-negative bacteria are more sensitive to irradiation than gram-positives (Thayer et al., 1993).

Food composition and preservation methods

The composition of food, including liquid or solids content, protein content, and thickness, influence the inactivation of microorganisms by irradiation. Solid foods offer greater protective effect to microorganisms against irradiation than liquid foods, phosphate buffer or other liquid media. Similarly, increasing amounts of protein in foods tend to provide a protective effect. Proteins and other food components, including natural antioxidants such as vitamin C and vitamin E, compete for free radicals formed from activated molecules and the radiolysis of water (Diehl, 1995). This competition for free

radicals minimizes the antimicrobial effect of irradiation. High hydrostatic pressure, heating, acidification, and certain chemical food preservatives increase the sensitivity of microorganisms to ionizing radiation by reducing the population of survivors. Alternatively, drying increases the radiation resistance of microorganisms. Increased microbial resistance to irradiation in dried foods is due to low water activity, which restricts the production of free radicals during the irradiation process (Thayer et al., 1995).

Temperature

Temperature is a major factor that affects the survival of microorganisms during irradiation. Studies on the effect of temperature on radiation inactivation of microorganisms have demonstrated that microbial sensitivity to radiation decreases with at colder temperatures. For example, radiation D-values for *E. coli* O157:H7 in mechanically deboned chicken meat were 0.28 kGy and 0.44 kGy at 5 °C and –5 °C, respectively (Thayer and Boyd, 1993). *E. coli* O157:H7 exhibited significantly higher radiation D-values in ground beef patties at –15 °C compared to 5 °C (Lopez-Gonzalez et al., 1999). Subfreezing temperatures in food produce conditions of low water activity, which increase irradiation resistance of microorganisms. Free radical production from the radiolysis of water is decreased as reaction rates are lowered at subfreezing temperature (Diehl, 1995). Also, the frozen state of food prevents free radicals from migrating to other areas beyond sites of free radical production (Taub et al., 1979). Free radicals such as the hydroxyl radical (OH·) and the hydrogen radical (·H), were associated with about 85% of the potential damage in irradiated *E. coli* (Billen, 1987).

Gaseous environment

The gaseous composition of the atmosphere to which microorganisms are exposed impacts their destruction by irradiation under certain conditions. Generally, microorganisms exhibit increased sensitivity to irradiation in the presence of oxygen (Epp

and Weiss, 1968; Samuni and Czapski, 1978). In contrast, some investigators have reported no significant differences in total bacterial counts or *E. coli* O157:H7 following electron beam irradiation of ground beef in air or under vacuum (Lopez-Gonzalez et al., 1999; Fu et al., 1995). These inconsistencies may be attributed to several factors. These include variations in methods used by researchers for recovering microbial survivors and variations in food composition, which can confer different amounts of protection to microorganisms during irradiation (Diehl, 1995).

Antimicrobial mechanism of action of ionizing radiation

During irradiation microorganisms are exposed to high energy rays, which collide with components of the microbial cells. These collisions produce changes in atoms and molecules. Changes in atoms occur when the energy from collisions is enough to remove an electron from the atomic orbit to produce ion pairs. Changes at the molecular level occur when the energy from collisions is sufficient to break chemical bonds between atoms to produce free radicals. Free radicals are very unstable because of their unpaired electrons; therefore, they tend to react with each other and with cellular molecules by pairing of their odd electrons. Ion pairs, free radicals, recombination of free radicals and reaction products from interaction of free radicals with cellular molecules, are involved in the antimicrobial mechanism of action of ionizing radiation (Mendonca, 2002).

The antimicrobial mechanism of action of ionizing radiation involves both direct and indirect effects of high-energy rays and particles on cellular components such as the cytoplasmic membrane and the DNA (Ingram and Roberts, 1980). The direct effect involves displacement of electrons from DNA to cause damage to this vital genetic material. Damage to the DNA could result in death of the microbial cell. Death of the cell could also result from an indirect action of irradiation that involves the radiolysis of

water in the cell or outside of the cell. The radiolysis of water results in the production of highly reactive hydrogen and hydroxyl radicals. These radicals can attack cellular components, including DNA, and break carbon-to-carbon bonds. Damage to DNA could involve single strand and/or double strand breaks in this important macromolecule. The inability of microorganisms to repair these lesions prevents them from replicating the DNA. The inability of the cells to replicate DNA and multiply results in death of the cell.

UV irradiation (non-ionizing)

Ultraviolet (UV) treatment is effective in destroying a wide variety of microorganisms and has been approved by the Food and Drug Administration for sterilization of water (Wright et al., 2000). UV light is useful for decontaminating surfaces (Sizer and Balasubramaniam, 1999). This form of radiation has been used on beef (Stermer et al., 1987), fish (Huang and Toledo, 1982) and poultry (Wallner-Pendleton et al., 1994) to control bacteria and increase shelf life with little effect on food quality. However, the poor penetration capacity of UV radiation and shadowing effects limit its widespread application in foods (Sizer and Balasubramaniam, 1999).

Because of the poor penetration capacity of UV radiation, its application has generally been limited to surface disinfection (Sizer and Balasubramaniam, 1999). However, it is useful for destroying microorganisms on the surface or near the surface of clear liquids, or within the clear liquid if an extremely thin film is irradiated within the wavelength range of 250 to 260 nm (Morgan, 1989). Typical penetration of UV radiation in juices is approximately 1 mm for 90% absorption of the light (Sizer and Balasubramaniam, 1999). This technology has been used successfully to increase the shelf life of refrigerated cider without affecting flavor (USDA, 1977).

The principal mechanism by which UV light exerts its lethal effect is through the formation of pyrimidine dimers in the DNA thus inhibiting DNA replication and cell reproduction (Perry et al., 2002). Exposure to 340 nm UV light also has a killing effect on microorganisms, although DNA does not strongly absorb light of this wavelength. This indicates that UV radiation may have other mechanisms of killing microorganisms (Altas, 1995).

Microbial repair of DNA damage caused by UV radiation

Bacteria have a number of repair systems that can be used to repair damaged DNA. Some of these repair systems are known to function in correcting UV-induced damage to DNA. One such repair system is the photo-reactivation process. This process in involved in the repair of adjacent pyrimidines that become cross-linked from the action of UV radiation. These pyrimidine dimers are recognized by the enzyme photolyase, which, in the presence of visible light, breaks the bond between the pyrimidine dimers to restore their original structure (Perry et al., 2002).

When mis-pairing of pyrimidines is extensive, these lesions can be repaired by an excision repair process. In excision repair, a multi-subunit endonuclease in *E. coli* hydrolyzes the phosphate backbone of DNA at two points on opposite sides of the damaged site. Enzymatic action of DNA helicase then causes the release of a 12-base oligonucleotide, which contains the damaged bases. The gap in the DNA created by the action of DNA helicase is filled in by DNA polymerase I, and the phosphate backbone is closed by DNA ligase (Perry et al., 2002; Prescott et al., 2002).

Another way by which the microbial cell removes pyrimidine dimers is recombination repair. In this repair process, DNA polymerase III, upon encountering a thymine dimer, would skip over it and continue synthesis of DNA on the other side of the

dimer. This action leaves a gap in the newly synthesized DNA. The opposite strand in the DNA undergoes normal replication. The gap formed around the damaged site is filled by excision of the corresponding fragment from the second strand. Then the gap is filled by a process analogous to recombination between the two related DNA molecules (Perry et al., 2002).

Stress Adaptation

The phenomenon of stress hardening or cross-protection, which refers to the increased microbial resistance to lethal factors after adaptation to environmental stresses, is an active area of research involving foodborne pathogens (Lou and Yousef, 1997). Bacteria such as *Salmonella typhimurium* (Rowe and Kirk, 1999), *Listeria monocytogenes* (Lou and Yousef, 1997), and *E. coli* O157:H7 (Buchanan et al., 1999), once subjected to a non-lethal stress become adapted and subsequently exhibit more resistance when the stress is reapplied (Rowe and Kirk, 1999). For example, it has been reported that *L. monocytogenes* developed thermotolerance following exposure to sublethal heating (Stephens et al., 1994). Acid-adapted *E. coli* developed increased resistance to weak organic acids (propionic, lactic, acetic, benzoic, sorbic, and trans-cinnamic) compared with non-adapted cell (Goodson and Rowbury, 1989). In the natural environment, many bacteria often confront limiting amounts of nutrients and, therefore, sustain only sporadic growth (Hengge-Aronis, 1993). Food-borne pathogens are also commonly stressed during food processing (Lou and Yousef, 1997).

There is a growing body of knowledge on the effects of stress-hardening on microbial resistance to heat (Stephens et al., 1994; Lihono et al., 2003). However, there is a scarcity of published information on the extent to which certain environmental stresses, including starvation, can increase the resistance of *E. coli* O157:H7 and other

foodborne pathogens to ionizing or non-ionizing radiation. The deficiency in the data cannot easily be corrected by extrapolating results generated with nonpathogenic strains of *E. coli* (Jenkins et al., 1990; Jenkins et al., 1988), because the organisms exhibit considerable strain variability, even within the O157:H7 serotype itself (Rowe and Kirk, 2000).

Starvation

Starvation-survival in bacteria is recognized as a physiological state, which results from inadequate amounts of nutrients (particularly energy) for growth and multiplication of the organism (Morita, 1982). Unlike gram-positive bacteria gram-negative bacteria such as *E. coli* generally do not form spores. However, when *E. coli* strains go into starvation they experience morphological changes in combination with changes in metabolism and physiology. Cells become much smaller and develop an almost spherical shape. Within the cell, the relative volume and disposition of the subcellular compartments changes, the cytoplasm is condensed and the volume of the periplasm is increased (Kolter et al., 1993). The cell envelope in the starvation phase undergoes some changes reflecting the need for protection and insulation from stressful environments. On their surfaces, starved cells are covered with more hydrophobic molecules that favor adhesion and aggregation. Membranes may become less fluid and less permeable as fatty acid composition changes. Also, the cell wall undergoes structural changes to increase resistance to autolysis (Kolter et al., 1993).

Among these starvation-induced physiological changes is the production of stress proteins such as heat shock proteins (Hsp), which are correlated with heat resistance in *E. coli*. The Hsp such as DnaK, GroL, HptG and other molecular chaperones may help denatured or damaged macromolecules to regain their native state. These chaperones

may be involved in protecting starved cells from the lethal effect of food preservation processes (Sanchez et al., 1992). Cheville et al. (1996) reported that *E. coli* O157:H7 in stationary growth phase or in starved conditions induced the synthesis of protective proteins coded for by the rpoS gene, which conferred resistance to a range of chemical and physical challenges. This phenomenon of cross-protection in *E. coli* O157:H7 is a major concern because its low infective dose, perhaps less than 50 organisms, could constitute an unacceptable risk (Rowe and Kirk, 1999). Stress-hardened pathogens may also have increased virulence, since pathogens may sense environmental stresses as signals for the expression of virulence factors (Lou and Yousef, 1997). Starvation should be considered as a stress that can induce cross-protection. This type of stress is relevant to the food industry because water, used to clean of rinse food contact surfaces or even incorporated directly into food is generally of a low nutrient status (Rowe and Kirk, 2000).

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CHAPTER 2. EFFECT OF STARVATION ON SURVIVAL AND INJURY OF *ESCHERICHIA COLI* O157:H7 FOLLOWING ELECTRON-BEAM IRRADIATION IN 0.85% SALINE AND IN APPLE JUICE

A paper to be submitted to the Journal of Food Protection

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ABSTRACT

This study evaluated the effect of starvation on the resistance of starved *Escherichia coli* O157:H7 to electron beam irradiation in 0.85% (w/v) saline and in apple juice. Exponential-phase cells of *E. coli* O157:H7, grown at 35°C in tryptic soy broth (TSB), were sedimented by centrifugation (10,000 x g, 10 min, 4°C), washed, then starved in 0.85% saline (25°C) for 10 days. Exponential- or stationary-phase cells grown in TSB at 35°C served as controls. Samples of 0.85% saline or pasteurized apple juice, inoculated with control cells or cells starved for 8 days, were exposed to electron beam irradiation at doses ranging from 0.0 to 0.7 kGy. *E. coli* survivors were enumerated by plating diluted samples on tryptic soy agar (TSA) or on Sorbitol McConkey agar (SMA) and counting bacterial colonies on agar plates after incubation (35°C) for 24 h. Starved cells consistently exhibited higher irradiation D-values than controls ($\alpha<0.05$). The D-values for control and starved *E. coli* O157:H7 in 0.85% saline were 0.11 and 0.26 kGy, respectively; D values in apple juice were 0.16, 0.19, and 0.33 kGy for exponential, stationary, and starved cells, respectively. Irradiation (0.7kGy) of *E. coli* O157:H7 in apple juice reduced numbers of exponential- and stationary-phase cells by ~ 4.32 and 3.74 log, respectively; starved cells were reduced by only 2.20 log. The results of this study indicate that starvation-induced stress cross-

protects *E. coli* O157:H7 from the irradiation treatment and it should be considered as an important factor when determining irradiation D- values for this pathogen.

INTRODUCTION

Since 1982 food-related diarrheal outbreaks associated with highly virulent strains of enterohemorrhagic *Escherichia coli* O157:H7 have been investigated in the United States (11). *E. coli* O157:H7 is a known cause of food poisoning that can result in severe complications such as hemorrhagic colitis, hemolytic uremic syndrome (HUS), or thrombotic thrombocytopenic purpura (TTP) (5). Particularly, Shiga-like toxins produced by enterohemorrhagic strains of *E. coli* during infections can cause death in young children and the elderly (11). Several outbreaks of hemorrhagic colitis and hemolytic uremic syndrome have been linked to *E. coli* O157:H7 associated with fresh-pressed apple cider and juice (2, 23) as well as ground beef and raw milk (3, 11).

The National Advisory Committee on Microbiological Criteria for Foods (15) recommended that production of fruit juices should include treatments capable of producing a cumulative 5-log-unit reduction in the levels of *E. coli* O157:H7 (3). In the apple juice industry, the target level of enterohemorrhagic *E. coli* inactivation has been already achieved by thermal pasteurization; however, producers and consumers of fresh apple juices are convinced that even relatively mild heat processing alters the unique flavor notes of the unpasteurized product (3). Therefore, there is a need for a non-thermal process to destroy *E. coli* O157:H7 in apple juice. Treatment with low-dose irradiation at refrigerated temperatures could be an attractive alternative technology for reducing *E. coli* O157:H7 (3).

Electron beam radiation ionizes molecules in its path and can inactivate foodborne microorganisms without increasing the temperature of the food (14). The

antimictobial action of radiation involves disruption of deoxyribonucleic acid (DNA) by causing single and/or double strand breaks. The inability to repair the damaged DNA results in cell death (16). The antimicrobial effectiveness of irradiation can be affected by factors such as temperature, radiation dose, food composition, and gaseous atmosphere (Mendonca, 2002). Also, changes in the physiological state of microorganisms due to prior stress may increase their resistance to irradiation. For example, Buchanan et al. (3) demonstrated that induced acid resistance in *E. coli* O157:H7 cross-protected this pathogen against gamma radiation in clarified apple juice.

In natural environments, microorganisms often endure a variety of stresses including intermittent periods of starvation (6). Cheville et al. (4) reported that *E. coli* O157:H7 in stationary growth phase or in starved conditions induced the synthesis of protective proteins coded for by the *rpoS* gene. Protective proteins are believed to be associated with cellular resistance to a range of chemical and physical challenges (20). Such increased microbial resistance to chemical and physical challenges can decrease the lethal effect of food preservation processes including acidification, heating and irradiation. Starvation-induced stress in foodborne pathogens is relevant to the food industry because water, used to clean or rinse food contact surfaces or even added directly to food, is generally of a low nutrient status (21). Also, pathogens may survive in water (without nutrients) on food contact surfaces long enough to induce the stress response, which may cross-protect them from subsequent food preservation methods (21).

There is a lack of published research on the extent to which starvation can induce cross-protection in *E. coli* O157:H7 against ionizing radiation. Therefore, the objective of this study was to determine the effect of starvation on radiation resistance and injury of *E. coli* O157:H7 following electron-beam irradiation in 0.85% saline and in apple juice.

MATERIALS AND METHODS

Microorganisms and culture conditions. Four strains of *E. coli* O157:H7 (ATCC43895, ATCC43894, FRIK 125, and C467) were used in the study. Strains ATCC43895, ATCC43894, and C467 were obtained from the culture collection of the Microbial Food Safety Laboratory of Iowa State University. Strain FRIK 125, an isolate from an apple cider outbreak, was provided by Dr. Charles Kaspar, University of Wisconsin. Stock cultures were stored at -70°C in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) supplemented with 10% glycerol until used. Each stock culture was transferred at least twice in 10 ml tryptic soy broth (TSB) (Difco) and incubated overnight at 35°C prior to each experiment.

Starvation of *E. coli* O157:H7. A portion (1.0 ml) of *E. coli* O157:H7 culture was transferred to TSB (100 ml) in a screw-capped 250-ml Erlenmeyer flask. The inoculated medium was incubated at 35°C with shaking at 150 rpm in a gyrorotary shaker incubator (New Brunswick Scientific Co. Inc., Edison, N.J.). Growth was monitored with a spectrophotometer (Spectronic 1201, Milton Roy Co., Rochester, NY) via optical density (OD) measurements at 600 nm. When *E. coli* O157:H7 grew to an optical density of 0.1 to 0.15, exponential phase cells were harvested by centrifugation (10,000 x g, 10 min, 4°C) and washed once in 0.85% (w/v) NaCl. The cell pellets were suspended in fresh saline to give a final cell concentration of approximately $2.0X10^7$ CFU/ml. The cells were starved by holding the cell suspension statically in a screw-capped 250-ml flask at 25°C for 10 days.

Determination of cell viability and radiation resistance. During starvation, samples of *E. coli* O157:H7 cell suspension were removed for determining cell viability and resistance to electron beam irradiation. Serial dilutions of the cell suspension were prepared in 0.1% peptone water and 0.1-ml aliquots of appropriate dilutions were surface

plated on TSA and Sorbitol MacConkey agar (SMA) (Difco). All inoculated agar plates were incubated aerobically at 35°C and bacterial colonies were counted at 24 h. Samples (10-ml) of cell suspension were placed into sterile screw capped test tubes and held in crushed ice before treatment with electron beam irradiation as described in the section on irradiation and dosimetry. Numbers of survivors in irradiated samples were determined as previously described for determination of cell viability.

Preparation and inoculation of test samples. Pasteurized, clarified apple juice was purchased from a local supermarket in Ames, Iowa. The apple juice was stored in a laboratory refrigerator (4°C) before inoculation. The level of background microflora in the apple juice was determined by surface-plating serial dilutions (0.1% peptone) on TSA. Washed exponential-phase cells of *E. coli* O157:H7 in saline were prepared from a TSB culture as previously described. Stationary-phase cells from an overnight (18 h) culture in TSB (35°C) were harvested by centrifugation (Sorvall® Super T21, Sorvall Product, L.P., Newtown, CT) at 10,000 x g for 10 min at 4°C, washed once in saline to removal residual growth medium, then suspended in fresh saline (4°C). Cells starved for 8 days in saline (25°C) were harvested by centrifugation, washed and suspended in fresh saline (4°C). Suspensions of starved or non-starved cells were adjusted to give approximately 10^8 CFU per ml. Tubes of sterile saline or apple juice (10 ml per tube) were each inoculated with 0.1 ml of *E. coli* O157:H7 cells (exponential, stationary or starved) to give approximately 10^6 CFU per ml. Screw-capped tubes of inoculated samples were held in a 50:50 ice/water mixture prior to irradiation.

Irradiation treatment and dosimetry. Inoculated tubes of saline or apple juice were irradiated at the Iowa State University Linear Accelerator Facility, which has a MeV CIRCE III Linear Electron Accelerator (MeV Industrie S.A., Jouy-en-Josas, France). All samples were irradiated in duplicate at five target doses (0, 0.15, 0.3, 0.5, 0.7 kGy) in

the electron beam mode at an energy level of 10 MeV. Dose rates were 12 kGy/min, 20 kGy/min, and 45kGy/min, for 0.15 and 0.3, 0.5 and 0.7, respectively. Dose rates differed because the linear accelerator had to be operated at a low power to apply the lower doses (0.15 and 0.30 kGy). Each target average dose represents an arithmetic average of doses measured at the top and bottom surfaces of the test samples.

Dosimeter alanine pellets, 5 mm (length) by 5 mm (diameter) (Bruder Analytische Messtechnik, Rheinstetten, Germany), were used to determine the maximum and minimum absorbed radiation doses. Alanine pellets were placed at the top and bottom surfaces of one of the duplicate tubes of saline or apple juice. Immediately after irradiation, the pellets were placed in a Bruker EMS 104 EPR Analyzer to measure absorbed doses by electron paramagnetic resonance. The average absorbed dose was derived from the arithmetic average of the top and bottom surface readings.

Microbiological analysis. All samples were held at 4°C and analyzed within 2 h after irradiation. Serial dilutions of saline or apple juice in 0.1% peptone were prepared and 0.1-ml aliquots of appropriate dilutions were surface-plated, in duplicate, onto plates of TSA (for saline samples) and on both TSA and SMA (for apple juice samples). In instances when increased sensitivity was required, 1.0-ml samples of undiluted saline or apple juice were plated directly onto appropriate agar plates. All inoculated plates were incubated aerobically at 35°C and bacterial colonies were counted at 24 h.

Calculation of D-values. The D-values (radiation dose that produces 90% reduction in viable cells) expressed in kGy, were determined by plotting the $\log_{10}$ number of survivors per ml versus radiation dose (kGy) using Microsoft Excel 98 Software (Microsoft Inc., Redmond, WA). Linear regression analysis (17) was used to determine the line of best fit for the data. The D-value was determined by calculating the negative reciprocal of the slope of the regression curve.

Determination of sublethal injury. For each replicate experiment survivor curves based on recovery of bacterial colonies on TSA and SMA were prepared. The percent sublethal injury for each strain at each radiation dose used (0.0 to 0.70 kGy) was calculated as follows:

$$\frac{\text{(CFU/ml on TSA-CFU/ml on SMA)}}{\text{(CFU/ml on TSA)}} \times 100$$

Statistical analysis. All experiments were replicated three times and results are reported as averages. The D-value data were compared using the Tukey method (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Figure 1 shows the viability of *E. coli* O157:H7 (ATCC 43895, ATCC 43894, FRIK 125, and C467) during starvation in 0.85% saline (25^{O}C) based on enumeration of bacterial colonies on TSA. Initial populations of *E. coli* ATCC 43895, ATCC 43894, and FRIK 125 decreased by about 0.5 log during the first two days of starvation. Initial populations of strain C467 decreased by about 0.3 log by day 4 of starvation. Numbers of culturable cells from all four strains remained relatively constant during days 6 through 10. These observations are consistent with those of Jouper-Jan et al. (7) who reported that after 9 days of multiple (energy and nutrient) starvation, only about 77% of the initial populations of *E. coli* and *Vibrio* spp. were culturable.

Not all cells in a starving bacterial population remain viable based on plate counts. Starved bacteria have been reported to actively transport and metabolize nutrients but fail to multiply and form colonies under standard plating conditions (19). Kurath and Morita (9) studied the physiology of a marine *Pseudomonas* sp. during starvation-survival of this organism. They demonstrated that within the population of

starved cells, numbers of respiring cells [based on active reduction of 2-(p-indophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT)] were 10-fold higher than the viable counts (based on plate counts). This difference between plate counts and INT-reducing counts indicates the presence of a sub-population of non-culturable but respiring cells that is about 1.0 log higher than culturable cells. Therefore, decreases in viability of *E. coli* O157:H7 reported in the present study and that of *Vibrio* spp. reported by Jouper-Jan et al. (7) may not reflect actual death of a part of the initial population. Further research is needed to determine if starvation induces a viable but non-culturable state in *E. coli* O157:H7.

The radiation resistance (D-value) of *E. coli* O157:H7 during starvation in 0.85% saline at 25^{o}C is shown in figure 2. The D-value for each of the four strains at day 0 of starvation was approximately 0.11 kGy. All D-values increased as starvation time increased and reached a maximum at about day 8 of starvation. Maximum D-values for starved strains ATCC 43895, ATCC 43894, C467, and FRIK 125 were 0.35, 0.23, 0.19, and 0.26 kGy, respectively. These results indicate that starvation increases the resistance of *E. coli* O157:H7 to electron beam irradiation. This increased resistance may be attributed to one or more starvation-induced physiological changes in this pathogen.

Physiological changes reported in starved bacteria include decreased membrane fluidity and permeability, decreased overall metabolism, rapid protein turnover, alterations in chromosome topology, and production of stress proteins (8, 12, 13). Among these starvation-induced physiological changes the production of certain stress proteins has been shown to correlate with increased thermal resistance in *E. coli* (13, 22). Stress proteins such as DnaK, GroL, HptG and other molecular chaperones may help denatured or damaged macromolecules to regain their natural conformation (13). These chaperones may also be important in enhancing the survival of starved *E. coli* O157:H7

cells following exposure to ionizing radiation.

Ionizing radiation inactivates microorganisms by inflicting lethal damage to DNA via a direct or indirect effect. The direct effect involves single and/or double strand breaks in DNA from direct deposition of energy into this macromolecule. The indirect effect involves the radiolysis of water in the cell and the cell's environment, producing free radicals and toxic oxygen derivatives that damage DNA (14). The starvation-induced radiation resistance in *E. coli* O157:H7 reported in this study suggests that starvation resulted in physiological changes that either resisted lethal DNA damage during irradiation or improved the pathogen's ability to repair damaged DNA. Further research is needed to determine the mechanism by which starvation increases resistance of *E. coli* O157:H7 to ionizing radiation.

Survivors of a four-strain mixture of *E. coli* O157:H7 (starved and non-starved exponential- and stationary-phase cells) following irradiation in pasteurized apple juice (pH 3.97) are shown in figures 3A and 3B. Populations of survivors following irradiation in apple juice are based on bacterial colony counts on TSA (figure 3A) and SMA (figure 3B). Populations of *E. coli* O157:H7 survivors on TSA were generally higher than those on SMA. Populations of the organism in apple juice decreased with increasing radiation dose irrespective of the plating medium used.

The higher numbers of survivors on TSA compared to SMA suggest that a portion of the surviving population may have been sub-lethally injured by irradiation. Depending on the extent of metabolic injury, injured cells can become sensitive to chemical agents in selective media (18). The colonies that developed on TSA represented both injured and non-injured cells, whereas only non-injured cells formed colonies on SMA. The ability to recover injured pathogens in foods during culturing procedures is important to the food industry because injured pathogens may recover in

foods and regain their pathogenicity (18). Therefore, in the present study, the use of TSA counts for estimating *E. coli* O157:H7 survivors is justified considering the fact that injured pathogens could potentially have the same health impact as non-injured ones. Based on TSA counts, 0.7 kGy reduced initial populations of exponential- and stationary-phase (control) cells by 4.32 and 3.74 log cycles, respectively whereas, starved cells were reduced by only 2.2 log cycles (Figure 3A). These results indicate the starved cells have a greater ability to survive the lethal effects of ionizing radiation compared to exponential- or stationary-phase cells.

The radiation resistance (D-value) for each of the four *E. coli* O157:H7 strains (exponential-phase or starved) in 0.85% saline and for the four-strain mixture (exponential-phase, stationary-phase, or starved) in apple juice is shown in Table 1. D-values for each of the four strains and the four-strain mixture were significantly higher in starved cells than in control (exponential- or stationary-phase) cells ($\alpha<0.05$). It has long been recognized that stationary-phase cells are generally more resistant to ionizing radiation than exponential-phase cells. Previous studies have demonstrated that *E. coli* O157:H7 cells in the stationary growth phase, and also those that endure starvation, synthesize specific sets of stress proteins. These stress proteins increase the cells' resistance to a range of chemical and physical challenges (1, 11).

The synthesis of a substantial amount of the stress proteins in starved or stationary-phase cells is controlled by the *rpoS* gene (4). By using an *rpoS* mutant, Rowe and Kirk (21) were able to demonstrate a central role for the *rpoS* gene in the induction of heat resistance following starvation stress in *E. coli* O157:H7. Even though the *rpoS* gene mediates the synthesis of protective proteins in stationary-phase and starved *E. coli* O157:H7, it is surprising that the starved cultures in the present study were much more resistant to radiation in apple juice than the stationary-phase cultures (Table

1). Further research is needed to fully explain the relatively high radiation resistance of starved *E. coli* O157:H7.

Figure 4 shows the levels of sub-lethal radiation injury in surviving populations of *E. coli* O157:H7 at each absorbed dose. Injury of exponential-phase cells were consistently higher than that of stationary-phase or starved cells at all doses. However, only at the higher doses (0.5 and 0.7 kGy) was the extent of this injury significantly greater that that of starved or stationary-phase cells ($\alpha<0.05$). In this study, sub-lethally injured cells represent cells which, at the time of analysis, were unable to form colonies on SMA. However, they were able to form colonies on TSA. These cells evidently endured severe damage. Severely injured cells require more intricate repair mechanisms which may need special environmental conditions to become fully functional. Stationary-phase cells or starved cells might have endured less sub-lethal injury or developed very efficient mechanism for repair of their lesions.

The starvation of *E. coli* O157:H7 in 0.85% saline was an attempt to simulate the survival of this pathogen in water (devoid of nutrients) on food contact surfaces for an extended period of time, sufficient to stimulate the stress response. The use of non-stressed pathogens in food safety experiments is likely to underestimate the full ability of these organisms to survive food preservation methods. Therefore, it would be practical to use D-values of pathogens in their most radiation-resistant state as a basis for establishing adequate food irradiation processes.

To our knowledge, the present study and that of Buchanan et al. (3) are the only ones that address the effects of certain environmental stress on the radiation resistance of *E. coli* O157:H7. The present study provides evidence that starvation in *E. coli* O157:H7 dramatically increases the resistance of this pathogen to electron beam irradiation. The development of irradiation processes for inactivating *E. coli* O157:H7

without altering the desirable sensory attributes of food products depends on accurate assessment of the organism's radiation resistance (D-value). Even small differences in D-values can have a significant effect on the antimicrobial efficacy of radiation processes because of the exponential nature of microbial destruction by irradiation. Further research on the radiation resistance of other stressed pathogens is needed to provide food processors with more realistic microbial resistance data. The acquisition of such data would permit the design of adequate processes that ensure the safety of irradiated foods.

ACKNOWLEDGMENTS

The authors thank Dr. Charles Kaspar, University of Wisconsin, Madison, for supplying one of the bacterial strains (FRIK 125) used in this experiment. The authors also thank Meijun Zhu for statistical assistance and Michael Holtzbauer, irradiator operator, for technical assistance. This project was supported by the USDA/CSREES National Research Initiative Competitive Grants Programs, Project No. 98-35502-6604.

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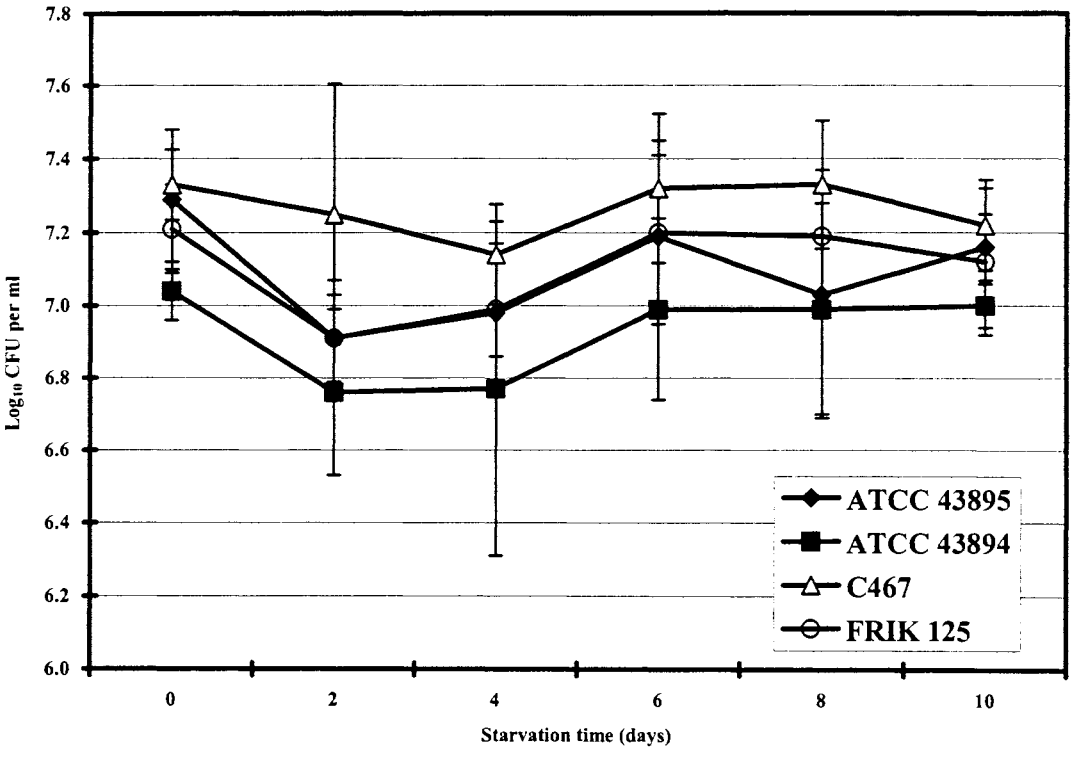


Figure 1. Viability of four strains of *E. coli* O157:H7 during starvation for 10 days in 0.85% saline (25°C).

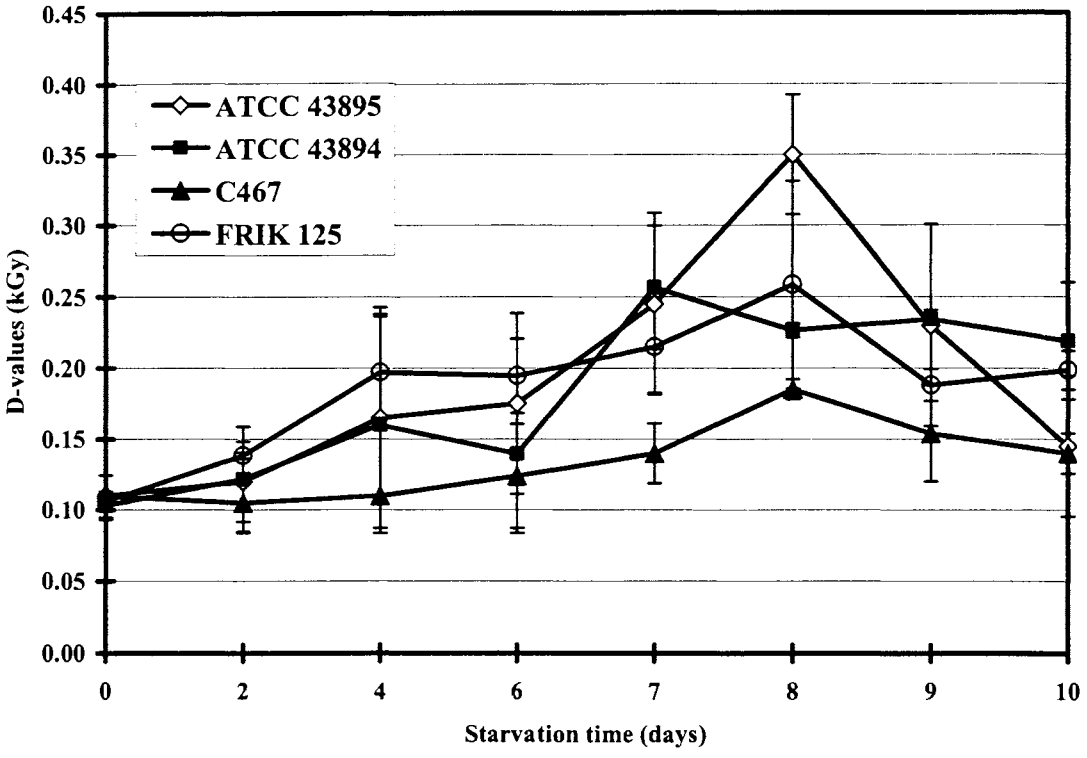
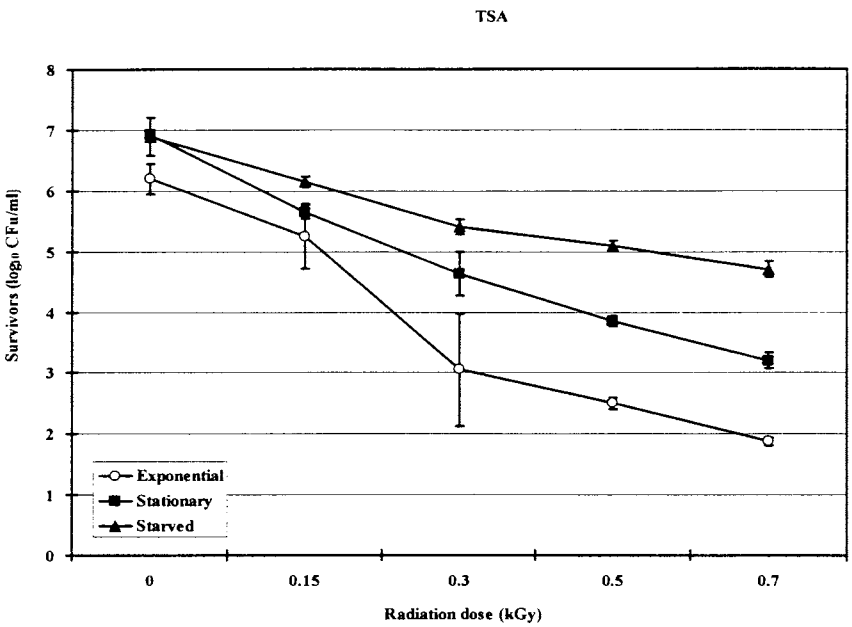


Figure 2. Radiation resistance (D-values) of four strains of *E. coli* O157:H7 during starvation for 10 days in 0.85% saline at 25°C.



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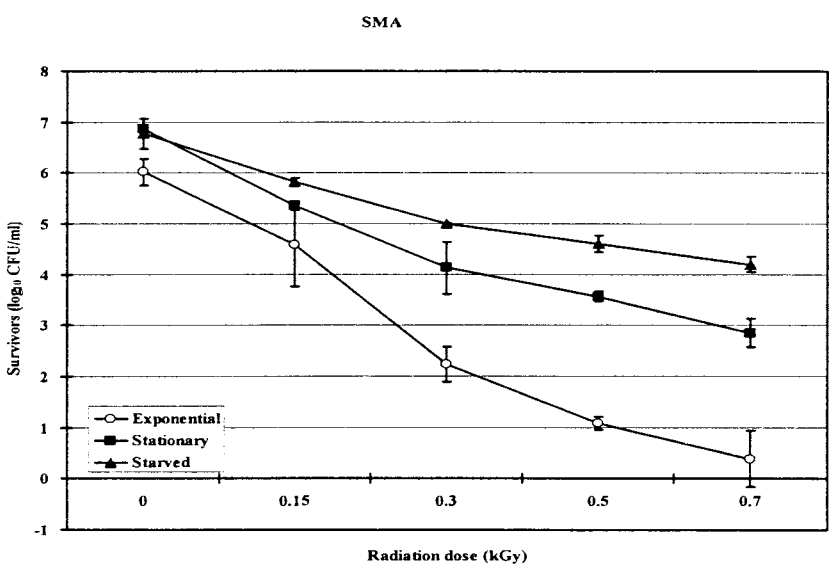

B

Figure 3. Survival of starved and non-starved (exponential- and stationary-phase) E. coli O157:H7 following irradiation in apple juice at 4 °C. Survivors were recovered on TSA (A) and SMA (B).

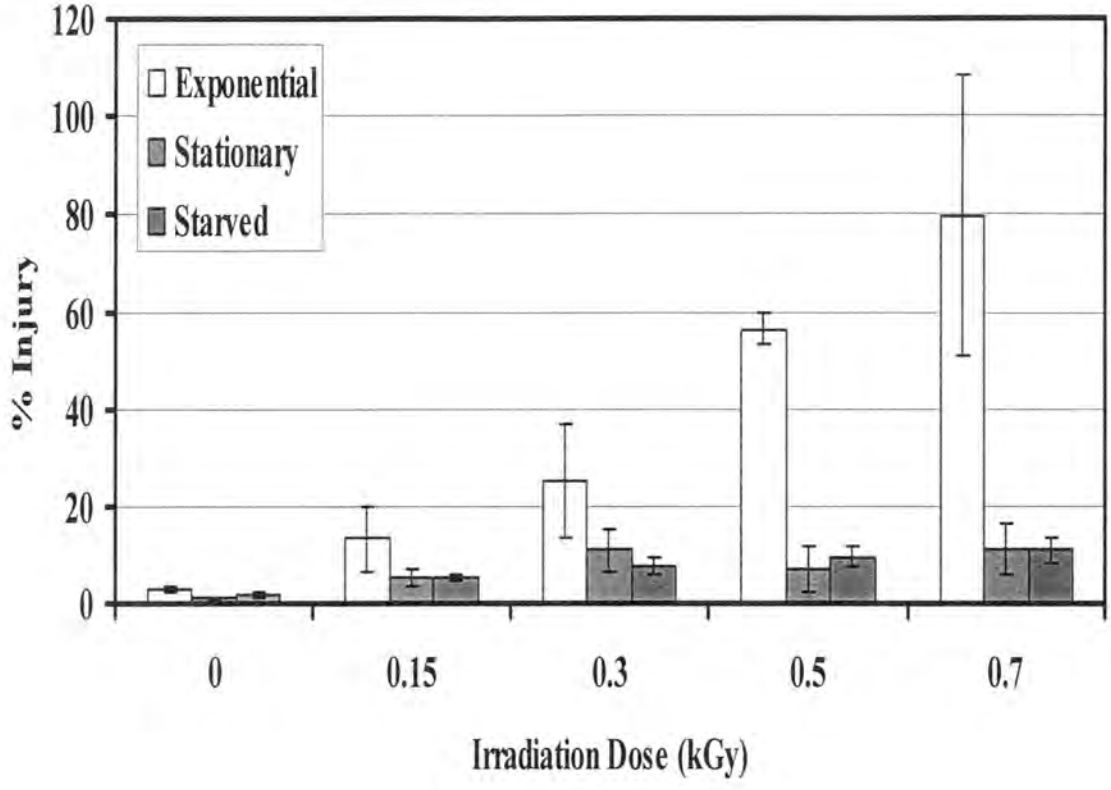

Figure 4. Sublethal injury of a four-strain mixture of *E. coli* O157:H7 following electron beam irradiation in apple juice.

Table 1. D-values[a] for each of four *E. coli* O157:H7 strains (in 0.85% saline) and the four-strain mixture (in apple juice) following electron beam irradiation. Survivors were recovered on TSA.

D-value (kGy)[b]

			0.85% saline		Apple Juice
Physiological state	ATCC 43895	ATCC43894	C467	FRIK 125	Mixture*
Exponential	0.11 (±0)A	0.10 (±0.01)A	0.11(±0.01)A	0.11 (±0)A	0.16 (±0.02)A
Stationary					0.19 (±0.01)B
Starved	0.35 (±0.04)B	0.23 (±0.01)B	0.19 (±0.01)B	0.26 (±0.07)B	0.33 (±0.05)C

[a]Means (± standard deviations) of 2 replicate experiments in saline and 3 replicates in apple juice.
[b]Means with different letters in the same column are significantly different ($\alpha<0.05$).

CHAPTER 3. EFFECT OF STARVATION ON RESISTANCE OF *ESCHERICHIA COLI* O157:H7 TO ULTRAVIOLET RADIATION IN 0.85% SALINE AND IN APPLE JUICE

A paper to be submitted to the Journal of Food Protection

Sujin Paik and Aubrey Mendonca

ABSTRACT

The effect of starvation on the resistance of *Escherichia coli* O157:H7 to ultraviolet (UV) radiation in 0.85% (w/v) NaCl (saline) and in pasteurized apple juice was investigated. *E. coli* O157:H7 cells, grown aerobically at 35°C in tryptic soy broth (TSB), were harvested by centrifugation (10,000 x g, 10 min, 4°C), washed, then starved in 0.85% saline (25°C) for 10 days. Exponential- or stationary-phase cells grown in TSB at 35°C served as controls. Starved cells and controls were harvested, washed once then used to inoculate 0.85% saline or apple juice. The inoculated media were exposed to UV radiation (254 nm) with a maximum intensity of 820 $\mu W/cm^2$ and samples were removed at set intervals for microbiological analysis. *E. coli* O157:H7 survivors were determined by plating diluted samples on tryptic soy agar (TSA) and counting bacterial colonies after 24 h incubation at 35°C. Cells starved for 8 days demonstrated significantly higher resistance to UV radiation than controls in 0.85% saline and in apple juice ($\alpha<0.05$). In 0.85% saline, UV D-values were 7.2 and 14.9 s for control (exponential-phase) and starved cells, respectively. In apple juice, D-values for exponential, stationary, and starved cells were 1.3, 1.8, and 3.2 min, respectively. Numbers of surviving stationary-phase cells were consistently higher than exponential-phase cells; however, differences were not significant ($\alpha>0.05$). These results indicate that starvation-induced stress increases the

resistance of *E. coli* O157:H7 to UV radiation and should be considered when determining the UV D-value for this pathogen.

INTRODUCTION

Escherichia coli O157:H7 is a foodborne pathogen that causes severe foodborne illness, which may include syndromes such as hemorrhagic colitis, hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (4). Most outbreaks have been associated with eating undercooked ground beef and drinking raw milk (2, 10). However, several *E. coli* O157:H7 outbreaks associated with unpasteurized apple juice and cider have drawn much attention to these food products as vehicles of *E. coli* O157:H7 infection (23).

The Food and Drug Administration (FDA) has proposed a regulation requiring that fruit and vegetable juice processors implement hazard analysis and critical control point (HACCP) programs. Processors of unpasteurized juices must adapt their processes to achieve a 5-log reduction of pathogens in the finished product (23). Many believe that heat pasteurization is the best means of destroying *E. coli* O157:H7 in apple juice products. However, the use of pasteurization is linked to increased costs and can result in undesirable changes in the sensory quality of juices (23). Therefore, there is an urgent need for a cost-effective alternative technology, which can achieve a 5-log reduction in fruit juices without altering the sensory quality of these products.

Treatment with ultraviolet light has been approved by the FDA for pathogen reduction in water (5) and is a promising, low-cost alternative to pasteurization. It has also been used successfully on beef (20), fish (7), and poultry (21, 22) to control bacteria and increase shelf life with little effect on food quality. The antibacterial action of UV involves damage to nucleic acids and the cell walls (21, 23), with the greatest effect at

wavelengths between 250 and 260 nm.

Microorganisms in the natural environment commonly encounter limiting amounts of nutrients unlike the nutrient rich conditions provided in laboratory media. Starvation stress caused by nutrient limitation has been reported to induce the production of protective proteins in microorganisms (6). Starved or stationary-phase *E. coli* O157:H7 cells produced the protective proteins coded by the *rpoS* gene, which cross-protected the microorganism against chemical and physical stresses (3). Starvation-induced stress in foodborne pathogens is important to the food industry because foodborne pathogenic bacteria are frequently exposed to low-nutrient status when water is used to clean or rinse food contact surfaces or is added directly to food (17). Pathogens may also survive in water (without nutrients) on food contact surfaces long enough to induce the stress response. This response may cross-protect them from subsequent food preservation methods, such as heating, electron beam irradiation, and UV radiation.

Rowe and Kirk (17) demonstrated that nutrient starvation in *E. coli* O157:H7 significantly increased the organism's heat resistance. Also a dramatic increase in heat resistance of starved *Listeria monocytogenes* has been reported (11, 12). While there is a growing body of knowledge on the effect of starvation on the heat resistance of microorganisms, research on how starvation impacts the radiation resistance of foodborne pathogens is scarce. Therefore, the purpose of this study was to determine the effect of starvation on the resistance of *E. coli* O157:H7 to UV irradiation in 0.85% saline and in apple juice.

MATERIALS AND METHODS

Microorganisms and culture conditions. Four strains, enterohemorrhagic *E. coli* O157:H7 of ATCC 43895, ATCC 43894, FRIK 125, and C467, were used throughout the

experiment. Strains ATCC 43895, ATCC 43895, and C467 were obtained from the culture collection of the Food Microbiology Laboratory at Iowa State University. Strain FRIK 125, an isolate from an apple cider outbreak, was provided by Dr. Charles Kaspar, University of Wisconsin. Stock cultures were stored at -70°C in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) supplemented with 10% glycerol. Each stock culture was transferred successively at least twice in 10 ml tryptic soy broth (TSB) (Difco) and incubated overnight at 35°C prior to each experiment.

Starvation of *E. coli* O157:H7. A portion (1.0-ml) of *E. coli* O157:H7 culture was transferred to pre-warmed (35°C) TSB (100 ml) in a screw-capped 250 ml Erlenmeyer flask. The inoculated medium was incubated at 35°C with shaking at 150 rpm in a shaker incubator (New Brunswick Scientific Co. Inc., Edison, N.J.). Optical density (OD) measurements (600 nm) were performed using a spectronic 1201 (Milton Roy Co., Rochester, NY), when the OD reached 0.1 to 0.15, exponential-phase cells were harvested by centrifugation (10,000 x g, 10 min, 4°C) and washed once in 0.85% (w/v) NaCl (4°C). The cell pellets were suspended in fresh saline and the cells were starved by holding the cell suspension statically in a screw-capped 250 ml flask at 25°C for 10 days.

Preparation and inoculation of test samples. Apple juice was obtained from a local supermarket in Ames, IA and stored at 4°C until used. The level of background microflora in the apple juice was determined by surface-plating serial dilutions (0.1% peptone) on TSA. Washed exponential phase cells of *E. coli* O157:H7 in 0.85% saline were prepared from a TSB culture as previously described. Stationary phase cells, harvested by centrifugation (10,000 x g, 10 min, 4°C) of an overnight (18 h) culture in TSB (35°C), were washed and suspended in fresh saline. Cells starved for 8 days in 0.85% saline (25°C) were centrifuged and suspended in fresh saline. Suspensions of

starved or non-starved cells were adjusted to give approximately 10^8 CFU per ml. Samples (100-ml) of sterile saline or apple juice were each inoculated with 1.0 ml of *E. coli* O157:H7 cells (exponential, stationary, or starved) to give a final concentration of approximately 10^6 CFU/ml.

UV treatment. Five-milliliter samples of inoculated saline or apple juice were placed into separate sterile petri dishes without lids. The depth of each 5-ml sample was abount 0.9 mm. All samples were irradiated using a XX-15F UV lamp (Spectronics Corp., Westbury, New York). The UV disinfection unit performed at a peak radiation of 254 nm with an approximate maximum intensity of 820 $\mu W/cm^2$. The distance between the sample and the UV light source was 10.7 inches. For each experiment, the UV lamp was turned on and allowed to warm up for about 3 min prior to exposure of cell suspensions to UV radiation to stabilize the intensity of the UV radiation. The intensity of the UV radiation was measured using a digital UV radiometer (Spectroline® DM-254XA, Spectonics Co., Westbury, New York).

Microbiological analysis. All samples were analyzed within 2 h after UV treatment. Saline or apple juice samples were diluted 1:100 in sterile 0.1% peptone water. Serial dilutions of saline or apple juice in 0.1% peptone were prepared and 0.1 ml aliquots of appropriate dilutions were surfaced-plated, in duplicate, onto plated of TSA for saline samples and on both TSA and Sorbitol McConkey Agar (SMA) (Difco) for apple juice samples. In instances when increased sensitivity was required, 0.1 ml samples of undiluted saline or apple juice were plated directly onto appropriate agar plates. All inoculated plates were incubated aerobically at 35 °C and bacterial colonies were counted in 24 h.

Calculation of D-values. The D-values, expressed in seconds (for saline) or minutes (for apple juice), were determined by plotting the $\log_{10}$ number of survivors per

ml versus exposure time using Microsoft Excel 98 Software (Microsoft Inc., Redmond, WA). Linear regression analysis (14) was used to determine the line of best fit for the data. The UV D-value was determined by calculating the negative reciprocal of the slope of the regression curve.

Statistical analysis. All experiments were replicated at least three times and results are reported as averages. The D value data were compared using analysis of the Tukey method (SAS Institute Inc, Cary, NC).

RESULTS AND DISCUSSION

Figure 1 shows the radiation resistance (D-value) of *E. coli* O157:H7 during starvation in 0.85% saline at 25°C. At day 0, D-values for the four *E. coli* O157:H7 strains ranged from 6 to 8 sec. The D-values increased steadily during starvation and reached a maximum at about 8 days. Strains ATCC 4895 exhibited the highest resistance to UV radiation; D-values were 25 sec, respectively. Maximum D-values for strains ATCC 43894, FRIK 125, and C 467 were 12, 12, and 11 sec, respectively. These results indicated that starvation enhances the resistance of *E. coli* O157:H7 to UV radiation.

The increased resistance of starved *E. coli* O157:H7 to UV radiation may be linked to one or more physiological changes triggered by starvation stress. Some physiological changes in starved bacteria include alterations in chromosome topology, decreased membrane fluidity and permeability, increased resistance to autolysis, decreased overall metabolism, rapid protein turnover, and production of stress proteins (8, 13). The production of certain stress proteins such as heat shock proteins (Hsp) in starved *E. coli* has been shown to enhance the heat resistance in this organism (13, 18). Hsp such as DnaK, GroL, HptG and other molecular chaperones seem to help repair

denatured or damaged macromolecules (13). Stress proteins may also be responsible for increasing the resistance of starved cells to the lethal effects of UV radiation.

UV radiation inactivates microorganisms via the covalent linkage of two adjacent pyrimidines in the DNA strand. The formation of thymine dimers is the most common lesion although thymine-cytosine and cytosine-cytosine dimers can be detected following UV treatment of DNA (15). These lesions inhibit DNA replication and function which can cause cell death if the damaged DNA is not repaired. In *E. coli* there are at least four different systems for repairing UV-induced damage to DNA: photoreactivation, excision (dark) repair, recombinational (post-replication) repair, and SOS-repair. In the present study, the increased resistance of starved *E. coli* O157:H7 to UV radiation suggests that starvation resulted in physiological changes, which either decreased the extent of UV-induced damage or improved the pathogen's ability to repair DNA damage. Further research is needed to determine the exact mechanism by which starved *E. coli* O157:H7 cells develop increased resistance to UV radiation.

Table 1 shows the UV radiation resistance (D-value) of exponential-phase and 8 days starved cells of each of four *E. coli* O157:H7 strains in physiological saline (25°C). UV radiation was administered at a maximum intensity of 820 $\mu W/cm^2$. D-values of exponential-phase cells ranged from 6.57 to 8.27 sec. Starvation for 8 days in 0.85% saline dramatically increased resistance to UV radiation. D-values ranged from 11.10 to 35.17 sec; strain FRIK 125 exhibited the highest radiation resistance (D-value = 35.17 sec). For each strain, the D-value for starved cells was significantly greater than that of exponential-phase cells ($\alpha<0.05$). Similar results were observed when a cocktail of all four strains of *E. coli* O157:H7 was exposed to UV radiation in apple juice (pH 3.97) (Table 2).

The UV radiation resistance of three physiological states of *E. coli* O157:H7

(exponential-phase, stationary-phase, and starved cells) were evaluated in apple juice (Table 2). Survivors were enumerated on both non-selective (TSA) and selective (SMA) media. Higher D-values were obtained when cells were exposed to UV radiation in apple juice compared to saline. Starved cells exhibited significantly greater radiation resistance than exponential-phase cells irrespective of the plating medium used for cell recovery ($\alpha<0.05$). The D-values for starved cells were consistently higher than those for stationary-phase cells; however differences were not statistically significant ($\alpha>0.05$). Generally, higher D-values for all three physiological states were obtained when TSA was used as the plating medium.

The observed increased resistance of *E. coli* O157:H7 in apple juice compared to saline might be due to the extent of UV penetration into the medium. The presence of pigments in a liquid has been reported to greatly reduce UV penetration (19). Since the apple juice was of a translucent gold color, it was not surprising that *E. coli* O157 had a higher UV resistance in apple juice compared to saline.

Stationary-phase cells are more resistant to radiation than exponential-phase cells. *E. coli* O157:H7 cells that enter the stationary growth phase as well as those in a starved state synthesize specific sets of protective proteins. The synthesis of protective proteins, called stress proteins, correlates with increased cellular resistance to a variety of chemical and physical challenges (1, 9). A substantial amount of the stress proteins produced in starved or stationary-phase cells are controlled by the *rpoS* gene (3). The *rpoS* gene plays a key role in the induction of heat resistance in starved *E. coli* O157:H7 (17). The role of the *rpoS* gene in mediating the synthesis of stress proteins in stationary-phase and starved *E. coli* O157:H7 may explain the higher D-values obtained for these two physiological states compared to exponential-phase cells.

The higher UV radiation resistance based on bacterial counts on TSA compared to

SMA indicates that a portion of the surviving population was sub-lethally injured by UV radiation. When bacteria endure metabolic injury by chemical or physical challenges, injured survivors can become sensitive to chemical agents in selective media (16). While colonies on TSA represented both injured and non-injured cells, only non-injured cells formed colonies on SMA. Injured pathogens in foods and their recovery following processing should be considered in the food industry because injured pathogens may recover in foods and regain their pathogenicity (16). Therefore, in this study, estimating *E. coli* O157:H7 survivors on TSA is justified considering the fact that injured pathogens can have the same health impact as non-injured ones.

Starvation of *E. coli* O157:H7 in saline is analogous to the survival of the pathogen in water (low nutrient) on food contact surfaces for an extended time period, enough to induce the stress response. The use of bacteria in nutrient-rich laboratory media for food safety experiments is likely to underestimate the survival capability of pathogens when they are exposed to food preservation methods. Although starved cells have been reported to be more resistant to chemical and physical stresses, the results of this study indicate that the resistance of starved *E. coli* O157:H7 to UV radiation is not significantly greater that the UV resistance of this same organism in the stationary-phase. Since overnight cultures (stationary-phase) are commonly use in food safety experiments, currently published data on UV resistance of *E. coli* O157:H7 in liquids should be a reliable index of the resistance of this organism in a starved state. Further research is needed to determine if other types of stress such as heat-shock, acid- or alkaline-adaptation or osmotic shock alter the UV resistance of this foodborne pathogen. As such data accumulate more reliable risk assessments can be conducted as an important part of hazard analysis and critical control point schemes. This in turn would help to improve the safety of foods for consumers.

ACKNOWLEDGMENTS

The authors thank Dr. Charles Kaspar, University of Wisconsin, Madison, for supplying one of the bacterial strains (FRIK 125) used in this study. The authors also thank Meijun Zhu for statistical assistance. This project was supported by the USDA/CSREES National Research Initiative Competitive Grants Program, Project No. 98-35502-6604.

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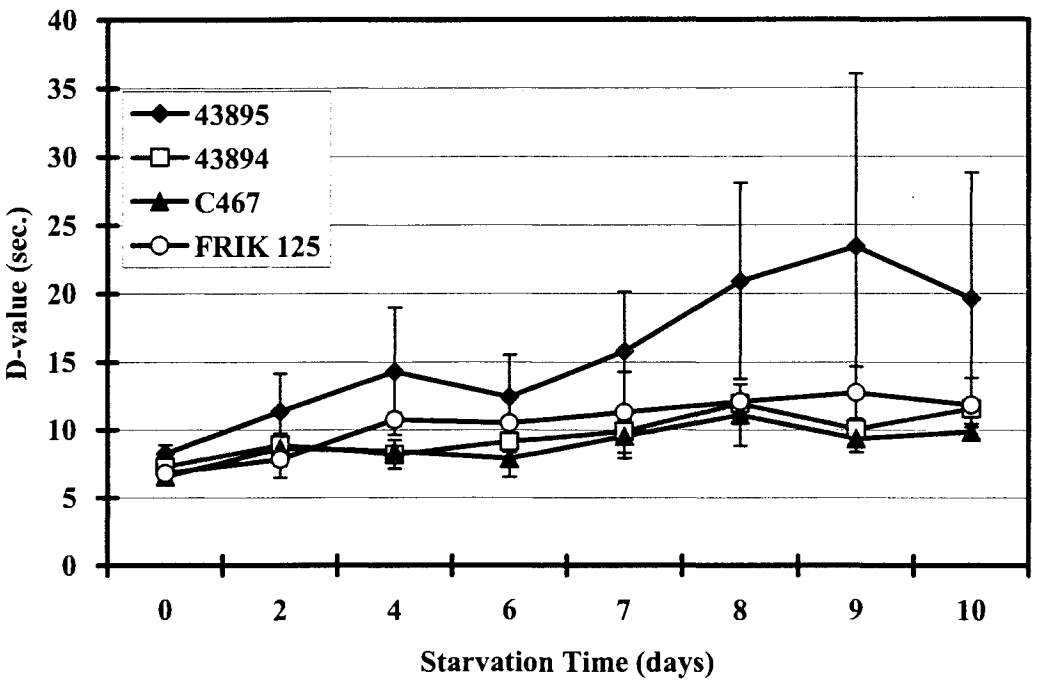

Figure 1. UV radiation resistance (D-values) of four strains of *E. Coli* O157:H7 during starvation for 10 days in 0.85% saline at 25°C.

Table 1. D-value[a] for each of four *E. coli* O157:H7 strains (in 0.85% saline) following UV irradiation. Survivors were recovered on TSA.

	D-value (sec.)[b]			
Physiological state	ATCC 43895	ATCC43894	C467	FRIK 125
Exponential	8.27 (±0.61)A	7.30 (±0.44)A	6.57 (±0.45)A	6.70 (±0.20)A
Starved	24.47 (±11.83)B	11.93(±0.55)B	11.10 (±2.26)B	12.1 (±0.42)B

[a]Means (± standard deviation) of 3 replicate experiments.

[b]Means with different letters in the same column are significantly different ($\alpha<0.05$).

Table 2. D-value[a] of the four-strain mixture *E. coli* O157:H7 (in apple juice) following UV irradiation. Survivors were recovered on TSA and SMA.

	D-value (min.)[b]	
	TSA	SMA
Physiological state	Mixture	Mixture
Exponential	1.29 (±0.48)A	1.14 (±0.43)A
Stationary	1.83 (±0.90)AB	1.63 (±0.75)AB
Starved	3.19 (±1.90)B	2.61 (±1.31)B

[a]Means (± standard deviation) of six replicate experiments

[b]Means with different letters in the same column are significantly different ($\alpha<0.05$)

CHAPTER 4. GENERAL CONCLUSIONS

The underlying objective of this research work was to evaluate the starvation in *E. coli* O157:H7 on the resistance of this pathogen against ionizing (electron-beam) and non-ionizing (ultraviolet) radiation. The work done in this thesis successfully meets the objectives.

The effect of starvation on radiation resistance and injury of *E. coli* O157:H7 following electron-beam irradiation in 0.85% saline and in apple juice was studied in the chapter 2. From this study, it can be concluded that starvation increased the resistance of *E. coli* O157:H7 to electron-beam radiation.

Chapter three evaluated the effect of starvation on the resistance of *E. coli* O157:H7 to UV irradiation in 0.85% saline and in apple juice. It was also concluded that the starvation enhanced the resistance of *E. coli* O157:H7 against the UV irradiation.

Results of microbial inactivation studies involving irradiation of starved *E. coli* O157:H7 could assist food processors in designing irradiation processes with an adequate margin of safety for control of this pathogen.

There is an urgent need for further research to determine the impact of other environmental stresses such as acidification and osmotic shock on the radiation resistance of foodborne pathogens.

For future studies on treatment of fruit juices by UV radiation, the use of UV light with a maximum intensity prescribed by the Food and Drug Administration is recommended.

ACKNOWLEDGMENTS

I sincerely thank my major professor, Dr. Aubrey Mendonca, for his tremendous support and scientific guidance. He gave me a second chance when I decided to change my major to Food Science and Technology. I also thank my graduate committee members Dr. Bonita Galtz and Dr. Mark Gleason for their scientific advice and help.

To my colleagues in the Microbial Food Safety Lab, it was fortunate for me to work with you all. I specially thank Ainura Orozaliva and Bledar Bisha for their great support and friendship, as well to Meijun Zhu for performing statistical analysis.

I dedicate my thesis to my parents. Their faith in me and emotional support have encouraged me to go through any difficulties and to finish this work. Finally I would like to thank God who is always with me and gives strength through my life.