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Inactivation of sanitizer-injured *Escherichia coli* O157:H7 on baby spinach using X-ray irradiation

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ABSTRACT

Chemical sanitizers are widely used to minimize cross-contamination during commercial flume washing of leafy greens. However, in addition to microbial inactivation, sanitizer exposure can also lead to sub-lethal injury. This study assessed the potential of sanitizer-induced cell injury to enhance resistance of *Escherichia coli* O157:H7 on baby spinach when X-ray irradiation was subsequently used as a microbial inactivation strategy. A 3-strain *E. coli* O157:H7 cocktail was exposed to a peroxyacetic acid-based sanitizer (PAS) at 5.2 ppm, a chlorine-based sanitizer (CS) at 5.2 ppm, both of which are commonly used in flume washing systems, or to a quaternary ammonium-based sanitizer (QAS) at 18 ppm to obtain 86–99% injury. Pre-irradiated, round-cut (2.54 cm diameter) baby spinach leaves were dip-inoculated by immersion in the injured cocktail for 5 min and then 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 Sorbitol MacConkey Agar (SMAC) overlayed with trypticase soy agar containing 0.6% yeast extract and SMAC. On inoculated spinach, *E. coli* O157:H7 injury decreased from 86–99 to 66, 63, and 1% for PAS-, CS-, and QAS-treated cells, respectively. D_{10}-values for PAS-, QAS-, and CS-injured *E. coli* O157:H7 on baby spinach were 0.014 ± 0.000, 0.022 ± 0.001, and 0.024 ± 0.001 kGy, respectively. Prior exposure to PAS significantly ($P < 0.05$) enhanced *E. coli* O157:H7 susceptibility to X-ray irradiation, while exposure to CS significantly ($P < 0.05$) reduced susceptibility. These results suggest that PAS may be preferred for irradiated baby spinach.

1. Introduction

Consumption of baby spinach and other leafy greens continues to be a topical issue among nutritionists who advocate increased consumption based on perceived health benefits. However, safety concerns have been raised regarding the increasing number of leafy green-associated outbreaks due to *Escherichia coli* O157:H7. During 1995–2007, *E. coli* O157:H7 was implicated in 13 mixed green salad and 12 lettuce outbreaks, resulting in a total of 652 illnesses (CSPI, 2009). 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 outbreaks in December traced to shredded lettuce that included a total of 152 confirmed cases in 5 north-eastern states, and 10 cases of HUS (FDA, 2007a; FDA, 2007b). These outbreaks, along with ongoing recalls of fresh-cut leafy greens confirm that currently used chemical sanitizers are not sufficiently effective, with bacterial populations decreasing only 1 to 2 logs on the product during commercial processing (Beuchat, Adler, & Lang, 2004; Keskinen, Burke, & Annous, 2009; Lopez-Galvez, Gil, Truchado, Selma, & Allende, 2010; Sapers, 2001). 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 on ionizing radiation has focused on gamma rays and E-beams as irradiation sources, with X-ray technology, until very recently, considered unfeasible economically. In previous work, gamma radiation respectively yielded D_{10}-values of 0.27 and 0.14 kGy for *E. coli* O157:H7 internalized in spinach and on the surface of lettuce (Niemira, 2007; Niemira, Sommers, & Fan, 2002). Similar *E. coli*

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O157:H7 inactivation values also have been reported using E-beam, with a D_{10}-value of 0.20 kGy for baby spinach (Neal, Cabrera-Diaz, Marquez-Gonzalez, Maxim, & Castillo, 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, Marks, Ryser, & Booren, 2007; Schilling et al, 2009), almonds and walnuts (Jeong, Marks, Ryser, & Harte, 2012), milk (Mahmoud, 2009b) various types of seafood (Mahmoud, 2009a; Mahmoud, 2009c; Mahmoud & Burrage, 2009; Robertson et al., 2006) and fresh produce (Jeong, Marks, Ryser, & Moosekian, 2010; Mahmoud, 2010a; Mahmoud, 2010b; Mahmoud, Bachman, & Linton, 2010; Moosekian, Jeong, Marks, & Ryser, 2010). Gamma and X-rays inactivate microorganisms primarily through breaks in single- or double-strand DNA that can occur directly or indirectly through high energy photons, secondary electrons, and free radicals generated in the food (Podgoršak, 2006). Generated at a lower energy level than gamma rays, X-rays differ in photon penetration and electron interaction. Using low-energy (70 kV) X-ray irradiation, we were previously able to decrease *E. coli* O157:H7 populations >5 log on fresh-cut iceberg lettuce, baby spinach and flat-leaf parsley at doses of 0.20, 0.18 and 0.26 kGy, respectively (Moosekian et al., 2010).

Bacterial inactivation by chlorine, peroxyacetic acid and quaternary ammonium compound-based sanitizers is well documented with their oxidative ability directed towards the cell membrane (Ioannou, Hanlon, & Denyer, 2007; Przestalski et al., 2000), and various metabolic functions (Bloomfield, 1996; Winter, Ilbert, Graf, Ozcelik, & Jakob, 2008). However, several previous reports have raised concerns regarding enhanced protection of *E. coli* following sublethal exposure to chemical sanitizers. Zook, Busta, and Brady (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. According to Demple and Halbrook (1983), prior treatment with peroxide doubled the survival rate of *E. coli* K-12 following exposure to gamma irradiation, with enhanced survival greatest at radiation doses <0.1 kGy. Given these concerns, this study aimed to assess the efficacy of X-ray irradiation against cells of *E. coli* O157:H7 that have been sublethally injured by chemical sanitizers during commercial washing of fresh-cut baby spinach.

2. Materials and methods

2.1. 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. After a second transfer in 200 ml of TSBYE, the strains were pelleted by centrifugation for 15 min at 2200 × *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 of *E. coli* O157:H7 as determined by plating on trypticase soy agar containing 0.6% (w/v) yeast extract (TSAYE; Becton Dickinson).

2.2. Peroxyacetic acid-based sanitizer (PAS) 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 PAS (Tsunami 100™, Ecolab, St. Paul, MN). After 2 min, the reaction was stopped by adding 1 ml of 38.5× neutralizing buffer (Difco neutralizing buffer, Becton Dickinson) to obtain ~90% injury. The injured cocktail was then pelleted by centrifugation at 2200 × *g* for 15 min and resuspended in PBS to obtain a population of ~8.0 log CFU/ml.

2.3. Quaternary ammonium-based sanitizer (QAS) 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 QAS (Whisper™, Ecolab). After 2 min, the reaction was stopped by adding 1 ml of 38.5× 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.

2.4. Chlorine-based sanitizer (CS) 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 initial exposure to a chlorine solution (CS) containing 22 ppm free chlorine (pH 7.5) (XY-12™, Ecolab). After 4 min, the inoculum was transferred to a sterile 400 ml beaker containing 10 ml of 77× 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.

2.5. Quantification of injury

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

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

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

N_{SMAC} = CFU/ml from SMAC plates

2.6. X-ray irradiator and dosimetry

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. The typical dose rate from this irradiator was 17 Gy/s in air at 10 cm from the source. Nominal surface dose was measured using radiochromic film dosimeters (GAF3001DS, GEX Corporation, Centennial, CO). The dosimeters were read 24 h after exposure using a standard spectrophotometric method (Spectronic Genesys 20, Thermo Fisher Scientific, Inc., Waltham, MA) based on calibration curves at 500/550 nm.