Food Research International 45 (2012) 1123–1128

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Comparison of multiple chemical sanitizers for reducing *Salmonella* and *Escherichia coli* O157:H7 on spinach (*Spinacia oleracea*) leaves

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ARTICLE INFO

Article history:
Received 21 February 2011
Accepted 13 April 2011

Keywords:
Lactic acid
Pathogen reduction
Spinach
Salmonella
E. coli O157:H7

ABSTRACT

Effectiveness of multiple chemical sanitizers on the reduction of *Salmonella* spp. and *Escherichia coli* O157:H7 on spinach was compared. Fresh spinach (*Spinacia oleracea*) was inoculated with a bacterial suspension containing multiple strains of rifampin-resistant *Salmonella* and *E. coli* O157:H7. Inoculated spinach leaves were treated with a water wash or water wash followed by 2% L-lactic acid at 55 °C, peroxyacetic acid (80 mg/L), calcium hypochlorite (200 mg/L), ozonated water (mg/L) or ClO_2 gas (1.2 or 2.1 mg/L). The L-lactic acid produced a 2.7 log CFU/g reduction for *E. coli* O157:H7 and a 2.3 log CFU/g reduction for *Salmonella*, statistically significant compared to water wash alone ($P<0.05$), which resulted in a reduction of 0.7 log CFU/g for both pathogens. These findings indicate that 2% L-lactic acid at 55 °C may be an effective treatment for reducing pathogens on spinach leaves.

1. Introduction

Once considered to be among the safest of foods, fresh vegetables have recently been recognized as an important vehicle of foodborne outbreaks in the US, and ready-to-eat leafy greens have been responsible for several outbreaks of *Salmonella* or *Escherichia coli* O157:H7 since 1998 (Tauxe, Doyle, Kuchenmueller, Schlundt, & Stein, 2010). This change can be attributed not only to changes in consumption patterns but also changes in production and processing technologies, new sources of produce as well as the manifestation of pathogens such as *Salmonella* and *E. coli* O157:H7 that have not been previously associated with raw produce (Burnett & Beuchat, 2001, Hanning, Nutt, & Ricke, 2009; Sivapalasingam, Friedman, Cohen, & Tauxe, 2004).

Disinfection is one of the most important processing steps in fresh-cut vegetable production and affects the quality, safety and shelf-life of the product. Fruit and vegetable growers and packers use chemical decontamination methods as interventions which are effective in reducing counts of bacteria as well as yeasts and molds. Numerous treatments have been developed to decontaminate leafy green vegetables including aqueous sanitizing agents; however, the effectiveness of these products depends on many factors including treatment conditions such as temperature, pH, water hardness, contact time, amount and rate of product throughput, type of vegetable, the volume of water per weight of produce, the amount of organic material present in the water and the resistance the pathogen has to the specific antimicrobial agent (FDA, 1998).

Despite these efficacy challenges for chemical sanitizers, the initial processing stages for minimally processed or bagged produce are critical for the removal or inactivation of pathogens because if they are not addressed at this preliminary period, they can potentially contaminate additional produce throughout processing (FDA, 2001).

Therefore, in an effort to reduce the number of foodborne illnesses associated with fresh produce, research must be conducted on each type of fruit or vegetable concerning specific pathogens as well as multiple types of interventions or treatment methods (Neal, Cabrera-Diaz, Marquez-Gonzalez, Maxim, & Castillo, 2008). Numerous studies have been performed to demonstrate the influence of chemical sanitizers on produce as well as the influence of hurdle treatments on minimally processed vegetables. Typically, these studies report the effectiveness of one or two chemical decontamination methods for a variety of produce items (such as bell peppers, strawberries or cantaloupes). In contrast, this study included a side by side comparison of various chemical sanitizers for the decontamination of one single commodity. No other reports are known that include the comparison of the wide range of different chemical sanitizers for reducing *Salmonella* or *E. coli* O157:H7 on spinach leaves. In addition, many of the decontamination studies use a spot inoculation method in which the suspension is allowed to dry on

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doi:10.1016/j.foodres.2011.04.011

the product surface before treatment. While this approach will yield excellent results for the efficacy of the decontamination method in a laboratory setting, it may not reflect the conditions of washing facilities.

Chorine is often used to sanitize produce, contact surfaces and facilities, as well as to diminish microbial loads in water used during cleaning and packaging (Cherry, 1999). Despite its popularity and convenience, the effects of chlorine on pathogens inoculated onto produce are inconsistent. Bershing, Winkler, Franz, and Premier (2000) described the efficacy of chlorine for inactivation of *E. coli* on lettuce and broccoli. Zhang and Farber (1996) reported that 200 mg/L for 10 min could reduce *L. monocytogenes* artificially inoculated onto shredded lettuce and cabbage by 1.7 and 1.2 log CFU/g, respectively. Takeuchi and Frank (2001) reported that 200 mg/L of chlorine treatment at 22 °C for 5 min achieved a significant reduction in the number of viable *E. coli* O157:H7 attached on leaf surfaces and in cut edge tissue (0.3 and 0.4 log CFU/g respectively) but high numbers of viable cells remained both at the cut edge and the surface. Delaquis, Stewart, Cazaux, and Toivonen (2002) reported on the fate and survival of *E. coli* O157:H7 and *L. monocytogenes* in ready-to-eat cut lettuce washed in cold and warm chlorinated water. One of the biggest challenges with using chlorine as a disinfectant is that it diminishes quickly upon contact with organic matter such as decaying vegetation which can be expected in the harvesting process (Beuchat, Nail, Alder, & Clavero, 1998; Li, Brackett, Chen, & Beuchat, 2001; Solomon, Potenski, & Matthews, 2002; Takeuchi & Frank, 2001).

Chlorine dioxide (ClO_2) has been proposed as a possible sanitizing agent. There are several advantages of using ClO_2 rather than hypochlorous acid (HOCl) such as ClO_2's reduced reactivity with organic material, it has a greater activity at neutral pH, however; its stability may be problematic (Benarde, Snow, Olivieri, & Davidson, 1967). Han, Selby, Schultze, Nelson, and Linton (2004) investigated the use of ClO_2 gas treatments for the decontamination of strawberries and reported a 5 log reduction in microbial counts however, it is important to note that they also stated that the effectiveness of ClO_2 gas treatments for reducing pathogens varies for different types of produce. Lee, Costello, and Kang (2004) reported that *E. coli* O157:H7 inoculated on lettuce leaves were reduced by 3.4, 4.4, and 6.9 CFU/g after treatment of ClO_2 gas for 30 min, 1 h and 3 h, respectively. Many pathogens cannot grow below pH levels of 4.5 thus; acidification by using organic acids may help prevent bacterial growth.

The antimicrobial mechanism by which organic acids reduce microbial numbers is due to several factors. These include a reduction in the environmental pH, disruption of cell membrane transport system and permeability, accumulation of ions, or a reduction in the internal cellular pH by the dissociation of hydrogen ions from the microorganism as it attempts to maintain homeostasis (Parish et al., 2003; Virto, Sanz, Alvarez, Condón, & Raso, 2005). Torriani, Orsi, and Vescovo (1997) reported that coliforms and fecal coliforms were reduced about 2 and 1 log CFU/g respectively, on mixed salad vegetables treated with 1% lactic acid. Alvarado-Casillas, Ibarra-Sánchez, Rodríguez-Garcia, Martínez-Gonzáles, and Castillo (2007) reported that lactic acid sprays can reduce bacterial pathogens by almost 3 log CFU/g on cantaloupes and 3.6 log CFU/g on produce with smooth surfaces such as bell peppers. Lin, Moon, Doyle, and McWatters (2002) studied the anti-bacterial efficacy of hydrogen peroxide with and without lactic acid at elevated temperatures on lettuce and reported that the combination of lactic acid and hydrogen peroxide was able to obtain reductions of >4 log CFU of *E. coli* O157:H7 and *Salmonella* per lettuce leaf and about 3 log CFU of *L. monocytogenes* per lettuce leaf was inactivated.

Peracetic acid is commonly used as a disinfectant treatment for fresh-cut vegetable process water for reducing yeast and mold counts which can improve the finished product's quality and extend the shelf-life. Beuchat, Adler, and Lang (2004) studied the efficacy of peroxyacetic acid in killing *L. monocytogenes* on iceberg and romaine lettuce with reductions not significantly different from lettuce leaves washed only with water. Wisniewsky, Glatz, Gleason, and Reitmeier (2000) reported that peroxyacetic acid achieved a 5 log reduction of *E. coli* O157:H7 inoculated on whole fresh apples, however; this was accomplished when the treatment was used at 2.1 to 14 times its recommended concentration depending on the length of the wash time.

Ozone is a highly reactive form of oxygen (O_3), which destroys microorganisms by reacting with oxidizable cellular components (Ramawamy, Rodriguez-Romo, Vurma, Balasubramanian, & Yousef, 2007). Rodgers, Cash, Siddiq, and Ryser (2004) compared different chemical sanitizers including ozone for reducing *E. coli* O157:H7 and *L. monocytogenes* on apples, lettuce, strawberries and cantaloupes and reported reductions in populations of both pathogens by ca. 5.6 log cycles using 3 mg/L.

The investigators of this study attempted to inoculate the spinach samples as well as decontaminate the samples in a manner similar to conditions within a produce washing facility. The objective of the present investigation was to determine the effectiveness of several chemical sanitizers on the reduction in populations of inoculated *Salmonella* and *E. coli* O157:H7 counts on spinach.

2. Materials and methods

2.1. Bacterial cultures

To facilitate the enumeration of pathogens on spinach, rifampin-resistant (Rif^+) *Salmonella* and *E. coli* O157:H7 were used. The Rif^+ mutants of *Salmonella*, corresponding to serotypes Agona, Gaminara, Michigan, Montevideo, Poona and Typhimurium, and 5 strains of *E. coli* O157:H7 were obtained from the Texas A&M Food Microbiology Laboratory culture collection. The Rif^+ mutants were obtained from pure cultures by following the method published by Kaspar and Tamplin (1993). *E. coli* O157:H7 expressing green fluorescent protein (GFP) and *Salmonella* expressing red fluorescent protein (RFP) were also obtained from the Texas A&M Food Microbiology Laboratory culture collection. The isolates had been previously transformed by electroporation to express GFP or RFP (Cabrera-Diaz et al., 2009).

Bacterial strains were cultured individually on tryptic soy agar slants (TSA; Difco, Becton Dickinson, Sparks, MD) and incubated at 37 °C for 24 h. Three days prior to each experiment the microorganisms were individually subcultured by two consecutive transfers to tryptic soy broth (TSB; Difco) and incubated at 37 °C for 12 h. Rifampin resistance was confirmed by streaking TSB cultures onto plates of TSA + 100 mg/L rifampin (rif-TSA; Sigma, St. Louis, MO) with incubation at 35 °C for 24 h.

2.2. Inocula preparation

Nine milliliters of a 12 h culture of each microorganism was dispensed into a sterile centrifuge tube (15 mL) and harvested by centrifugation at 1623 ×*g* in a Jouan B4i centrifuge (Thermo Electron Corp., Madison, WI) for 15 min at 21 °C. The pellet for each microorganism was resuspended in 5 mL of 0.1% peptone water (Difco) and then 1 mL aliquots of each were subsequently combined to generate a bacterial cocktail in a sterile bottle containing 89 mL 0.1% peptone water. The prepared inoculum containing each pathogen at a concentration of approximately 8 log CFU/mL was used within 2 h of preparation and was kept at room temperature (23 to 24 °C) during the experiment.

2.3. Sample preparation and inoculation of spinach

Fresh spinach leaves typical of leafy greens entering the US food supply were kindly provided by the Winter Garden Spinach Producers Board (Crystal City, TX). The spinach was harvested at approximately 45 days and placed in coolers with an internal temperature of 4 °C for 6 h and transported for 250 miles to the Texas A&M Food Microbiology Laboratory, where it was stored at 4 °C for up to 24 h. In the laboratory,