Research Note

Reduction of Escherichia coli O157:H7 in Fresh Spinach, Using Lactic Acid Bacteria and Chlorine as a Multihurdle Intervention

S. E. GRAGG AND M. M. BRASHEARS*

Department of Animal and Food Sciences, Texas Tech University, Box 42141, Lubbock, Texas 79409-2141, USA

MS 09-206: Received 7 May 2009/Accepted 17 September 2009

ABSTRACT

A 12-day shelf life study was conducted at 7°C to determine whether Escherichia coli O157:H7 on spinach can be controlled effectively by selected strains of lactic acid bacteria (LAB) alone or in combination with chlorine as a multihurdle intervention. The multihurdle intervention consisted of both LAB and chlorine and was applied to spinach as a rinse and evaluated in comparison to LAB alone and chlorine and water rinses. Reductions achieved by all treatments were compared with those observed for an inoculated control. The spinach was inoculated by submersion in a solution containing an E. coli O157:H7 cocktail at 1.0 × 10⁸ CFU/ml. LAB were applied postharvest at a concentration of 2.0 × 10⁸ CFU/ml and 200 ppm of chlorine was used for the chlorine rinse. All spinach samples were packaged in commercial packaging, held in a retail display case, and tested for E. coli O157:H7 on days 0, 1, 3, 6, 9, and 12 using the Neo-Grid filtration system and CHROMagar. Survival of LAB throughout the shelf life also was determined. Significant reductions in pathogen populations were achieved by water (P = 0.0008), LAB (P < 0.0001), chlorine (P < 0.0001), and multihurdle (P < 0.0001) treatments when compared with controls. The multihurdle treatment produced the greatest reduction from control populations, a reduction of 1.91 log CFU/ml. This reduction was significantly greater than that achieved with water (P < 0.0001), LAB (P = 0.0025), and chlorine (P < 0.0001) alone, indicating that the application of chlorine and LAB is most effective as a combination treatment. The results obtained from this study indicate that the industry standard chlorine wash may be more effective when applied in combination with LAB.

Since its recognition as a significant foodborne pathogen in 1982 (9), Escherichia coli O157:H7 has been associated with raw or undercooked ground beef (13). When multiple outbreaks of E. coli O157:H7 infection occurred in the mid-1990s, fresh produce was identified as a vector for the transmission of the pathogen (5). Since then, awareness of the potential for fruits and vegetables to be associated with foodborne illness has increased (7).

Fresh spinach is classified as a minimally processed vegetable because it has undergone minimal amounts of processing, such as shedding, peeling, or slicing, before packaging (11). The lack of a thermal processing step results in reliance on effective postharvest interventions to remove any associated pathogens (17). Currently, disinfection of produce by washing is the only processing step targeted at reducing microbial levels on fresh-cut vegetables (14).

Sodium hypochlorite used in a cold chlorinated water rinse is the most common postharvest intervention utilized in the fresh fruit and vegetable industry (2, 19). However, the efficacy of this intervention for inactivating pathogens on the surfaces of fresh produce is minimal, depending on various conditions including the microbial load and presence of organic material (11). Implementation of postharvest chlorine washes is effective for reducing the total microbial population by only 1 to 2 log units (21). Therefore, a more effective postharvest intervention is needed to improve the safety of bagged leafy greens.

Numerous characteristics of lactic acid bacteria (LAB) are antagonistic to other bacteria, including some pathogens (1, 4). The metabolic activities of LAB result in the production of antimicrobial compounds such as hydrogen peroxide, bacteriocins, carbon dioxide, and organic acids (8, 15, 16). Success has been achieved using LAB to control E. coli O157:H7 and other pathogens in raw meat products (8, 18), in cooked meat products (1), and in live cattle (3, 22, 23). Because these cultures produce inhibitory compounds (i.e., acid, hydrogen peroxide, and bacteriocins) under refrigerated conditions but do not grow (12, 18), they may be able to reduce pathogens in fresh spinach.

The objective of this study was to determine whether Bovamine Meat Cultures, a commercially produced product containing freeze-dried LAB cultures, and chlorine either alone or in combination as a multihurdle approach could be used as an effective postharvest intervention for reducing total numbers of E. coli O157:H7 in fresh spinach during refrigerated storage.

MATERIALS AND METHODS

Bacterial strains. A cocktail of four E. coli O157:H7 strains was used for this study: A4 966, A5 528, A1 920, and 966. All strains were isolated from cattle and maintained in the stock culture,* Author for correspondence. Tel: 806-742-2805, Ext 235; Fax: 806-742-4003; E-mail: mindy.brashears@ttu.edu.
collection at Texas Tech University. The cocktail was prepared by making frozen concentrated cultures as described by Brashears et al. (4).

Bovamine Meat Cultures (Nutrition Physiology Corporation, Guyon, OK) is a commercially available LAB product comprising four LAB strains: Lactobacillus acidophilus (NP 51), Lactobacillus crispatus (NP 35), Pediococcus acidilactici (NP 3), and Lactobacillus lactis subsp. lactis (NP 7) (18). The culture was prepared by a commercial manufacturer and packaged in 10-g portions in a freeze-dried form.

**Treatment preparation.** An LAB wash with a concentration of 2.0 × 10^8 CFU/ml was prepared by combining one 10-g packet of freeze-dried Bovamine meat culture with 990 ml of buffered peptone water (BPW; Oxoid, Basingstoke, UK) containing 1% glucose. The concentration of LAB was determined by making serial dilutions in BPW and plating on lactobacilli deMan Rogosa Sharpe (MRS) agar (EMD, Gibbstown, NJ). To metabolically activate the bacteria, the LAB culture was held in a 37°C incubator for 1 h. The concentration of the LAB wash was reevaluated postincubation by plating on MRS agar. A 200 ± 10-ppm chlorine wash was prepared by combining 7.6 ml of sodium hypochlorite germicidal bleach (Clorox Company, Oakland, CA) with 2.0 liters of sterile tap water at room temperature. The concentration of total chlorine was determined using an HI 95771 ultrahigh range meter (Hanna Instruments, Woonsocket, RI). The pH of the chlorine solution was determined with a pH meter (model 550A, Orion, Beverly, MA) to be 8.6. A 1.0-liter sterile tap water wash also was prepared.

**Sample preparation.** Fresh spinach was obtained from a commercial grower in California. The product was shipped overnight on the day of harvest. A total of 1,500 g of the spinach was weighed into sterile plastic bags (VWR, West Chester, PA). The four-strain cocktail of E. coli O157:H7 was diluted 1:1,000 in BPW to obtain a final concentration of 1.0 × 10^6 CFU/ml and an inoculum volume of 13 liters. The preweighed spinach was submerged in the inoculum and allowed to soak for 20 min to facilitate attachment. The inoculated spinach was spread evenly with sterile tongs across sterile drying racks in a biological safety level II hood (model 54L925, Fisher Hamilton, Two Rivers, WI) and allowed to dry for 1 h. After 30 min of drying, the spinach was turned over to ensure uniform air exposure and dried for an additional 30 min.

Upon completion of drying, 200 g of the dry inoculated spinach was added to a sterile rinsate bag (bird rinse bag, 15 by 20 in. [38 by 51 cm]; 3M, St. Paul, MN) and set aside to serve as the control. The remainder of the dry spinach was weighed into four sterile bags, with 200 g in each bag. Spinach in each of the four bags was ultimately exposed to a different treatment.

All treatments were added to the rinsate bags and agitated by hand for 1 min. Each treatment was applied at a ratio of 500 ml/200 g of spinach. The rinse treatments were as follows: 500 ml of the 2.0 × 10^8-CFU/ml LAB solution, 500 ml of 200 ppm of sodium hypochlorite, 500 ml of sterile tap water, and a multihurdle intervention that was initially rinsed with 500 ml of 200 ppm of sodium hypochlorite followed by 500 ml of sterile tap water and 500 ml of the 2.0 × 10^8-CFU/ml LAB solution. After agitation, all samples were drained in a sterile colander and transferred to a sanitized salad spinner (Farberware, Garden City, NY). The spinach was spun 20 times, transferred to a new sterile rinsate bag, and set aside.

Plastic oxygen-permeable rollstock used in the packaging of fresh spinach was utilized in this study. The rollstock was cut and sealed to create bags approximately 26.0 cm long and 11.45 cm wide. The seal function of a vacuum sealer (FoodSaver Gamesaver Deluxe Plus, Sunbeam, Boca Raton, FL) was used to create all seals on the bags.

A spinach sample (25 ± 1 g) was added to each premade bag with sterile tongs. The bags were sealed, labeled, and placed in a retail display cooler set at 7°C. Samples from each treatment were randomized across all three shelves and throughout the entire length of the cooler to reduce bias. The temperature of the retail display case was continuously recorded using a continuous temperature recorder (MKII, Temprecord International, Auckland, New Zealand). Before beginning the study, the temperature was set to 7°C and was monitored throughout storage.

**Microbiological analysis.** On days 0, 1, 3, 6, 9, and 12, one bag was randomly selected from each treatment group and the control in the retail display cooler, and 10 g of spinach was collected. The samples were homogenized with 90 ml of BPW at 230 rpm for 2 min in a stomacher (model 400, Seward, Bohemia, NY). Homogenized samples were serially diluted and quantitatively analyzed for E. coli O157:H7 using the Neo-Grid method (Neogen, Lansing, MI). Neo-Grid filters were placed on CHROMagar (CHROMagar, Paris, France) containing tellurite, cefixime, cefsulodin, and novobiocin at 2.5 mg/liter, 25 μg/liter, 5 mg/liter, and 5 mg/liter, respectively. CHROMagar plates were incubated at 37°C for 24 ± 2 h. Mauve colonies were counted as presumptively positive for E. coli O157:H7. Random colonies were subjected to confirmation using a latex agglutination kit (Remel, Lenexa, KS). The survival of LAB also was determined by spread plating on lactobacilli MRS agar plates, which were incubated for 24 to 48 h at 37°C. All colonies were counted and presumed to be LAB.

**Experimental design and analysis.** A complete randomized block design with three independent replicates was used for this work. The Statistical Analysis System (SAS Institute, Cary, NC) software was used to analyze the data. All data was subjected to the PROC MIXED and PROC UNIVARIATE commands. The least-squares means obtained from the PROC MIXED procedure were used to identify significant differences between each individual treatment in comparison to the control and to evaluate the survival of LAB for the multihurdle and LAB treatments at each sampling point. The least-squares means of each rinse treatment also was compared with one another to determine whether one treatment was significantly more effective than the other.

**RESULTS**

Table 1 presents the survival of LAB throughout the shelf life study. The LAB did not grow during storage and thus would not contribute to the spoilage of the product. The time × treatment interaction was not significant at P < 0.05 but was significant at P = 0.1. Figure 1 illustrates the general trend of all treatments employed in this study and depicts the effectiveness of each treatment for controlling E. coli O157:H7 throughout the shelf life. E. coli O157:H7 cells steadily grew from day 0 through day 6 and then gradually declined in the nontreated and water-treated samples, indicating that the cells entered into death phase. The pathogen cells in the hurdle treatment were reduced initially and remained in stationary phase after day 6. Most likely, the chlorine reduced the pathogen initially and the LAB kept levels low, resulting in an extension of the lag phase. When applied alone, both LAB and chlorine...
TABLE 1. LAB populations recovered from spinach subjected to LAB and multihurdle treatments a

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAB</td>
<td>7.61</td>
<td>7.65</td>
<td>7.48</td>
<td>7.23</td>
<td>7.11</td>
<td>6.88</td>
</tr>
<tr>
<td>Hurdle</td>
<td>7.53</td>
<td>7.48</td>
<td>7.47</td>
<td>7.16</td>
<td>7.12</td>
<td>6.89</td>
</tr>
</tbody>
</table>

a Samples were held in a retail display cooler at a target temperature of 7 °C for 12 days.

FIGURE 1. Populations of E. coli O157:H7 in each spinach treatment sample held in a retail display cooler at a target temperature of 7 °C for 12 days. 1LAB treatment with Bovamine Meat Cultures. 2Multihurdle treatment with chlorine, water, and Bovamine Meat Cultures. a Standard error for all treatments is 0.3082.

FIGURE 2. Composite least-squares means of E. coli O157:H7 populations in each spinach treatment sample held in a retail display cooler at a target temperature of 7 °C for 12 days. Treatment means (bars) with different letters are significantly different (P < 0.10). 1LAB treatment with Bovamine Meat Cultures. 2Multihurdle treatment with chlorine, water, and Bovamine Meat Cultures.

appeared to extend the lag phase. However, these treatments were not completely successful for controlling the pathogen, as indicated by increases in E. coli O157:H7 populations on day 9 and day 12 in LAB and chlorine treatment groups, respectively. The composite data for each treatment are illustrated in Figure 2; there were no interactions at the P < 0.05 level. These data indicate that significant reductions were achieved by water (P = 0.0008), LAB (P < 0.0001), chlorine (P < 0.0001), and the multihurdle (P < 0.0001) treatments when compared with the control. However, the multihurdle treatment resulted in the greatest decline of E. coli O157:H7 populations in comparison to the control, with a reduction of 1.91 log units. The reduction achieved by the multihurdle intervention was significantly greater than the reductions achieved by water (P < 0.0001), LAB (P = 0.0025), and chlorine (P < 0.0001) treatments alone.

The improved effectiveness of the multihurdle intervention is likely due to cell injury caused by the chlorine in the first wash, resulting in cells with increased susceptibility to the inhibitory action of the LAB. Although the metabolites produced by LAB are antagonistic toward E. coli O157:H7, the effect of bacteriocins on gram-negative bacteria is limited (8). However, the ability of bacteriocins to act on gram-negative bacteria is improved when these bacterial cells are injured (5). Therefore, the hypothesis was that the synergistic action of chlorine and LAB in combination most likely results in greater pathogen reduction due to bacteriocin and metabolite activity on weakened E. coli O157:H7 cells.

The effective use of LAB (Bovamine Meat Cultures in particular) has been demonstrated in fresh meat products. Smith et al. (18) utilized the same four LAB strains found in Bovamine Meat Cultures as an intervention for ground beef inoculated with E. coli O157:H7 at a concentration of 1.0 × 10^6 CFU/ml and reported significant reductions of 2.0 and 3.0 log units compared with controls after 3 and 5 days of storage, respectively. These reductions are similar to our results obtained for the multihurdle intervention.

Temperature and E. coli O157:H7 inoculation levels are two parameters in this study that deserve further investigation. Because Bovamine Meat Cultures consist of LAB strains specifically chosen for their ability to produce inhibitory substances without growth at refrigeration temperatures, reducing the storage temperature of LAB-treated spinach may improve the success of both the LAB and multihurdle treatments. An inoculation level of 1.0 × 10^6 CFU/ml is most likely significantly higher than the level of inoculation in our study; however, we used a concentration of 1.0 × 10^5 CFU/ml as the inoculation method may more accurately reflect inoculation by rain or irrigation and should also be considered for future research. Although a preliminary sensory study indicated no significant difference between water-treated and LAB-treated spinach on the day of application (data not shown), future research should be conducted to evaluate the sensory properties of LAB-treated spinach throughout the shelf life.
The multihurdle treatment was more effective than chlorine alone and resulted in the most significant reductions of *E. coli* O157:H7 populations in comparison to control populations. However, numerous variables must be further evaluated and may yield information that would greatly improve the success of the LAB and multihurdle treatments.

**ACKNOWLEDGMENTS**

Bovamine Meat Cultures and funding for this research were provided by the Nutrition Physiology Corporation. We also thank numerous industry representatives for their contributions to this study.

**REFERENCES**