Inhibition of *Salmonella* Typhimurium and *Listeria monocytogenes* in Mung Bean Sprouts by Chemical Treatment

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ABSTRACT

This study was undertaken to compare the efficacies of chlorous acid (268 ppm), sodium hypochlorite (200 ppm), and lactic acid (2%) in eliminating total mesophilic microorganisms, *Salmonella* Typhimurium, and *Listeria monocytogenes* on commercial mung bean sprouts immediately after treatment and during posttreatment refrigerated storage. Treatment with sodium hypochlorite for 10 min did not reduce the total aerobic count by 0.6 and 0.8 log CFU/g, respectively, and maintained the same level or a lower level of the total aerobic count during the storage time. Treatment with chlorous acid reduced *Salmonella* Typhimurium from 5.0 log to undetectable levels (<0.48 log CFU/g), and the pathogen remained undetectable over a 9-day storage period. Treatment with lactic acid resulted in an initial 3-log reduction and further reduced the number of *Salmonella* Typhimurium cells to undetectable levels after 3 days. For *L. monocytogenes*, treatment with chlorous acid resulted in an initial 5-log reduction, and treatment with lactic acid resulted in a 2-log reduction at the beginning and undetectable levels after 9 days. When chemically injured cells were investigated by the selective overlay method, no statistical difference was observed (*P* < 0.05) between the number of injured cells recovered following treatment with chlorous acid and the number of bacteria when treated with lactic acid. These data suggest that treatment with chlorous acid may be useful in reducing total mesophilic microorganisms, *Salmonella* Typhimurium, and *L. monocytogenes* in commercial mung bean sprouts.

An increase in the consumption of fresh fruits and vegetables in the United States has been paralleled by an increase in the number of foodborne illnesses attributed to fresh produce (1). Since 1995, sprouts have been increasingly implicated in foodborne outbreaks (1, 3, 11, 13-15). Many microbiological surveys have indicated the presence of a variety of foodborne pathogens in sprouts (1, 2, 5, 11, 13-15, 21, 22). For instance, *Salmonella* spp. and *L. monocytogenes* have been isolated from sprouted seeds, including alfalfa, mung bean, cress, soybean, and mustard seeds (2, 16, 17, 19).

In 1997, the Centers for Disease Control and Prevention submitted a request to the Food and Drug Administration/Center for Food Safety and Applied Nutrition asking for expert consultation regarding the recent foodborne illness outbreaks associated with sprouts (10). Subsequently, the Food and Drug Administration asked the National Advisory Committee on Microbiology Criteria for Food to review the current literature on sprout-associated outbreaks, identify the organisms and production practices of the greatest public health concern, prioritize research needs, and provide recommendations for intervention and prevention strategies (10). In August 1998, a sprouts task force initiated research at the National Center for Food Safety and Technology in Illinois to study the effects of commercial sprouting conditions on pathogens and to evaluate potential seed treatment interventions (including chemical, heat, and irradiation treatments). Several treatments show potential for significant reduction, but not elimination, of pathogens (13). However, there are relatively few studies applicable to food processors pertaining to the reduction or elimination of pathogenic bacteria on seeds and sprouts. In 1999, the Food and Drug Administration issued guidance documents to the sprout industry aimed at producing safer sprouts. Good agricultural and manufacturing practices, seed disinfection, and testing of every production batch for *Salmonella* spp. and *Escherichia coli* O157:H7 were recommended (23).

Seeds are generally recognized as the source of the microbial menagerie present in the final product (18). Foodborne pathogens including *Salmonella* spp. and *L. monocytogenes* have been isolated from seeds. Most research with sprouts has dealt with disinfection procedures to eliminate pathogens while preserving the germinability of the seed. Treatment of seeds with 20,000 mg of calcium hypochlorite per ml (ppm) has been recommended to reduce the size of the microbial population (13), although in some cases this treatment was unable to completely prevent the regrowth of *E. coli* O157:H7 during the sprouting of inoculated seeds (9, 20). It is possible that the pathogens were protected from the disinfectant in some way, perhaps by their location in or on the seeds (9, 20). Besides, seed sprouting provides an excellent environment for the growth of many types of microorganisms. If foodborne pathogens...
are present on or in the seed, they are likely to grow to significant population levels in the finished sprout (13, 19).

Therefore, antimicrobial treatments applied after sprouting offer the best promise for reducing the numbers of several foodborne pathogens in finished commercial mung bean sprouts.

Chlorous acid (HClO₂; Alcide Co., Redmond, Wash.) is a newly developed disinfectant and has been approved by the Food and Drug Administration for use on raw agricultural commodities (4). However, to date, no research studies have compared the effects of chlorous acid on sprouts with those of other widely used disinfectants. Thus, this study was undertaken to compare the efficacies of chlorous acid, sodium hypochlorite, and lactic acid in eliminating total mesophilic microorganisms, Salmonella Typhimurium, and L. monocytogenes on commercial mung bean sprouts.

Following antimicrobial treatments, sublethally injured foodborne pathogens assume added significance because they are potentially as dangerous as their uninjured counterparts (10). Thus, after chemical treatment, we also determined the occurrence of injured foodborne pathogens in sprouts by using the overlay method (10).

**MATERIALS AND METHODS**

**Cultures and cell suspension.** Three *Salmonella* Typhimurium cultures (ATCC 19585, ATCC 14028, and DT104 Killercow) and three *L. monocytogenes* cultures (ATCC 19114, ATCC 19113, and ATCC 7644) obtained from the Food Science and Human Nutrition bacteria collection at Washington State University (Pullman, Wash.) were used to inoculate mung bean sprouts. Each strain of *Salmonella* Typhimurium or *L. monocytogenes* was cultured in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C for 24 h, harvested by centrifugation at 4,000 × g for 20 min at 4°C, and washed three times with buffered peptone water. The final pellet was resuspended in buffered peptone water, corresponding to approximately 10⁷ to 10⁸ CFU/ml. Next, each group of foodborne pathogens was mixed to produce culture cocktails. The mixed culture cocktails (*Salmonella* Typhimurium or *L. monocytogenes*) were used for further experimentation.

**Sprout preparation and inoculation.** Commercial mung bean sprouts were purchased from local stores and inoculated with *Salmonella* Typhimurium or *L. monocytogenes* as follows. Prepared culture cocktails of *Salmonella* Typhimurium or *L. monocytogenes* were diluted in 5-liter sterile deionized water to a concentration of 10⁸ to 10⁹ CFU/ml. Two 500-g bunches of mung bean sprouts were immersed in 5 liters of aqueous suspension containing *Salmonella* Typhimurium or *L. monocytogenes* for 20 min at room temperature and then dried in a laminar-flow biosafety hood for 30 min with the fan running.

**Chemical treatments and storage.** Each 500-g bunch of inoculated mung bean sprouts was submerged in 5 liters of 200 ppm sodium hypochlorite (Food Science of America Inc., Seattle, Wash.), 2% lactic acid (pH 2.0; Fisher Scientific, Pittsburgh, Pa.), or 268 ppm chlorous acid (working strength Sanova, pH 2.5; Alcide Corporation, Redmond, Wash.) for 10 min at room temperature (22°C). Sterile deionized water served as a control. Following treatments, 500 g of inoculated and treated sprouts was placed in UV sterile plastic zip lock bags (G. T. Bag Company, Novato, Calif.) and stored at 4°C for further experiments.

**Bacterial enumeration.** Inoculated and chemically treated mung bean sprouts (25 g) were placed in a stomacher bag containing 50 ml of buffered peptone water and homogenized for 2 min with a model 400 circulator Seward stomacher (Seward, London, UK). After homogenization, the sample was serially 10-fold diluted with 9 ml of sterile buffered peptone water. Total mesophilic microorganisms were enumerated after spread plating 10⁶ and 10⁷ CFU per plate on XLD agar and OAB, respectively. Tryptic soy agar was used as a nonselective medium to repair and enumerate injured cells. After the solidification of tryptic soy agar in a petri dish, chemically injured microorganisms were enumerated by spread plating 10⁶ and 10⁷ CFU per plate on XLD agar and OAB, respectively.

**Enumeration of injured Salmonella Typhimurium and L. monocytogenes.** The overlay (OV) method (10) was used to enumerate injured cells of *Salmonella* Typhimurium and *L. monocytogenes* on XLD agar and OAB, respectively. Tryptic soy agar was used as a nonselective medium to repair and enumerate injured cells. After the solidification of tryptic soy agar in a petri dish, chemically injured *Salmonella* Typhimurium or *L. monocytogenes* was inoculated directly onto the medium. After incubation at 37°C for 2 h, to allow injured microorganisms to recover and resuscitate, petri dishes were overlaid with 7 ml of selective medium (OAB or XLD agar). After solidification, the plates were incubated further for an additional 22 h at 37°C. Following incubation, typical black colonies were enumerated. These experiments were duplicated and repeated three times.

**Statistical analysis.** All experiments were repeated three times with duplicate samples. Data were analyzed by analysis of variance by the ANOVA procedure of SAS (SAS Institute, Cary, NC).
N.C.) for a completely randomized design (treatment, storage time, and treatment × storage). When the effect was significant (P < 0.05), means were separated by Duncan’s multiple-range test.

RESULTS AND DISCUSSION

Table 1 shows the populations of total mesophilic microorganisms treated by water or disinfectants (sodium hypochlorite, lactic acid, or chlorous acid). Commercial mung bean sprouts contained about 10⁹ CFU/g of total mesophilic microorganisms per g, and that number was not reduced by submersion in distilled water for 10 min (control group). When stored for 9 days at 4°C, total mesophilic microorganisms increased by more than 2 log units and reached >10⁶ CFU/g. Treatment with sodium hypochlorite for 10 min did not significantly reduce the population of total mesophilic microorganisms (P > 0.05). However, when sprouts were treated with lactic acid or chlorous acid for 10 min, levels of total mesophilic microorganisms were statistically lower (P < 0.05). These two chemicals showed a residual effect in reducing levels of total mesophilic microorganisms after storage for 3 and 6 days at 4°C, resulting in reductions of almost 2 log units after 3 days and 1.5 log units after 6 days (Table 1). Levels of total mesophilic microorganisms on sprouts treated with lactic acid or chlorous acid after 9 days of storage were kept lower than those on sprouts that were not chemically treated (Table 1). These results show that lactic acid and chlorous acid not only initially reduce levels of total mesophilic microorganisms and continue to reduce the population, but also produce a preservative effect, in contrast to the control and sodium hypochlorite treatments, which resulted in rapid population growth under refrigeration.

The effects of sodium hypochlorite, lactic acid, and chlorous acid on the survival of Salmonella Typhimurium and L. monocytogenes on sprouts is shown in Tables 2 and 3. The initial levels of each pathogen were around 5 log CFU/g and were not changed by treatment with distilled water (Tables 2 and 3). Treatment with 200 ppm sodium hypochlorite decreased both pathogens by 1 to 2 log units and maintained this level of reduction for 9 days (Tables 2 and 3). Lactic acid and chlorous acid significantly reduced Salmonella Typhimurium populations immediately after treatment and during cold storage (Table 2). Chlorous acid initially reduced Salmonella Typhimurium to undetectable levels (<0.48 log CFU/g) on XLD agar plates and maintained undetectable levels over 9 days of storage. Treatment with lactic acid resulted in an initial 3-log reduction and further reduction to undetectable levels after 3 days (Table 2).

To date, there have been no research reports that have evaluated the effects of disinfectants on commercial mung bean sprouts after seed sprouting and compared chlorous acid antimicrobial activities with those of other disinfectants. Most studies concerning sprouts have tested seeds, particularly alfalfa seeds, before sprouting and investigated the efficacy of calcium and sodium hypochlorite, hydrogen peroxide, ethanol, and other disinfectants (3,13). Treatment with 2,040 ppm active chlorine reduced the population of Salmonella Stanley on alfalfa seed from a level of 10⁶ CFU/g to undetectable levels (<0.3 log CFU/g) (6). In another study, solutions containing calcium hypochlorite (1,800 ppm), sodium hypochlorite (2,000 ppm), 6% hydrogen peroxide, or 80% ethanol reduced Salmonella on alfalfa seed 1,000-fold after 10 min (3,13). Significant reductions

<table>
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<th>Treatment</th>
<th>Count before treatment</th>
<th>Count on storage day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Water</td>
<td>5.02 ± 0.28 Aa</td>
<td>4.90 ± 0.20 Aa</td>
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<tr>
<td>Sodium hypochlorite (200 ppm)</td>
<td>4.96 ± 0.12 Aa</td>
<td>2.73 ± 0.44 Ab</td>
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<tr>
<td>Lactic acid (2%)</td>
<td>4.97 ± 0.18 Aa</td>
<td>1.88 ± 1.03 Ab</td>
</tr>
<tr>
<td>Chlorous acid (268 ppm)</td>
<td>5.01 ± 0.15 Aa</td>
<td>&lt;0.48 Bc</td>
</tr>
</tbody>
</table>

Data represent means ± standard deviations of three measurements. Means with the same small capital letter in the same row are not significantly different (P < 0.05). Means with the same lowercase letter in the same column are not significantly different (P < 0.05).

Control group; sterile deionized water was used.

TABLE 2. Populations (log₁₀ CFU/ml) of Salmonella Typhimurium following chemical treatments of mung bean sprouts stored at 4°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Count before treatment</th>
<th>Count on storage day:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Water</td>
<td>5.28 ± 0.22 Aa</td>
<td>5.26 ± 0.21 Aa</td>
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<tr>
<td>Sodium hypochlorite (200 ppm)</td>
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<td>4.23 ± 0.53 Ab</td>
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<td>Lactic acid (2%)</td>
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<td>3.33 ± 0.485 Ab</td>
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<tr>
<td>Chlorous acid (268 ppm)</td>
<td>5.24 ± 0.25 Aa</td>
<td>0.75 ± 1.30 Bc</td>
</tr>
</tbody>
</table>

Data represent means ± standard deviations of three measurements. Means with the same small capital letter in the same row are not significantly different (P < 0.05). Means with the same lowercase letter in the same column are not significantly different (P < 0.05).

Control group; sterile deionized water was used.
REDUCTION OF FOODBORNE PATHOGENS IN MUNG BEAN SPROUTS

FIGURE 1. The recovery of injured Salmonella Typhimurium with XLD agar medium used both directly and with the OV method. ■ XLD with agar overlay (OV-XLD); □ XLD used directly; NA, not detected. Bars with different letters are significantly different (P < 0.05).

FIGURE 2. The recovery of injured L. monocytogenes with OAB used both directly and with the OV method. ■ OAB with agar overlay (OV-OAB); □ OAB used directly. Bars with different letters are significantly different (P < 0.05).

in Salmonella populations were usually observed when the concentration of the test chemical was increased (3). Our results showed that lactic acid and chlorous acid strongly reduced or eliminated Salmonella and preserved mung bean sprouts during storage. In particular, treatment with chlorous acid resulted in a significant reduction compared with other treatments without changing the visual quality of the sprouts (data not shown).

Treatment with 268 ppm chlorous acid also produced a strong reduction of L. monocytogenes populations (Table 3). After treatment, a 5-log reduction was observed initially, and a ~4.5-log reduction was maintained during storage (Table 3). When sprouts were treated with lactic acid, they showed a 2-log reduction in L. monocytogenes levels initially, and a further reduction to undetectable levels occurred during storage for 9 days (Table 3).

This study shows that chlorous acid is an outstanding food-grade disinfectant because it is highly effective in reducing or eliminating total mesophilic microorganisms, Salmonella Typhimurium, and L. monocytogenes in the finished product. Moreover, the potential for pathogen growth during the sprouting process underscores the importance of treatments to eliminate pathogens after sprouting, because there is no inherent step to reduce or eliminate pathogens in raw sprouts (13). However, total mesophilic microorganisms showed less reduction than did cultured Salmonella Typhimurium and L. monocytogenes, perhaps because the native microflora on sprouts have more resistance to the antibiotics in selective media than do those cultured in a laboratory.

After antimicrobial treatments, sublethally injured foodborne pathogens assume added significance because they are potentially as dangerous as their uninjured counterparts (7, 8, 10, 12). Thus, in this study, chemically injured Salmonella Typhimurium and L. monocytogenes were also investigated by using the selective OV method after chemical treatments (8, 10). Selective media such as XLD agar for Salmonella Typhimurium and OAB for L. monocytogenes contain agents that can inhibit injured target microorganisms. Nonselective media such as tryptic soy agar can recover sublethally injured microorganisms but do not have the selectivity to isolate specific foodborne pathogens (10). For the OV method, selective agar is added to the top of tryptic soy agar, allowing selective agents to come in contact with recovered microorganisms (10). Thus, this method can enumerate chemically injured cells as well as healthy cells (8, 10). Figure 1 shows the comparison of XLD and OV-XLD media for the enumeration of chemically injured Salmonella Typhimurium. Treatments with sodium hypochlorite and lactic acid resulted in injured Salmonella Typhimurium cell levels of 1.3 and 0.6 log CFU/g, respectively (Fig. 1). However, when inoculated sprouts were treated with chlorous acid, undetectable levels were observed both on OV-XLD plates and on directly plated XLD agar (Fig. 1). There were no significant levels of chemically injured L. monocytogenes cells on OV-OAB plates. Treatment with sodium hypochlorite resulted in 0.4 log CFU of chemically injured L. monocytogenes per g, and treatment with chlorous acid resulted in very few chemically injured cells (Fig. 2). In general, Salmonella Typhimurium was more sensitive to chemical injury than was L. monocytogenes, and treatment with sodium hypochlorite resulted in more chemical injury than did other chemical treatments (Figs. 1 and 2). These results show that chlorous acid can significantly reduce the foodborne pathogens Salmonella Typhimurium and L. monocytogenes. Chlorous acid has the added benefit of not producing chemically injured bacterial cells. Chlorous acid should be tested on other foodborne pathogens.

REFERENCES

4. Food and Drug Administration. 1999. Secondary direct food addi-