Efficacy of Disinfectants To Reduce *Listeria monocytogenes* on Precut Iceberg Lettuce

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MS 06-028: Received 12 January 2006/Accepted 24 March 2006

ABSTRACT

The efficacy of water, chlorinated water (100 ppm), peracetic acid solution (0.05%), and commercial citric acid–based produce wash (0.25%) to reduce the population of *Listeria monocytogenes* on precut lettuce was tested. Samples were inoculated with a mixture of equal amounts of five *L. monocytogenes* strains at a level of 4.7 log CFU/g, and analyzed on the day of washing and after 3 and 6 days of storage at 6°C. Sanitizer reduced the number of *L. monocytogenes* at maximum 1.7 log CFU/g and number of *L. monocytogenes* reached the inoculation level during 6 days of storage. Thus, disinfectants do not eliminate *L. monocytogenes* on precut lettuce and cannot be solely relied on in producing precut lettuce safely. The inoculated *L. monocytogenes* strains were recovered at different rates after 6 days of storage; one of these strains was not recovered at all. Thus, strain-specific differences exist in the ability of *L. monocytogenes* to survive the washing treatments of the lettuce.

The presence of *Listeria monocytogenes* on fresh and preprepared lettuce has been documented in several studies (13, 20, 29, 32). Moreover, the number of outbreaks associated with vegetable consumption has increased in recent years (11) and listeriosis cases have also been associated with vegetables (7, 10, 19, 21, 31). The rise in outbreaks is possibly due to the increasing number of packed, minimally processed vegetables available, and the new packing methods, which have enabled the shelf lives of these products to be extended.

*L. monocytogenes* is able to grow on packed lettuce at relatively low temperatures (15, 24). In addition, increased handling in the preparation of ready-to-eat products intensifies the risk of contamination and consumption without prior heating further increases the risk to consumers. A wide range of sanitizers has been developed to eliminate pathogenic bacteria from fresh produce, and they have been studied extensively for their efficacy to reduce the *L. monocytogenes* population on lettuce (12, 14, 15, 26, 30, 33, 36). The most common sanitizer used to wash preprepared lettuce is chlorine. However, chlorine rapidly loses its effectiveness in contact with organic matter. Furthermore, chlorine can react with trace amounts of organic material to form carcinogenic byproducts (23). Peracetic acid has broad-spectrum antimicrobial activity, even in the presence of heterogeneous organic matter, and it does not produce toxic or mutagenic residuals or byproducts (22). A few studies are available on peracetic acid as a disinfectant for lettuce (28, 30, 36) and it may be a promising alternative to chlorine wash.

Differences exist between *L. monocytogenes* strains in their ability to survive treatment with disinfectants (27) and in their capacity to attach to plant material (17), suggesting that some strains are more difficult to eliminate from lettuce than others. Different *L. monocytogenes* strains have been observed to be more strongly associated with certain food types (9, 18); thus, some *L. monocytogenes* strains may be particularly well adapted to survive on lettuce.

This study was designed to compare the effectiveness of water, chlorine, peracetic acid, and the commercial citric acid–based sanitizer in reducing the populations of five *L. monocytogenes* strains on precut iceberg lettuce and to examine the growth of these strains during subsequent storage. An evaluation was also conducted to verify the effect of the sanitizers on the sensory quality of the lettuce.

MATERIALS AND METHODS

Preparation of lettuce for inoculation tests. Iceberg lettuce imported from Spain was purchased from a Finnish supplier on the day of examination. The heads of lettuce were prepared for treatment by discarding the outer leaves, the heart, and any poor quality parts and then shredding the lettuce with a sharp knife into pieces of approximately 5 by 1 cm.

Inoculation of lettuce samples with *L. monocytogenes*. The inoculum consisted of a mixture of five *L. monocytogenes* strains: NCTC7973, NCTC5214, ATCC 19116, LM206, and LM168. Strains LM206 and LM168 were originally isolated from mixed salads. Each strain was cultured onto blood agar and incubated for 24 h at 37°C. One colony of each strain was then cultured in a brain heart infusion (BHI) broth (Difco, Sparks, Md.) and incubated overnight at 37°C to yield a culture containing approximately 10⁹ bacteria per ml. The inocula were prepared by mixing equal amounts of each culture in a BHI broth followed by dilution with sterile peptone water (0.1%, wt/vol). The inoculation volume was 0.5 ml/100 g of lettuce, and the final number of inoculated bacteria was approximately 4.7 log CFU/g. Amounts of 100 to
300 g of shredded lettuce were placed into sterile plastic bags and weighed, and the inoculum was pipetted evenly onto the lettuce. The bags were shaken to ensure even distribution of bacteria. Inoculated lettuce was allowed to stand 2 h at 5°C before washing treatments commenced.

**Washing procedure.** Lettuce was treated with potable water (control), chlorinated water (100 ppm), 0.05% peracetic acid solution (Fluka, Steinheim, Germany), and a 0.25% solution of commercial citric acid–based produce wash (Fresh Produce Wash, Vegetec International Ltd., Suffolk, UK). The chloride solution was prepared using sodium hypochlorite (Finnish Chemicals Oy, Joutseni, Finland). Commercial citric acid–based produce wash, containing fatty acid saccharose esters, glutamate, monosodium citrate, and glycerol, was used according to the manufacturer’s instructions. The concentrations tested were selected on the basis of the results of the sensory evaluation, literature (1), and the product information of the disinfectant suppliers.

A plastic strainer in a metal bucket was used to wash 500 g of lettuce per 10 liters of washing solution. The procedure consisted of a prewash in tap water for 30 s, followed by a wash in washing solution for 60 s. Water rinsing was performed for 60 s after washes with peracetic acid solution and chlorinated water. Lettuce was dried in a vegetable spin dryer for 60 s.

The experiments were repeated four times. There were duplicate inoculated samples in the first and last trials and uninoculated samples in all but the first trial. Each wash was therefore performed six times for inoculated samples and three times for uninoculated samples.

**Packaging and storage.** Lettuce was packaged in 100-g lots in plastic bags (10 by 15 cm) made of Cryovac (Sealed Air Corporation, Saddle Brook, N.J.) packaging film (O2 permeability 5,300 cm3/m2·day, 101.3 kPa, 23°C) and sealed using a heat sealer. Packages were stored at 6°C for 3 and 6 days.

**Microbiological analyses.** A qualitative procedure was used to verify raw materials for the presence of *L. monocytogenes* and total mesophilic populations were estimated from the aerobic plate count (APC). The inoculation level was tested and the APC was measured in inoculated lettuce before washing. Samples were analyzed on packing day (within 4 h) and after 3 and 6 days of storage for *L. monocytogenes* and APC. In all microbiological analyses, the portions (25 g) of lettuce were aseptically transferred into 225 ml of Fraser broth base (Oxoid, Basingstoke, Hampshire, UK) without added supplement and homogenized in a stomacher blender for 1 min.

Quantitative analyses were carried out to estimate *L. monocytogenes* populations in inoculated samples and the qualitative procedure was applied to the analysis of uninoculated samples. Quantitative *L. monocytogenes* analysis was performed according to the International Organization for Standardization (ISO) method (4), with minor modifications. The initial suspension was prepared in Fraser broth base and decimal dilutions in buffered peptone water. Samples were plated onto *L. monocytogenes* blood agar (LMBA; LAB M, Bury, UK) instead of PALCAM agar, and incubated at 37°C for 48 h. Qualitative *L. monocytogenes* analysis was also performed according to the ISO method (3), with slight modifications. After primary enrichment in half Fraser broth (Oxoid) and secondary enrichment in Fraser broth (Oxoid), the cultures were plated onto PALCAM agar (Oxoid) and LMBA, instead of Oxford agar, and incubated at 37°C for 48 h.

APC was determined according to the NCFA method (5) by preparing a decimal dilution in sterile peptone water and plating onto plate count agars (Difco), which were then incubated at 30°C for 3 days.

**Identification of inoculated *L. monocytogenes* strains with PFGE.** Altogether, 160 *L. monocytogenes* strains from the inoculated lettuce samples were collected after 6 days of storage (i.e., 40 strains after each trial, including 10 strains from each wash). Strains were subjected to pulsed-field gel electrophoresis (PFGE) analyses for identification. In situ DNA isolation and PFGE were performed as described by Autio et al. (8, 9), using Pronase (Roche Diagnostics GmbH, Mannheim, Germany) instead of proteinase K. Digestion was carried out by using restriction endonuclease *AciI* (New England Biolabs, Beverly, Mass.). Samples were electrophoresed in a Gene Navigator system with a hexagonal electrode (Pharmacia, Uppsala, Sweden). The pulse time was ramped from 1 to 35 s for 18 h. Low Range PFG marker (New England Biolabs) was used for fragment size determination. The gels were stained with ethidium bromide and digitally photographed with an Alpha Imager 2000 documentation system (Alpha Innotech, San Leandro, Calif.).

**Sensory evaluation.** The preparation of the lettuce and the washing procedures of the samples for the sensory evaluation were the same as in the inoculation tests, but for shredding a Hälleleo RG-200 (AB Hällede Maskiner, Sweden) shredder was used and washing was performed using a Meiko GK60 (Meiko Maschinenbau GmbH & Co., Germany) vegetable washer. The amount of lettuce not inoculated with *L. monocytogenes* was ca. 6 kg in 56 liters of washing solution in each treatment. After washing, the lettuce was dried in a JMD C-45 spin dryer (500 rpm; Duchateau Food Machinery, The Netherlands), after which it was packed in Cryovac packaging film in 200-g lots and stored at 6°C. The sensory evaluation was performed after 6 days of storage. Sensory characteristics of the lettuce samples were evaluated using descriptive analysis (25). All sensory work was carried out in separate booths at the sensory laboratory, which fulfills the requirements of the ISO standards (2, 6). An experienced sensory panel of five members developed the vocabulary and assessed the samples once. The assessors of the internal sensory panel have passed the basic taste test, the odor test, and the color vision test, and they have been trained in different sensory methods over several years. Attribute intensities were rated on 10-point graphic intensity scales, anchored at their ends. The middle of the scale was marked as 0, indicating the reference sample. The value -5 was anchored as, for example, “less fresh than the reference sample” and the value +5 correspondingly as “more fresh than the reference sample.” The odor was evaluated directly after opening the package. The other attributes were evaluated from samples presented on white disposable plates, without the package. Each assessor received each sample both packed and on a disposable plate. The blind-coded samples (each sample portion was 200 g) were presented to the panelists in random order, except the reference sample, which was marked and presented separately to the panelists. The reference sample used in the evaluations was lettuce from the same batch as the actual samples, cut and washed with potable water on the day of the sensory evaluation and packed in a typical polypropylene film (O2 permeability 900 cm3/m2·day, 50% relative humidity and 23°C). The data were collected using computerized data-gathering system (CSA, Computerized Sensory Analysis System, Compusense 5, version 4.0, Compusense Inc., Guelph, Canada).

**Statistical analysis.** Data from the inoculation tests were subjected to SPSS for Windows for analysis of variance and to Student’s t test.
TABLE 1. Listeria monocytogenes counts on precut lettuce samples inoculated with a mixture (4.7 log CFU/g) of equal amounts of five L. monocytogenes strains before and after washing treatment and after 3 and 6 days of storage at 6°C

<table>
<thead>
<tr>
<th>Washing solution</th>
<th>Before wash</th>
<th>After wash</th>
<th>Reduction by wash</th>
<th>3 days’ storage</th>
<th>6 days’ storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>4.36 ± 0.16</td>
<td>3.87 ± 0.18 A</td>
<td>0.5</td>
<td>4.18 ± 0.47 A</td>
<td>5.42 ± 0.23 A</td>
</tr>
<tr>
<td>Chlorinated water</td>
<td>4.36 ± 0.16</td>
<td>3.62 ± 0.18 B</td>
<td>0.7</td>
<td>3.81 ± 0.47 AB</td>
<td>4.82 ± 0.26 B</td>
</tr>
<tr>
<td>Peracetic acid</td>
<td>4.36 ± 0.16</td>
<td>2.64 ± 0.26 C</td>
<td>1.7</td>
<td>3.07 ± 0.76 B</td>
<td>4.70 ± 0.34 B</td>
</tr>
<tr>
<td>Commercial wash</td>
<td>4.36 ± 0.16</td>
<td>3.37 ± 0.39 B</td>
<td>1.0</td>
<td>3.63 ± 0.26 B</td>
<td>4.74 ± 0.15 B</td>
</tr>
</tbody>
</table>

a Results are reported as means ± standard deviations (n = 6).

b Means in the same column with different letters are significantly different (P < 0.05).

c Commercial citric acid–based produce wash.

RESULTS

L. monocytogenes was not recovered from any of the raw iceberg lettuce. The measured inoculation level of the mixture of five L. monocytogenes strains varied between 4.2 and 4.6 log CFU/g. APCs on inoculated and uninoculated lettuce samples before washing ranged from 4.8 to 5.6 log CFU/g and from 3.8 to 5.7 log CFU/g, respectively (Tables 1 through 3).

Chlorinated water, peracetic acid solution, and commercial citric acid–based produce wash reduced the numbers of L. monocytogenes by 0.7, 1.7, and 1.0 log CFU/g, respectively (Table 1). Water was significantly less effective (P < 0.05) than any of the disinfectants in decreasing the number of L. monocytogenes. Peracetic acid solution reduced the number of L. monocytogenes significantly more (P < 0.01) than commercial citric acid–based produce wash or chlorinated water. APCs on inoculated samples were reduced by 1.0, 1.7, and 0.8 log CFU/g with chlorinated water, peracetic acid, and commercial citric acid–based produce wash, respectively (Table 2). Peracetic acid reduced the APCs significantly more (P < 0.001) than the other disinfectants. On uninoculated samples, the reduction of APCs was 1.2, 2.1, and 1.3 log CFU/g with chlorinated water, peracetic acid, and commercial citric acid–based produce wash, respectively (Table 3).

The number of L. monocytogenes remained lower than inoculation levels during 3 days of storage at 6°C, being significantly lower (P < 0.05) on samples washed with commercial citric acid–based produce wash and peracetic acid than on those washed with water (Table 1). After 6 days of storage, the number of L. monocytogenes had reached the inoculation level on all samples. Samples washed with water had significantly higher numbers (P < 0.05) of L. monocytogenes at the end of the storage period than the samples washed with disinfectants. The size of the initial bacterial flora had no effect on the reduction of L. monocytogenes by washes or the number of L. monocytogenes after 6 days of storage (P > 0.05).

L. monocytogenes strain LM206 was recovered most frequently after 6 days of storage, and it was the most prevalent strain after washes with chlorinated water and peracetic acid (Table 4), whereas strain ATCC 19116 was the most frequent after washes with water and commercial citric acid–based produce wash. Strain NCTC7973 was not present among the 160 strains analyzed after 6 days of storage.

The results from the sensory evaluation of the samples showed that chlorinated water, peracetic acid solution, and commercial citric acid–based produce wash all had fairly minor effects on the sensory quality of lettuce (Fig. 1).

DISCUSSION

All of the washing solutions evaluated decreased the populations of L. monocytogenes on precut iceberg lettuce, peracetic acid being the most effective. Zang and Farber (36) reported peracetic acid to reduce L. monocytogenes by 0 to 0.2 log CFU/g more than tap water. In our study, the reduction was higher, the difference between peracetic acid

TABLE 2. Aerobic plate counts on lettuce samples inoculated with a mixture (4.7 log CFU/g) of equal amounts of five L. monocytogenes strains before and after washing treatment and after 3 and 6 days of storage at 6°C

<table>
<thead>
<tr>
<th>Washing solution</th>
<th>Before wash</th>
<th>After wash</th>
<th>Reduction by wash</th>
<th>3 days’ storage</th>
<th>6 days’ storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>5.21 ± 0.4</td>
<td>4.46 ± 0.31 A</td>
<td>0.8</td>
<td>5.18 ± 0.94 A</td>
<td>7.05 ± 0.59 A</td>
</tr>
<tr>
<td>Chlorinated water</td>
<td>5.21 ± 0.4</td>
<td>4.25 ± 0.26 A</td>
<td>1.0</td>
<td>5.08 ± 0.54 A</td>
<td>6.85 ± 0.54 A</td>
</tr>
<tr>
<td>Peracetic acid</td>
<td>5.21 ± 0.4</td>
<td>3.51 ± 0.36 B</td>
<td>1.7</td>
<td>4.42 ± 0.98 A</td>
<td>6.33 ± 0.87 A</td>
</tr>
<tr>
<td>Commercial wash</td>
<td>5.21 ± 0.4</td>
<td>4.39 ± 0.29 A</td>
<td>0.8</td>
<td>4.75 ± 0.58 A</td>
<td>6.54 ± 0.74 A</td>
</tr>
</tbody>
</table>

a Results are reported as means ± standard deviations (n = 6).

b Means in the same column with different letters are significantly different (P < 0.05).

c Commercial citric acid–based produce wash.
and water being 1.2 log CFU/g. The reduction of *L. monocytogenes* with chlorine wash was about 1 log CFU/g, which is consistent with the results of previous studies (15, 33). The commercial citric acid–based produce wash at 0.25% was as effective as 100 ppm chlorinated water against *L. monocytogenes*. Regardless of the statistically significant differences, the total reduction of *L. monocytogenes* was at maximum 1.7 log CFU/g and none of the sanitizers eliminated *L. monocytogenes* from produce.

The number of *L. monocytogenes* rose during storage, reaching the initial inoculation level prior to 6 days of storage at 6°C. *L. monocytogenes* is reported to be able to grow on lettuce (15, 33), with growth increasing at higher temperatures (29). The optimal upper limit for refrigerated products is lower than in our study, being about 4°C, but in practice the optimal temperature limits are frequently abused before consumption of the produce (34). Our results show that *L. monocytogenes* is able to grow, regardless of the disinfectant used, at typical household refrigerator temperatures. This stresses the importance to allowing only good-quality products into the market to ensure the quality and safety of fresh-cut lettuce for the consumer. Initial inoculation levels of *L. monocytogenes* were achieved during storage and, although the counts of *L. monocytogenes* were lower on samples washed with disinfectants than on those washed with water, the differences reduced during the storage. Therefore, using disinfectants to wash lettuce does not solely assure the safety of the product. Venkitanarayanan et al. (35) stated that, compared with water wash, disinfectants prevent the build-up of pathogens in washing solution, and thus, the use of sanitizers is advisable. The disinfectants studied are usable in practice because they had only minor effects on the sensory characteristics of the product.

The APCs were reduced more with 0.05% peracetic acid treatment than with the other washing solutions. Some researchers have found peracetic acid to be less effective in reducing APCs on lettuce than most of the other disinfectants tested, including chlorine (28, 30), but they had used peracetic acid at a lower concentration (80 ppm). The concentration that we used (500 ppm) appears to be more optimal for washing lettuce.

The initial size of the background microflora did not affect the reduction or the growth of *L. monocytogenes*. Thus, the size of the native flora probably has no effect on growth of *L. monocytogenes* on lettuce, a conclusion supported by Delaquis et al. (15) and Francis and O’Beirne (16). However, the composition of the initial bacterial flora, which may be affected by the washing procedures, might have an impact on growth of *L. monocytogenes* (16).

Recovery of the inoculated *L. monocytogenes* strains after 6 days of storage clearly showed that there were strain-specific differences in the ability to survive on precut lettuce. The differences may be due to variable ability to tolerate different disinfectants or to differences in attachment to the lettuce surface, or to a combination of these. Different *L. monocytogenes* strains are known to have variable resistance to disinfectants (28) and this may lead to better survival of certain strains during the washing of the lettuce. Gorski et al. (17) observed that strain-specific differences exist in the ability of *L. monocytogenes* to attach to the surface of alfalfa sprouts and the poorest colonizing strain was unable to attach to the sprout. In our study, one

### TABLE 3. Aerobic plate counts on uninoculated lettuce samples before and after washing treatment and after 3 and 6 days of storage at 6°C

<table>
<thead>
<tr>
<th>Washing solution</th>
<th>Before wash</th>
<th>After wash</th>
<th>Reduction by wash</th>
<th>3 days’ storage</th>
<th>6 days’ storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>4.67 ± 0.82</td>
<td>3.51 ± 0.65</td>
<td>1.2</td>
<td>4.85 ± 0.77</td>
<td>6.28 ± 0.93</td>
</tr>
<tr>
<td>Chlorinated water</td>
<td>4.67 ± 0.82</td>
<td>3.48 ± 0.77</td>
<td>1.2</td>
<td>3.77 ± 0.99</td>
<td>5.79 ± 1.19</td>
</tr>
<tr>
<td>Peracetic acid</td>
<td>4.67 ± 0.82</td>
<td>2.55 ± 0.32</td>
<td>2.1</td>
<td>3.27 ± 0.75</td>
<td>5.31 ± 0.42</td>
</tr>
<tr>
<td>Commercial wash</td>
<td>4.67 ± 0.82</td>
<td>3.36 ± 0.93</td>
<td>1.3</td>
<td>3.62 ± 0.98</td>
<td>5.11 ± 0.73</td>
</tr>
</tbody>
</table>

*a* Results are reported as means ± standard deviations (*n* = 3).

*b* Commercial citric acid–based produce wash.

### TABLE 4. Number of different *L. monocytogenes* strains isolated from lettuce samples, inoculated with a mixture of equal amounts of five *L. monocytogenes* strains, after different washes and 6 days of storage at 6°C

<table>
<thead>
<tr>
<th>Inoculated <em>L. monocytogenes</em> strain</th>
<th>Water (<em>n</em> = 40)</th>
<th>Chlorinated water (<em>n</em> = 40)</th>
<th>Peracetic acid (<em>n</em> = 40)</th>
<th>Commercial wash* (<em>n</em> = 40)</th>
<th>Total (<em>n</em> = 160)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM206</td>
<td>14 (35)</td>
<td>21 (53)</td>
<td>22 (55)</td>
<td>11 (28)</td>
<td>68 (43)</td>
</tr>
<tr>
<td>ATCC 19116</td>
<td>16 (40)</td>
<td>9 (23)</td>
<td>5 (13)</td>
<td>18 (45)</td>
<td>48 (30)</td>
</tr>
<tr>
<td>LM168</td>
<td>4 (10)</td>
<td>10 (25)</td>
<td>11 (28)</td>
<td>10 (25)</td>
<td>35 (22)</td>
</tr>
<tr>
<td>NCTC5214</td>
<td>6 (15)</td>
<td>0 (0)</td>
<td>2 (5)</td>
<td>1 (3)</td>
<td>9 (6)</td>
</tr>
<tr>
<td>NCTC7973</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*a* Commercial citric acid–based produce wash.
strain (NCTC7973) could not be detected on lettuce after storage, and this strain may not have been able to attach to the lettuce surface, thus being easily removed with all of the washing methods. Gorski et al. (17) did not find any relation between attachment and source of the strains, but they had no strains originating from vegetables, unlike the present study. In our study, both of the strains originally isolated from salads were recovered, with one of these being the most recovered strain of all. This may indicate that strains originating from salads possess some characteristics that make them more adapted to environmental conditions encountered in packed freshly cut lettuce. To our knowledge, the strain-specific ability of \textit{L. monocytogenes} to survive on lettuce has not been studied previously. However, more studies are needed to elucidate the factors influencing the ability of certain \textit{L. monocytogenes} strains that grow better on lettuce than others. Clearly, the detected difference between strains provides evidence that single strain observations are inadequate.

With regard to the production of the precut lettuce, our data demonstrates that the number of \textit{L. monocytogenes} is reduced by the sanitizers, but none of the washing solutions can eliminate \textit{L. monocytogenes} from produce, and therefore, high-quality raw material and good manufacturing practices remain important. The ability of some \textit{L. monocytogenes} strains to survive well on lettuce raises a challenge for the food production industry because these strains are especially difficult to remove from the products.

**ACKNOWLEDGMENTS**

We are grateful to Rajia Keijama and Jaana Nieminen for excellent technical assistance. The Ministry of Agriculture and Forestry in Finland is acknowledged for financial support.

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