Fate of *Listeria monocytogenes* on Ready to Serve Lettuce

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ABSTRACT

The ability of *Listeria monocytogenes* to survive and grow on head lettuce obtained from a retail outlet over a period of 10 months was determined. Lettuce was torn into bite sized pieces, inoculated with *L. monocytogenes* ATCC 7644, placed into plastic bags, and held under a variety of storage conditions. Samples stored at 5°C and 12°C were subjected to aerobic plate count analysis, and levels of *L. monocytogenes* were determined immediately after inoculation and after 7 and 14 d of storage. Samples stored at 25°C were sampled after inoculation and after 4 and 8 h storage. Lettuce juice was inoculated, stored at 5°C and sampled as described for head lettuce. Aerobic plate counts on lettuce stored at 5°C and 12°C increased greatly during the 14 d of storage. Behavior of *L. monocytogenes* was variable. In most trials, numbers increased by several log cycles during 14 d of storage, but in several trials growth never occurred or did not persist for 14 d. The same general growth patterns were observed in lettuce held at 25°C. Aerobic plate counts increased by 1 or 2 log cycles and *L. monocytogenes* increased by 1 log cycle, except for occasional trials where the organisms did not grow or survive. Lettuce juice held at 5°C was also able to support growth of *L. monocytogenes*. *L. monocytogenes* serotype 1 was isolated from some uninoculated samples indicating that the organism was naturally present on some of the lettuce heads purchased from retail outlets.

Emphasis on the consumption of fresh vegetables for a healthful diet has increased during recent years. Fresh vegetables are exposed to microorganisms and conditions which further microbial multiplication during growth, transport and preparation for consumption (9,14,18,22,23,27,34). Vegetable exudate released through handling and shipping damage and/or salad preparation creates an available source of nutrients to encourage microbial growth (4,21,23,27).

Fresh lettuce is a widely used salad vegetable. The number and variety of microorganisms associated with lettuce indicate it provides them a good growth environment (10,12,14,18,20,23,34). *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella* have been isolated from salads of unspecified ingredients (9,28,29). Disease causing organisms, including *Salmonella*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus*, *Erwinia herbicola*, *Hafnia alvei* and *Yersinia intermedia* have been isolated from lettuce or lettuce containing salads (5,7,18,19,34).

Fresh salad vegetables are becoming a matter of concern as a potential source of *L. monocytogenes* in human infections. *L. monocytogenes* is widespread in nature and has been isolated from soil and vegetation (32,33) as well as from sick and healthy individuals and animals (3,11,17,26). *L. monocytogenes* has been reported to survive from nine months to ten years under various circumstances (13,31). This wide dissemination of *L. monocytogenes* coupled with its long term survival provide ample opportunity for the contamination of fresh salad vegetables. In fact, Hofer (16) detected three untypable *L. monocytogenes* strains on lettuce. Ho et al. (15) implicated raw celery, tomatoes and lettuce in an outbreak of *L. monocytogenes* among hospital patients. Cabbage is another salad vegetable implicated as a source of human listeriosis (26).

Enhancing the appearance of salads and controlling microbial growth by sulfiting agents is no longer permitted (30). Therefore, environmental temperature control becomes more important in controlling microbial growth. However, *L. monocytogenes* have been reported to grow in tryptose broth at 6°C (1) and in cabbage juice and raw shredded cabbage at 5°C (2,8). The organism has survived in cottage cheese stored at 3°C (24) and in Mexican-style cheese assumed to have been held under normal refrigeration conditions (6). Thus, *L. monocytogenes* has the potential to survive and grow at normal refrigeration temperatures for salad vegetables.

The potential for association of *L. monocytogenes* with lettuce, combined with the possibly lethal effects of listeriosis (6,15,26) create a potential public health concern when lettuce, whole or ready-to-serve, is refrigerated or held at salad bar ambient temperatures prior to consumption. The purpose of this work was to determine the fate of *L. monocytogenes* on lettuce prepared and stored for use in salads.

MATERIALS AND METHODS

Culture of *L. monocytogenes*

A representative isolate, *Listeria monocytogenes* ATCC

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inoculum that allowed for manual L. monocytogenes provide a growth medium was diluted 1:100 in sterile distilled water to

The uninoculated control was similarly treated except sterile distilled water. The lettuce was drained and torn into bite sized pieces. The pieces were tossed 24 times. The uninoculated control was similarly treated except sterile distilled water was used in place of the inoculum. Samples were stored in sealed plastic bags and held at one of three temperatures. Storage was at 5°C to simulate normal, good preservation and at 12°C to represent normal mishandling. Lettuce held at 5°C and 12°C were sampled immediately after inoculation and after 7 and 14 d storage. To simulate ambient storage conditions, inoculated and uninoculated lettuce in sealed and open plastic bags was held at 25°C. These samples were plated immediately and after 4 and 8 h storage. Unless otherwise indicated, the procedure was based on five replications of each treatment. 

**Selection and Identification of Isolates**

Random 11-g subsamples were selected for plating and dispersed in 99 ml phosphate-magnesium chloride buffer (25) via a Stomacher (Tekmar Co., Cincinnati, OH). Total Plate Counts were obtained by surface plating decimal dilutions on Plate Count Agar (PCA; Difco) and incubating for 48 h at 35°C. To determine the population of L. monocytogenes, five isolates were chosen from each of the duplicate countable PCA plates and streaked on McBride Listeria Agar (MLA). These were selected by a statistically unbiased method by making a cross on the bottom of each plate and then making a circle approximately 60 mm in diameter. One colony was chosen based on the proximity to the center of each intercept. This method allowed evaluation of population density of L. monocytogenes by proportionality. MLA was prepared by adding 1% glycine anhydride and 0.05% lithium chloride to Phenylethanol Agar (Difco). The L. monocytogenes culture was used as a control. Plates were incubated 24 h at 35°C. Inoculated sample isolates were examined for L. monocytogenes by observing for a blue appearance of colonies on MLA with oblique transmitted light, Gram stain, catalase and oxidase production and rotating tumbling motility in wet mounts. Control isolates from 5°C and one trial at 12°C were screened by Gram stain and MLA growth. Isolates from uninoculated lettuce which were small Gram positive rods produced blue MLA colonies were examined for catalase, oxidase and urease production, wet mount and SIM motility, nitrate reduction, methyl red, Voges-Proskauer and Triple Sugar Iron Agar reactions, utilization of dextrose, esculin, maltose, rhamnose, mannitol and xylose, and B-hemolysis by the CAMP test on human blood. Listeria O Antisera types 1 and 4 were used for serotyping by the rapid slide test (Difco).

**Preparation of lettuce juice**

Lettuce juice was prepared as previously reported by processing in an Acme Juicerator (Acme Juicer Mfg. Co., Sierra, CA) (21). Two 100-ml samples were taken with one serving as the control. L. monocytogenes inoculum was prepared as before and 0.1 ml added to the other sample. Both samples were plated immediately following inoculation and after 7 and 14 d incubation at 5°C. Sample plating, selection and examination of isolates was identical to that for head lettuce.

**RESULTS AND DISCUSSION**

L. monocytogenes grew in the variety of conditions employed to simulate good preservation, normal mishandling and ambient serving conditions. Results of L. monocytogenes growth in lettuce juice at 5°C are given in Table 1. In three of five trials, L. monocytogenes competed effectively with the inherent flora in lettuce juice and averaged 1.9 x 10^7 Colony Forming Units (CFU)/ml after 7 d incubation and 9.9 x 10^7 CFU/ml after 14 d incubation. In two other trials, L. monocytogenes was not detected in the lettuce juice at any time. Apparently the inoculum was not high enough to be detected in these experiments. These data were not used in assessing the growth of L. monocytogenes. In these trials the Aerobic Plate Counts (APC) for the uninoculated lettuce and lettuce inoculated with L. monocytogenes were comparable to those for the trials in which L. monocytogenes grew.

L. monocytogenes grew on the lettuce stored at 5° and 12°C producing 10^4 CFU/g after 7 d incubation and 10^6 CFU/g after 14 d at either temperature (Tables 2 and 3). However, this represented slightly over one log growth in 14 d at 5°C and slightly over three logs growth at 12°C for 14 d. In an additional trial at 12°C L. monocytogenes failed to grow. In another trial at 12°C L. monocytogenes grew to 1.2 x 10^7 CFU/g at 7 d and then decreased to undetectable levels at 14 d. In two trials at 5°C, L. monocytogenes grew to 2.9 x 10^6 and 4.2 x 10^5 CFU/g after 7 d and declined to undetectable levels after storage for 14 d. In trials at 5° and 12°C, the APC for uninoculated and L. monocytogenes inoculated lettuce were comparable to those for the trials where L. monocytogenes grew.

L. monocytogenes grew on lettuce stored at 25°C in sealed or open bags (Tables 4 and 5) producing a one and one-half log increase in CFU/g in sealed samples and a log increase in CFU/g in open samples. With another sealed sample, L. monocytogenes grew to 1.5 x 10^4 CFU/g at 4 h and was then undetectable after 8 h incubation. L. monocytogenes multiplied throughout the storage period in only three of the five trials run. Data represent results from only those three trials.

**TABLE 1. Fate of natural flora and Listeria monocytogenes in lettuce juice at 5°C.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>CFU/ml</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated lettuce</td>
<td>1.0x10^3</td>
<td>3.7x10^3</td>
<td>9.0x10^7</td>
<td></td>
</tr>
<tr>
<td>Lettuce inoculated with L. monocytogenes</td>
<td>9.3x10^7</td>
<td>7.6x10^7</td>
<td>1.7x10^7</td>
<td></td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>7.2x10^7</td>
<td>1.9x10^7</td>
<td>9.9x10^7</td>
<td></td>
</tr>
</tbody>
</table>

*L. monocytogenes* multiplied throughout the storage period in only three of the five trials run. Data represent results from only those three trials.

* Aerobic plate count represented as antilog of average log.
* CFU calculated on the basis of relative frequency of occurrence of *L. monocytogenes* among CFU on plate count agar from lettuce inoculated with *L. monocytogenes*. 

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TABLE 2. Fate of natural flora and Listeria monocytogenes on lettuce at 5°C.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated lettuce</td>
<td>5.3x10^5</td>
<td>2.1x10^7</td>
<td>1.4x10^7</td>
</tr>
<tr>
<td>Lettuce inoculated with L. monocytogenes</td>
<td>3.2x10^6</td>
<td>1.3x10^6</td>
<td>1.1x10^7</td>
</tr>
</tbody>
</table>

*L. monocytogenes* multiplied throughout the storage period in only two of the four trials run. Data represent results from only those trials.

aAerobic plate count represented as antilog of average log.

bCFU calculated on the basis of relative frequency of occurrence of *L. monocytogenes* among CFU on plate count agar from lettuce inoculated with *L. monocytogenes*.

cL. monocytogenes* multiplied throughout the storage period in only three of the five trials run. Data represent results from only those three trials.

dAerobic plate count represented as antilog of average log.

eCFU calculated on the basis of relative frequency of occurrence of *L. monocytogenes* among CFU on plate count agar from lettuce inoculated with *L. monocytogenes*.

TABLE 3. Fate of natural flora and Listeria monocytogenes on lettuce at 12°C.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated lettuce</td>
<td>5.9x10^5</td>
<td>8.7x10^6</td>
<td>6.3x10^6</td>
</tr>
<tr>
<td>Lettuce inoculated with L. monocytogenes</td>
<td>6.7x10^5</td>
<td>1.4x10^6</td>
<td>2.2x10^8</td>
</tr>
</tbody>
</table>

*L. monocytogenes* multiplied throughout the storage period in only three of the five trials run. Data represent results from only those three trials.

aAerobic plate count represented as antilog of average log.

cCFU calculated on the basis of relative frequency of occurrence of *L. monocytogenes* among CFU on plate count agar from lettuce inoculated with *L. monocytogenes*.

TABLE 4. Fate of natural flora and Listeria monocytogenes on lettuce at 25°C (sealed sample).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated lettuce</td>
<td>3.0x10^4</td>
<td>7.8x10^5</td>
<td>1.3x10^6</td>
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<tr>
<td>Lettuce inoculated with L. monocytogenes</td>
<td>5.9x10^4</td>
<td>1.5x10^5</td>
<td>1.8x10^6</td>
</tr>
</tbody>
</table>

*L. monocytogenes* multiplied throughout the storage period in only four of the five trials run. Data represent results from only those four trials.

aAerobic plate count represented as antilog of average log.

cCFU calculated on the basis of relative frequency of occurrence of *L. monocytogenes* among CFU on plate count agar from lettuce inoculated with *L. monocytogenes*.

dAerobic plate count represented as antilog of average log.

eCFU calculated on the basis of relative frequency of occurrence of *L. monocytogenes* among CFU on plate count agar from lettuce inoculated with *L. monocytogenes*.

TABLE 5. Fate of natural flora and Listeria monocytogenes on lettuce at 25°C (open sample).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated lettuce</td>
<td>1.4x10^5</td>
<td>5.8x10^5</td>
<td>6.9x10^5</td>
</tr>
<tr>
<td>Lettuce inoculated with L. monocytogenes</td>
<td>1.4x10^5</td>
<td>2.8x10^5</td>
<td>1.2x10^6</td>
</tr>
</tbody>
</table>

*L. monocytogenes* multiplied throughout the storage period in only three of the four trials run. Data represent results from only those three trials.

aAerobic plate count represented as antilog of average log.

cCFU calculated on the basis of relative frequency of occurrence of *L. monocytogenes* among CFU on plate count agar from lettuce inoculated with *L. monocytogenes*.

L. monocytogenes failed to grow in one open sample stored at 25°C. Again, in both of these trials, the APC for uninoculated and *L. monocytogenes* inoculated lettuce were comparable to those for trials where *L. monocytogenes* grew.

Over 1300 random isolates were examined from the combined trials for uninoculated and inoculated lettuce. These isolates formed the basis upon which the relative frequency of occurrence of *L. monocytogenes* among CFU was calculated.

An interesting phenomenon of this experiment was the failure of *L. monocytogenes* to initiate or maintain growth equally well in multiple trials under conditions which were identical so far as could be determined. No relation could be found between the phenomenon and several experimental factors, including: the season of the year, appearance of the lettuce at the end of the trial (taken as an indication of general spoilage), the original normal flora count versus the final *L. monocytogenes* count or the difference between the original normal flora and *L. monocytogenes* counts versus the final *L. monocytogenes* count. The most likely explanation was a low inoculum in these trials. However, two factors which may be of importance were not examined during the experiment - the pH of the stored lettuce and the identity of the inherent flora on the lettuce where *L. monocytogenes* failed to initiate or maintain growth. Since lettuce can support the growth of a wide variety of microorganisms, it is possible that in these trials *L. monocytogenes* failed to compete with the inherent flora. Additionally, the presence of organisms able to ferment lettuce may have caused the pH to fall to a level where growth of *L. monocytogenes* could have been affected. Furthermore, the cause of the phenomena may be inherent in the characteristics of the lettuce or *L. monocytogenes*. Lettuce provides many discrete microenvironments. These conditions contribute to variable results and no two samples would be expected to be identical. Fowler and Foster (12) reported total plate counts ranging from 10^3 to 10^5 CFU/g for fresh lettuce prepared to go on a serving line. Survival of *L. monocytogenes* soil samples also showed wide variation (31). Thus, the phenomenon of variability extends beyond fresh vegetables. The reasons for this variation are not known, but the information could be useful in inhibiting the growth of *L. monocytogenes*.

*L. monocytogenes* was isolated from two uninoculated samples, indicating that the organism was naturally present on some of the lettuce heads purchased from retail outlets. From over six hundred randomly chosen isolates from uninoculated lettuce, four were identified as *L. monocytogenes* serotype 1. Three of the isolates came from one head of lettuce. *L. monocytogenes* were found on the uninoculated lettuce in mid-July and early August when the warm humid environment would encourage bacterial growth. It can be speculated that more *L. monocytogenes* may have been isolated if the outside lettuce leaves had not been removed as part of the experimental procedure. It should be noted that a number of lettuce samples appeared...
acceptable for human consumption in spite of a high *L. monocytogenes* count. *L. monocytogenes* serotype 1 has been found on other vegetation (32) and isolated from lettuce purchased at the retail outlet. Although this sample may be too small to extrapolate to retail lettuce as a whole, it raises the question of the public health significance of *L. monocytogenes* on lettuce.

Lettuce, celery and tomatoes were implicated in the Boston hospital outbreak of listeriosis (15) and assumed to have involved only small amounts of vegetables since they are referred to as accompanying other foods. A small infective dose may be all that is required to produce listeriosis in debilitated patients. *L. monocytogenes* has been found on lettuce and can multiply under good refrigeration, mishandling and ambient serving conditions. Viewed in light of the potential adverse effects of listeriosis, the public health significance of *L. monocytogenes* in nature is questionable.

REFERENCES


