Coliforms and Prevalence of *Escherichia coli* and Foodborne Pathogens on Minimally Processed Spinach in Two Packing Plants

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**ABSTRACT**

Minimally processed spinach has been recently associated with outbreaks of foodborne illnesses. This study investigated the effect of commercial minimal processing of spinach on the coliform and *Escherichia coli* counts and the prevalence of *E. coli* O157:H7, *Salmonella*, *Shigella* spp., and *Listeria monocytogenes* on two types of spinach before and after minimal processing. A total of 1,356 spinach samples (baby spinach, n = 574; savoy spinach, n = 782) were collected daily in two processing plants over a period of 14 months. Raw spinach originated from nine farms in the United States and three farms in Canada. Overall, the proportion of samples positive for coliforms increased from 53% before minimal processing to 79% after minimal processing (P < 0.001). Average total coliform counts also increased significantly after processing, especially in baby spinach (mean ± standard deviation, 1.16 ± 0.14 log CFU/g to 2.37 ± 0.08 log CFU/g following processing; P < 0.001). *E. coli* was isolated from 8.9% of the samples (mean ± standard deviation, 1.81 ± 1.4 log CFU/g), and no difference in prevalence or CFU counts after processing (P > 0.1) was observed. *E. coli* O157:H7 and *Shigella* spp. were not isolated from any of the samples. *Salmonella* and *L. monocytogenes* were isolated from 0.4 and 0.7% of samples, respectively. Results demonstrate that commercial minimal processing of spinach based on monitored chlorine washing and drying may not decrease microbial load on spinach leaves as expected. Further research is needed to identify the most appropriate measures to control food safety risk under commercial minimal processing of fresh vegetables.

Consumption of minimally processed vegetables has grown in the last decade due to their convenience and to healthier diet trends. Fresh spinach consumption alone grew by 130% between 1999 and 2006 in the United States (45). Despite numerous nutritional benefits (32, 49), the consumer’s choice to include fresh vegetables in daily diet is currently influenced by the awareness of an increasing number of reported outbreaks of foodborne disease (14).

Outbreaks of foodborne illness attributed to consumption of fresh produce have increased from 1% in the 1970s to 12% in the 1990s (36). From 1990 to 2004, fresh produce was responsible for the largest number of foodborne illness cases caused by any single commodity, constituting 21% in the Center for Science in the Public Interest database (37). A recent outbreak linked to fresh spinach caused 205 illnesses and three deaths (3) and led to significant losses for the fresh spinach industry (21). The exact source of contamination in that outbreak was never clearly identified. The identification of a precise source of contamination of fresh spinach is often difficult, as contamination can occur anywhere in the farm-to-fork continuum. Risk management tools used currently for controlling contamination of fresh green leafy vegetables are based on prevention and are mostly centered on good agricultural practices and good manufacturing practices, which provide guidelines to prevent and reduce biological contamination of fresh vegetables from known sources (4, 16, 43). While these guidelines set up a risk management framework based on current understanding of potential hazards, there are insufficient data for validation of specific targets during minimal processing of vegetables as a measure of success for these risk mitigation strategies.

Several studies have reported coliform and *Escherichia coli* counts on leafy green vegetables at the farm level (19, 22) and at the retail and catering level (10, 31). However, information regarding the impact of processing practices is limited. Although there are reports assessing other produce (13, 38), only one study reporting coliform count changes before and after washing of leafy greens in an on-site packaging facility is available (26).

This study investigated the effect of a commercial minimal processing practice on the bacterial load (coliforms and *E. coli*) and prevalence of potential foodborne pathogens (*E. coli*, *Salmonella*, *Shigella* spp., and *Listeria monocytogenes*) in two types of fresh spinach in a processing plant setting over a period of 14 months. The effects of seasonality and product type were also determined.

**MATERIALS AND METHODS**

Samples. Savoy (curly leaves) and baby (flat leaves) spinach (*Spinacea oleracea*) samples were collected in two processing/
packing plants from January 2006 through March 2007. Spinach originated from nine farms in the United States (four in Texas, and one each in Arizona, California, Colorado, Maryland, and New Jersey) and three farms in Canada (two in Ontario and one in Quebec). Spinach samples were collected daily before and after minimal processing (washing and packing) in paired and unpaired fashion. Upon arrival, a 500-g composite sample of raw material was collected from every incoming truck load for each farm. Each truck load represented approximately 900 crates of 225 ml was added to 25 g of sample and homogenized in a stomacher for 2 min at 230 rpm. Samples were diluted in 0.1% peptone water, inoculated on Petrifilm and read after 24 h of incubation at 35°C.

Analysis of E. coli O157:H7 was conducted using an enzyme-linked immunosorbent assay (ELISA; Reveal, Neogen Corporation, Lansing, Mich.) on 20-h broth (modified tryptic soy broth with novobiocin) enrichment cultures of 50-g samples. ELISA-positive samples were cultured for isolation of E. coli O157:H7 as previously described (47).

The detection of Salmonella was conducted using an immunomagnetic separation method (34). Suspect colonies were further biochemically and serologically confirmed following a previously described protocol (7). Presumptive-positive Salmonella isolates were subsequently serotyped by the Health Canada Laboratory for Foodborne Zoonoses, Guelph, Ontario, Canada. The detection of Shigella was conducted following a previously described protocol (18).

Listeria spp. and L. monocytogenes detection was performed as previously described (28). Confirmation of suspect colonies was performed through biochemical and serological tests as previously described (24). When a specimen tested positive, the enumeration of Listeria monocytogenes in the original samples was performed (27).

Statistical analysis. Microbial counts were expressed as log CFU per gram. Associations between plants, processed and unprocessed spinach, product type, and microbial indicator counts were investigated by using a generalized linear model. The differences in proportions of samples positive for E. coli before and after minimal processing were compared using the chi-square test. The distribution of coliform counts was analyzed using the Kolmogorov-Smirnov test (35). Statistical data analysis was performed in Minitab statistical software (version 15.1.2007, Minitab Inc.).

RESULTS

In total, 1,356 samples (baby spinach, 574; savoy spinach, 782) were collected between January 2006 and March 2007. Data for January 2007 were not available. When available, paired samples (before and after processing) were collected. Minimally processed spinach made up 68.7% (n = 931) of all tested samples. Four hundred twenty-five (31.7%) samples were collected upon arrival and did not undergo further processing. Forty percent of baby spinach and 95% of savoy spinach samples were processed in plant A, while the remainder was processed in plant B. The number of samples tested per organism of interest is shown in Table 1.

When the results for baby spinach minimally processed in plants A and B were analyzed, no differences in coliform counts (P = 0.69) or E. coli-positive samples (P = 0.52) between the two plants were observed. No comparison was made for savoy spinach because most of it was processed in plant A.

A significant increase in coliform counts on spinach following minimal processing was observed for both types of product (P < 0.001). The increase of coliform counts was particularly large for baby spinach (mean ± standard deviation, from 1.16 ± 0.14 log CFU/g to 2.37 ± 0.08 log CFU/g). Overall, 53% (n = 215) of unprocessed spinach was coliform positive whereas minimally processed product tested positive for coliforms 79% (n = 688) of the time. The observed difference was significant (P < 0.001).

After processing, 43 and 20.8% of total samples had coliform counts of >10³ and >10⁴ CFU/g, respectively. The corresponding counts for unprocessed spinach were 25 and 12.4%.

Analysis of paired data showed that coliform counts decreased after processing in only 20% of cases. For the majority of cases (80%) either no difference (27%) or an increase (53%) in coliform counts was observed after processing (Fig. 1).
TABLE 1. Number of spinach samples analyzed for bacterial contamination

<table>
<thead>
<tr>
<th>Organism(s)</th>
<th>Spinach product</th>
<th>Commercial process</th>
<th>Total no. tested/organism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baby Savoy</td>
<td>Unprocessed</td>
<td>Processed</td>
</tr>
<tr>
<td>Coliforms</td>
<td>564</td>
<td>712</td>
<td>405</td>
</tr>
<tr>
<td>E. coli/E. coli O157:H7</td>
<td>574</td>
<td>782</td>
<td>425</td>
</tr>
<tr>
<td>Salmonella/Shigella</td>
<td>561</td>
<td>750</td>
<td>404</td>
</tr>
<tr>
<td>Listeria spp.</td>
<td>409</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

*a Only processed baby spinach was tested for Listeria.

*b NT, not tested.

Minimal processing did not have a significant effect on the counts of E. coli \( (P = 0.12) \). Of all tested samples, 8.9\% were contaminated with E. coli. The difference between samples positive for E. coli before (8.4\%) and after (9.0\%) minimal processing was also not significant \( (P = 0.64) \). Most of the contaminated samples yielded very low numbers (mean of positive samples \pm standard deviation, 1.81 \pm 0.8 \log \text{CFU/g}). Only in 2.8\% (38 of 122) and 2.4\% (33 of 122) of positive samples was E. coli present in numbers greater than \( 10^2 \) and \( 10^3 \) \text{CFU/g}, respectively. There was no significant difference between the two types of spinach in the E. coli counts detected \( (P = 0.65) \).

Of all samples tested for Listeria spp., 1.2\% (5 of 409) were positive. Listeria seeligeri was detected in two samples. L. monocytogenes was isolated from three samples (0.7\%), one unprocessed and two minimally processed. The positive samples originated from the same farm and had counts of L. monocytogenes of \(<100 \text{CFU/g}. All positive samples (five of five) had coliform counts of \( >1,000 \text{CFU/g}\) and were negative for E. coli and all other pathogens. No further molecular typing analyses were performed with these isolates.

Salmonella was detected in 0.4\% (5 of 1,311) of tested samples. All Salmonella isolates were recovered from savoy spinach; one from unprocessed and four from processed spinach samples. All five samples had different origins and contained coliform counts of \( >1,000 \text{CFU/g} \) (five of five) and were positive for E. coli (three of five). Salmonella enterica serovar Kentucky and Salmonella enterica serovar Copenhagen were among the serovars isolated. Shigella spp. or E. coli O157:H7 organisms were not isolated in this study.

Throughout the study, month-to-month variability was observed in coliform counts and prevalence of E. coli as shown in Figure 2 \( (P < 0.001) \).

DISCUSSION

In this study, minimal processing resulted in either no change or increased coliform contamination in most (>80\%) lots of processed product despite rigorous control of chlorine concentrations and ORP. Reduction of microbial populations on produce depends on the type of the produce surface being washed, the type of natural microflora present (25), and whether the contaminant is located on the surface, in the form of biofilms, or internalized. Laboratory-based studies have shown that pathogens, spoilage bacteria, and
thermotolerant coliforms do not survive longer than 30 s at ORP values of 665 mV or higher (39). As the chlorine activity in this study was maintained under constant ORP (750 to 800 mV) and pH (6.8 to 7.0) and the wash water was regularly tested and was negative for coliforms and E. coli, it was unlikely that the increase occurred through contaminated input water. The equipment was sanitized daily and tested for organic residues with ATP swabs, so the likelihood that the increase can be attributed solely to cross-contamination from equipment was also low. The most logical and parsimonious explanation is that coliforms and other organisms were transferred from some of the more heavily contaminated product. It is also possible that the washing step might have enhanced coliform proliferation on the leaves by increasing water content on the leaf surfaces.

Furthermore, it is possible that the microbial populations naturally present on leaf surfaces have different susceptibility to chlorine treatment and that chlorine treatment may create the conditions for growth of certain bacterial populations that favor or limit coliform and pathogen survival. Population dynamics of specific coliforms was not tested in this study. According to previously published findings, the presence of some bacterial populations on leaf surfaces or environment contributed to the observed reduction or increase of pathogens (5, 12). Various microorganisms have been shown to interact with pathogens in different environments at the growing, processing, and storage levels (9, 21, 33, 48).

Previous reports on the effects of minimal processing on microbial loads on fresh vegetables are too conflicting to make an objective comparative analysis. Although no studies of spinach in commercial plants are available, results of the present study are in agreement with those of Johnston et al. (17), who found a significant increase in coliform counts in cilantro (1.4 log) and parsley (1.7 log) during processing, but the details of processing methodology were not given by the authors. Likewise, increase of coliforms has been observed for other produce after washing. For instance, Gagliardi et al. (13) reported an increase of coliforms on melons from a range of 2.5 to 3.5 log CFU/g before washing to a range of 4 to 5 log CFU/g after washing. In contrast, other studies demonstrated that minimal processing, specifically washing, reduces microbial loads on fresh produce (11, 25, 26).

Reported reduction with chlorine washes is usually 1 to 2 log for pathogens on various commodities (25). Discrepancies in coliform counts and changes that occur during washing may be attributed to differences in experimental conditions including temperature, storage, washing treatment, and type of vegetables. In addition, most of the reported results are based on experiments performed in laboratory. Although this study was based on only two processing plants, the changes in coliform contamination observed in this study indicate that similar outcomes could be observed in other plants using similar processing practices. Further studies are required to investigate the limited efficacy of these processing protocols to control microbial counts on the product.

Chlorine washes are most commonly used by the fresh-vegetable processing industry, and their primary role is to maintain the sanitary conditions of wash water and prevent cross-contamination, while the reduction of microbial role is their secondary role. Phillips and Harrison (26) reported a 0.62- to 1.11-log reduction of coliform counts after washing a spring mix with 5 ppm of chlorine wash. The efficacy of chlorine washes depends on the activity of chlorine (ORP) in washes, contact time, and the susceptibility of the contaminating microflora.

Results from this study suggest that seasonal variations exist with a higher level of contamination in summer and fall months. This is of interest, as this time period seems to coincide with major U.S. outbreaks of E. coli O157 recently reported in association with consumption of spinach and lettuce (37). However, spinach processed during the summer was of a different origin than spinach processed during the winter season, and this may be a confounding factor for seasonal variations.

The incidence of E. coli was consistent with a number of other studies reporting the presence of E. coli in 8.9% of lettuce samples (20) and 8% of leafy green vegetables (22) and in 14 of 214 spring mix samples (26). Lower and higher prevalences were also found, for instance, 1.5% in the United Kingdom (30) and 0.8% in the United States (17). Higher prevalence of E. coli on lettuce, parsley, and dill (29.4%) was reported in Turkey by Aycicek et al. (1) and on conventionally grown leafy green vegetables (24%) by Mukharjee et al. (23). Garcia-Villanova Ruiz et al. (29) in 1987 found E. coli in 86% of tested fresh vegetables. These differences can be attributed to multiple factors, such as seasonality and location. Moreover, the prevalence of E. coli depends upon the type of vegetables (23). Although there is some inconsistency in the literature regarding what is referred to as leafy green vegetables (17), in general, leafy greens and lettuce carry higher microbial loads than other fresh vegetables (22).

This study demonstrated that the prevalence of foodborne pathogen contamination in commercially available products is minimal but may exist in commercially processed products and represents a risk for serious outbreaks.
TABLE 2. Prevalence of Salmonella and L. monocytogenes in various salad vegetables

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Product</th>
<th>Prevalence (%)</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella</td>
<td>Ready-to-eat vegetables</td>
<td>0.2</td>
<td>United Kingdom</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>0.3</td>
<td>United States</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Leafy greens</td>
<td>0.7</td>
<td>United States</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Salad vegetables</td>
<td>3.0</td>
<td>Brazil</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Salad vegetables</td>
<td>7.5</td>
<td>Spain</td>
<td>29</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>Salad vegetables</td>
<td>0.6</td>
<td>Brazil</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>1.1</td>
<td>Norway</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Fresh vegetables</td>
<td>1.4</td>
<td>France</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Lettuce</td>
<td>2.5</td>
<td>Australia</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Ready-to-eat vegetables</td>
<td>6.1</td>
<td>Canada</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td>0.7</td>
<td>United States/Canada</td>
<td>This study</td>
</tr>
</tbody>
</table>

if postprocessing proliferation is not prevented with appropriate handling and storage practices. The presented data are in agreement with previous reports for Salmonella and L. monocytogenes (Table 2).

Low numbers of isolated pathogens preclude any powerful modeling of coliform and E. coli counts with pathogen presence. Despite the increase in coliform counts, E. coli levels were in general low, and the occurrence of pathogens was sporadic. The relevance of coliform and E. coli levels on the food safety of fresh produce remains unclear. These organisms are used as indicators of food safety risk in water and various foods, where they are generally associated with pathogens that originate from similar environments (e.g., intestinal pathogens) and are also able to survive in foods (44). Coliform counts have been recently used to indicate food safety of raw milk in legislative microbial standards (2). While they certainly are indicators of microbial contamination, coliforms may not be appropriate indicators in the risk assessment of food safety of minimally processed vegetables because they may not exhibit the same behavior during processing.

The interpretation of the presence and number of coliform on produce is unclear at this time. The presence of coliforms does not necessarily indicate fecal contamination (8). Nevertheless, evidence of coliforms is a measure of microbial contamination of the produce from unknown sources such as the environment and possibly animal and human waste.

This study has provided valuable information on the effectiveness of commercial minimal processing of spinach and a base for further research of complex relationships between total coliforms, E. coli, and pathogens on leaf surfaces to determine effective measures of treatments for reduction of microbial load and removal of pathogenic microorganisms in minimal processing of vegetables.

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