IS LOCAL PRODUCE SAFER?

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Abstract

There is a growing demand for fresh, safe, high-quality, and locally grown vegetables. This study compared microbial populations in Romaine lettuce, Bibb lettuce, and spinach procured from grocery stores and farmers' markets throughout the course of a summer. Standard microbial techniques were used to analyze 42 samples for the presence of total aerobic mesophilic and psychrophilic bacteria; yeasts and molds; surface and internalized coliforms and *Escherichia coli*; and the pathogens *E. coli* O157:H7 and *Salmonella* spp. Large variations in counts were found between produce types, sampling days, and between grocery and farmers' market samples. The average highest microbial loads were associated with spinach samples from the grocery store, with both total aerobic mesophilic and psychrophilic counts greater than 7.1 log CFU/g. Average psychrophilic counts were higher than mesophilic microorganisms in all samples tested. In general, lettuce from farmers' markets had more bacterial, yeast, and mold presence than lettuce from grocery stores.

Mentors

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coliforms, *E. coli*, grocery store, farmers' market, internalization, lettuce, mesophilic bacteria, molds, psychrophilic bacteria, spinach, yeasts

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IS LOCAL PRODUCE SAFER?:

Microbiological Quality of Fresh Lettuce and Spinach from Grocery Stores and Farmers' Markets

Emiria Soendjojo, Food Science

INTRODUCTION

Raw vegetables and ready-to-eat salads are reservoirs of microorganisms, including bacteria, molds, and yeasts, which can be introduced into the plant environment during cultivation, harvest, transport, marketing, and even by the consumer. Many of these microorganisms are not harmful and are part of the natural background microflora of the plant (Brandl, 2006). However, human pathogenic bacteria, such as *Escherichia coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, and *Shigella* spp., have been associated with foodborne outbreaks involving fresh produce (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004; Tauxe, 1997). The number of reported outbreaks that involved fresh produce as the known vehicle for transmission has been increasing. Among outbreaks reported in the US in the 1970s, less than 1% were associated with fresh produce; however, this increased to over 6% in the 1990s (Sivapalasingam et al., 2004). Many factors, including an overall increase in the consumption of fresh fruits and vegetables, have contributed to the frequency of produce-related outbreaks (Pollack, 2001). Increased research efforts in this area of food safety are developing a better understanding of not only what pathogenic bacteria can be associated with fresh produce, but also how the bacteria are introduced onto or into the plant and methods to minimize contamination (Avila-Quezada, Sanchez, Gardea-Bejar, & Acedo-Felix, 2010; Brandl, 2006; Critzer & Doyle, 2010; Deering, Mauer, & Pruitt, 2012a; Lynch, Tauxe, & Hedberg, 2009).

Many microorganisms can be found on the surface of the plant, as well as internalized in the inner tissues of

the plant (Deering et al., 2012a). Bacteria are able to reach the interior portions of the plant through natural openings, such as stomata, lenticels, broken trichomes, and areas of emergence of lateral roots (Kroupitski et al., 2009; Quadt-Hallmann, Benhamou, & Kloepper, 1997; Saldana, Sanchez, Xicohtencatl-Cortes, Puente, & Giron, 2011). In addition, human pathogenic bacteria (such as *E. coli* O157:H7 and *Salmonella* spp.) are able to internalize within the plant tissue following contamination that has occurred to the seed (Deering, Pruitt, Mauer, & Reuhs, 2011, 2012b; Warriner, Ibrahim, Dickinson, Wright, & Waites, 2003), seedling (Warriner, Spaniolas, Dickinson, Wright, & Waites, 2003), soil (Bernstein, Sela, & Neder-Lavon, 2007; Beuchat, Scouten, Allen, & Hussey, 2003; Franz et al., 2007; Hora, Warriner, Shelp, & Griffiths, 2005), and wash/irrigation water (Buchanan, Edelson, Miller, & Sapers, 1999; Hintz, Boyer, Ponder, Williams, & Rideout, 2010; Ibarra-Sanchez, Alvarado-Casillas, Rodriguez-Garcia, Martinez-Gonzales, & Castillo, 2004; Mootian, Wu, & Matthews, 2009; Penteado, Eblen, & Miller, 2004). Bacteria that are internalized within the plant are problematic because they are protected from the effects of sanitizers that are routinely used in the fresh produce industry to reduce the number of bacteria that are associated with the plants (Buchanan et al., 1999; Zhuang, Beuchat, & Angulo, 1995). Because they cannot be washed off, if pathogenic bacteria are present in the internal structures of fresh produce and they survive the sanitization process, then they may cause illness following consumption of the contaminated produce. In 2006, there were *E. coli* O157:H7 outbreaks associated with both spinach and lettuce, resulting in 71 illnesses in

the lettuce outbreak and 204 illnesses and three deaths in the spinach outbreak (Zimmer, 2008). If high levels of spoilage-causing bacteria have internalized in the plant, even in the absence of pathogens, then the shelf life and overall quality of the produce will be decreased.

Fresh produce can also be contaminated with various yeasts and molds. These organisms, like bacteria, can be introduced to the plant at any time during the cultivation and distribution process (Tournas, 2005). Some of these organisms, such as *Alternaria*, *Rhizopus*, and *Aspergillus*, can contribute to an increased rate of spoilage in various vegetables that ultimately reduces the shelf life and/or quality of the products (Banwart, 1979). Some yeasts and molds can also produce toxic metabolites, called mycotoxins, that are pathogenic to humans if consumed (Tournas, 2005). This is of greatest concern when the populations are high, and, given that many molds can grow in refrigerator storage conditions normal for fresh produce, even a low starting population present on a plant may be sufficient to cause illness (Tournas, 2005). Sanitizers and washing steps are routinely used in the fresh produce industry to reduce the number of microorganisms that are associated with the plants (Lee & Baek, 2008; Neal et al., 2011; Vandekinderen, Devlieghere, Meulanaer, Ragaert, & Van Camp, 2009). Having an assessment of the mycological profile of fresh produce allows for a better determination of the overall quality of the produce.

The objective of this study was to compare the microbial populations in Romaine lettuce, Bibb lettuce, and spinach procured from grocery stores and farmers' markets throughout the course of a summer. Standard techniques were used to enumerate the populations of total aerobic mesophilic and psychrophilic bacteria, yeasts and molds, and coliforms. Following a surface sterilization technique, the number of coliforms and *E. coli* internalized within the inner tissues of the leaves were also determined. In addition, selective plating media were used to identify the presence of the human bacterial pathogens *E. coli* O157:H7 and *Salmonella* spp. from the fresh produce samples. This work provides a baseline assessment of the populations of microorganisms present on fresh produce samples that originated from different sources (grocery store vs. farmers' market).

MATERIALS AND METHODS

Sampling and Preparation of Produce Samples

A total of 42 produce samples (Romaine lettuce, Bibb lettuce, and spinach) were obtained from grocery stores

and farmers' markets in West Lafayette, Indiana, from May through August. No additional washing steps were applied to the produce after purchase, and the samples were stored at 4°C until the analysis was performed. The samples included 13 Romaine lettuce samples from the grocery store, 8 Bibb lettuce samples from the grocery store, 8 Romaine lettuce samples from the farmers' market, 6 Bibb lettuce samples from the farmers' market, and 7 spinach samples from the grocery store. No spinach samples from the farmers' market were included in the study due to the inconsistent availability of spinach. Only leaves that were undamaged were used for the microbial analyses.

Aerobic Mesophilic and Psychrophilic Plate Counts

Following the methods described in the *Bacteriological Analytical Manual* for determination of total aerobic bacteria (Maturin & Peeler, 2001), 25 g of leaf sample was weighed and transferred into 225 mL of sterile 1% buffered peptone water in a sterile stomacher bag. The sample was homogenized in a stomacher (Stomacher 400 Laboratory Blender, Seward Laboratory Systems, Bohemia, NY, USA) for 120 s. Dilutions were made by transferring 1 mL of sample into 9 mL of sterile 1% buffered peptone water as the diluent and repeating until all dilutions (10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5}) were made. Duplicate 100 μ L samples from each dilution were spread plated on Plate Count Agar (PCA; BD Diagnostic Systems, Franklin Lakes, NJ, USA) for each type of analysis. For the mesophilic plate counts (also called total plate counts), all plates were incubated at 37°C for 24 hours and then the number of colonies was recorded (colony forming units, CFU). For the psychrophilic plate counts, plates were stored at 7°C for 5 days and then the number of colonies was recorded.

Yeast and Mold Count

Following the methods described in the *Bacteriological Analytical Manual* for determination of yeasts and molds (Tournas, Stack, Mislivec, Koch, & Bandler, 2001), 25 g of leaf sample was weighed and transferred to 225 mL of 1% buffered peptone water in a sterile stomacher bag. The sample was homogenized in a stomacher for 120 s. The samples were diluted using sterile 1% buffered peptone water as the diluent, as described above, and 100 μ L of the appropriate dilutions (10^{-2} , 10^{-3} , 10^{-4}) was spread plated on Potato Dextrose Agar (PDA; BD Diagnostic Systems, Franklin Lakes, NJ, USA) in duplicate. All plates were held at room temperature for 72 hours and then the number of colonies was recorded.

Enumeration of Coliforms and *E. Coli*

Total coliforms and total *E. coli* were enumerated by following the 3M Petrifilm manufacturer's procedure (3M Petrifilm Coliform Count Plates; 3M, St. Paul, MN, USA). Plates were prepared in duplicate and inoculated with 1 mL of sample dilutions 10^{-1} , 10^{-2} , and 10^{-3} . The plates were incubated at 37°C for 24 hours. Confirmed coliforms presented as red colonies associated with gas bubbles. Levels of contamination were calculated as colony-forming units per gram (CFU/g).

Surface Sterilization

In addition to total coliform and *E. coli* counts, it was also of interest to determine what fraction of the coliform population was not on the surface, but instead internalized in the plant tissue. Following a modified protocol for surface sterilization of produce leaves (Sharma et al., 2009), 25 g of leaf sample was weighed and washed using a 0.6% hypochlorite solution (sodium hypochlorite, Sigma-Aldrich, St. Louis, MO, USA) for 30 seconds, followed by washing with sterile water. The samples were then washed briefly with 70% ethanol and rinsed thoroughly with sterile water. Once the surfaces were sterilized, then the method described above for enumerating coliforms was followed.

Enrichment for Presumptive *Salmonella* spp. and *E. Coli* O157:H7

Twenty-five grams of produce sample were weighed and transferred to 225 mL of pre-enrichment media (buffered peptone water for *Salmonella* spp. and modified *E. coli* broth for *E. coli* O157:H7) in sterile stomacher bags. The sample was homogenized in a stomacher for 120 s and incubated at 37°C for 16 hours.

Isolation of *E. Coli* O157:H7 and *Salmonella* spp. Using Dynabeads

Isolation of *E. coli* O157:H7 and *Salmonella* spp. was performed using Dynabeads per the manufacturer's instructions (Dynabeads, Invitrogen). A 1 mL sample (prepared as described previously) was aseptically transferred to a sterile tube and 20 µL of Dynabeads anti-*E. coli* O157 or anti-*Salmonella* spp. was then added. The tube was inverted several times and held for 3 minutes at room temperature. The beads were concentrated using a magnetic particle concentrator (Dynal MPC) and were washed with sterile PBS-Tween (PBS; 0.15M NaCl, 0.01M Sodium-Phosphate buffer, pH 7.4, with 0.05 % Tween-20). Finally, the beads were suspended in 100 µL of 0.1% NaCl.

The supernatant was removed and 1 mL of washing buffer was added into each tube. This was repeated 2 times, and then 100µL of 0.1% NaCl was added to each tube. For identification of *E. coli* O157:H7, 100 µL of sample was spread plated on Sorbitol MacConkey agar supplemented with Cefixime-Tellurite plates (CT-SMAC; BD Diagnostic Systems). For identification of *Salmonella* spp., 100 µL of sample was spread plated on Xylose Lysine Desoxycholate Agar (XLDA; BD Diagnostic Systems). All plates were incubated at 37°C for 24 hours and the number of positive colonies recorded.

Multiplex Polymerase Chain Reaction (PCR)

Positive colonies were picked into 3 ml of Luria Bertani (LB) broth and incubated overnight at 37°C with shaking at 100 rpms. Amplification reactions were performed in a final volume of 20 µL containing 2 µL of the liquid culture (whole cells), 200 µM dNTPs (Promega),

<i>E. coli</i> O157:H7 Target	Primer Sequence	<i>Salmonella</i> spp. Target	Primer Sequence
eaeA	5'-CAGGTCGTCGTGTCTGCTAAA-3'	ompF	5'-CCTGGCAGCGGTGATCC-3'
	5'-TCAGCGTGGTTGGATCAACCT-3'		5'-AAATTTCTGCTGCGTTTGCG-3'
uidA	5'-TGATGCTCCATAACTTCCTG-3'	iroB	5'-TGC GTATTCTGTTTGTCGGTCC-3'
	5'-GCGAAACTGTGGAATTGGG-3'		5'-TACGTTCCCACCATTCTTCCC-3'
rfb	5'-CATTGGCATCGTGTGGACAG-3'	hist	5'-ACTGGCGTTATCCCTTCTCTGGTG-3'
	5'-AAGATTGCGCTGAAGCCTTTG-3'		5'-ATGTTGTCCTGCCCTGGTAAGAGA-3'
fliC	5'-GCGCTGTGCGAGTTCTATCGAGC-3'	hilA	5'-CTGCCGCAGTGTTAAGGATA-3'
	5'-CAACGGTGACTTTATCGCCATTCC-3'		5'-CTGTGCGCCTTAATCGCATGT-3'

Table 1. List of primer sets used for the multiplex PCR verification of presumptive *E. coli* O157:H7 and *Salmonella* spp. from lettuce samples.

Type and Source of Produce	Total Mesophilic Plate Count	Total Psychrophilic Plate Count	Total Yeast and Mold	Total Coliform	Internalized Coliform	Internalized <i>E. coli</i> Coliform
Romaine Lettuce Grocery	3.5×10^5	7.5×10^6	2.7×10^3	6.5×10^0	1.2×10^4	0
Bibb Lettuce Grocery	2.0×10^5	1.4×10^7	2.8×10^3	2.7×10^2	5.8×10^1	0
Romaine Lettuce Farmers' Market	1.1×10^6	6.4×10^6	2.1×10^4	5.9×10^3	1.3×10^4	0
Bibb Lettuce Farmers' Market	1.6×10^6	7.9×10^6	6.8×10^3	3.4×10^3	1.1×10^3	0
Spinach Grocery	$>3 \times 10^6$	$>3 \times 10^7$	2.6×10^3	4.1×10^1	4.5×10^3	0

Table 2. Total number of microorganisms present (mean CFU/g) on Romaine lettuce, Bibb lettuce, and spinach obtained from grocery stores and farmers' markets.

Figure 1. Representative LB plate showing the various types of microorganisms (identified by different colors and size/shape of colonies) present on the surface of a lettuce leaf following plating for the enumeration of total mesophilic aerobic bacteria.

PCR Buffer (50 mM KCl, 10 mM Tris pH 9.0, 0.1% Triton X-100, 2mM $MgCl_2$), 0.5 units of Tag DNA polymerase (Bioron), and 5 pM each of the forward and reverse primers (Table 1; Integrated DNA Technologies, Inc.). PCR amplification was performed using a PTC-100 programmable thermal controller (MJ Research, Inc.) with the temperature cycling as follows: 95°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 25 seconds, and extension at 68°C for 1 minute, with a final extension at 68°C for 10 minutes. The PCR products were size-separated by Tris-Borate-Ethylenediaminetetraacetic acid (TBE)-buffered agarose gel electrophoresis. Agarose (2%) gels were run for 1 ½ hours at 120V in 1X TBE running buffer (89mM Tris-Base, 89mM Boric Acid, 2mM EDTA pH 8.9) (modified from Lolle, Hsu, & Pruitt, 1998).

RESULTS AND DISCUSSION

Many different microorganisms were visible (identified by differences in color and shape/size of the colonies) following plating for the enumeration of the total number of aerobic bacteria present on the lettuce and spinach leaves (Figure 1). It was hypothesized that produce sampled from the farmers' market would have fewer bacteria due to a shorter amount of time between harvesting and selling. This is in comparison to most fresh produce at the grocery store that has a longer time between harvest and sale, and possibly more handling steps in the distribution

chain compared to farmers' market produce. However, the grocery store produce has also likely been treated with various chemicals that are designed to increase the shelf life of the product (Park, Alexander, Taylor, Costa, & Kang, 2008; Singh, Singh, Bhunia, & Stroshine, 2002). As the time between harvest and consumption increases, there is more time for microorganisms to grow in the samples. Differences were observed between the microbial quality of the different types of produce and the location (grocery store vs. farmers' market) at which the produce was purchased (Table 2).

Contrary to our hypothesis, the lettuce obtained from the farmers' market had a higher number of microbes present in total mesophilic plate counts compared to grocery store samples. The total bacteria enumerated from Bibb lettuce obtained from the farmers' market was 1.6×10^6 CFU/g compared to 2.0×10^5 CFU/g (Figure 2) enumerated from Bibb lettuce from the grocery store. The Bibb lettuce from the farmers' market contained approximately 8 times more bacteria than the Bibb lettuce from the grocery store. This trend was also true for the Romaine lettuce samples where the total bacteria enumerated from the farmers' market samples was 1.1×10^6 CFU/g compared to 3.5×10^5 CFU/g (Figure 2) enumerated from Romaine lettuce from the grocery store. The Romaine lettuce from the farmers' market

contained approximately 3 times more bacteria than the Romaine lettuce from the grocery store. Together these data indicate that the lettuce available at the farmers' market contains a much greater number of bacteria compared to the lettuce obtained at the grocery store. This could be due to the sterilization treatments used to increase the shelf life of the produce available at the grocery store (Park et al., 2008; Singh et al., 2002), and this could indicate that these treatments are effective at reducing the microbial population on the produce.

The spinach from the grocery store had the highest number of total bacteria ($> 3 \times 10^6$ CFU/g, Figure 2) out of all of the samples tested. Due to the inconsistent availability of spinach from the farmers' market, we do not have a comparison of spinach from grocery stores and farmers' markets. The high number of bacteria enumerated from the spinach compared to the lettuce samples may be the result of differences in the surface composition and morphology between the plants. It has been shown that differences in how well a bacterium is able to attach and colonize a plant can vary depending on the type of plant examined (Patel & Sharma, 2010). For example, differences in attachment were observed for various *Salmonella enterica* serovars that were examined on both lettuce and cabbage plants. This may be attributed to the differences in composition and structure

Figure 2. Total mesophilic bacteria plate count (log CFU/g) results for Romaine lettuce, Bibb lettuce, and spinach obtained from the grocery store and farmers' market.

Figure 3. Psychrophilic bacteria plate count (log CFU/g) results for Romaine lettuce, Bibb lettuce, and spinach obtained from the grocery store and farmers' market.

Figure 4. Total yeasts and molds counts (log CFU/g) for Romaine lettuce, Bibb lettuce, and spinach obtained from the grocery store and farmers' market.

Figure 5. Total coliform plate counts (log CFU/g) for Romaine lettuce, Bibb lettuce, and spinach obtained from the grocery store and farmers' market.

Figure 6. Total internalized coliform plate counts (log CFU/g) present within Romaine lettuce, Bibb lettuce, and spinach leaves obtained from the grocery store and farmers' market. Analyses were conducted on leaves that had been surface sterilized.

of the waxy cuticle covering the leaf surface (Patel & Sharma, 2010). Similar differences in the cuticle between the spinach and lettuce cultivars examined in this study may be present that could account for the variation in the total number of bacteria present between samples. In addition, differences between cultivation, harvesting, and handling practices of spinach and lettuce could also be contributing factors to the differences in microbial populations enumerated.

The total number of psychrophilic bacteria was similar between each of the 5 types of produce samples tested (Figure 3), although there was a trend that the grocery sample of a type of lettuce had a higher psychrophilic count than its farmers' market counterpart. Psychrophilic counts identify the level of microorganisms that are able to survive and grow in refrigeration temperatures (7°C), where consumers store most fresh produce. The trend in having higher numbers of psychrophilic microbes in grocery samples would be consistent with the refrigerated distribution and storage of produce that ends up on the grocery store shelf. This time in the refrigerator could enable the psychrophilic microbes to grow. If the farmers' market samples had not received the same temperature treatment prior to purchase, then it is feasible that the psychrophilic counts would be lower in these samples. Interestingly, the number of psychrophilic bacteria

present in every sample was higher than the number of mesophilic bacteria. The reason for this is not fully understood.

The total number of yeasts and molds are also similar between samples, with the exception that the Romaine lettuce from the farmers' market had approximately 8 times more yeasts and molds compared to the other samples tested (Figure 4). A previous study found that yeasts were the most prevalent organisms found in minimally processed vegetables, with yeast counts ranging from less than 100 to 4×10^8 CFU/g and mold counts ranging from less than 100 to 4×10^4 CFU/g (Tournas, 2005). The combined yeast and mold counts in our study were well below the highest levels reported by Tournas (2005). The higher number of yeasts and molds present in the farmers' market sample may be a reflection of the growing environment of the plant, as well as how the plants were handled post-harvest. Yeasts and molds can be introduced onto the produce from workers' hands during harvest and handling (Tournas, 2005). If the farmers' market samples did not receive the same level of sanitization treatment as the grocery samples, then the higher yeast and mold counts could be expected. Since the trend in higher counts on farmers' market samples is consistent between mesophilic aerobic plate counts, molds, and yeasts, this is a likely scenario.

Figure 7. Representative plate showing presumptive positive *E. coli* O157:H7 colonies present on a CT-SMAC plate.

Figure 8. Representative plate showing presumptive positive *Salmonella* spp. colonies visible on a XLDA plate.

All of the samples tested had coliforms present on the surface, as well as internalized within the interior portions of the leaves (Figures 5 and 6). A study that examined the presence of coliforms on fresh produce (lettuce, cabbage, cucumber, tomato, and green pepper) from both organic and conventional farms reported that 92% of the produce was positive for coliforms and the mean counts were similar between the two types of farming operations (Mukherjee, Speh, Dyck, & Diez-Gonzalez, 2004). This indicates that the results obtained for the presence of coliforms in this study are similar to what has been observed previously. The number of internalized coliforms present in both lettuce and spinach indicates that these bacteria are able to internalize and survive within the plant tissue. Internalized bacteria would also likely survive post-harvest sanitization measures and be present within the samples that are sold to consumers. However, no internalized *E. coli* coliforms were found in any of the samples (Table 2). *E. coli* is typically used as a reference indicator for fecal contamination (Jay, 2000). Although there are many studies that have reported *E. coli* isolation from fresh produce (Jay, 2000; Mukherjee et al., 2004), the absence in these samples indicate that good agricultural practices were utilized at the farms to produce lettuce and spinach that are safe for human consumption.

There were several presumptive *E. coli* O157:H7 (Figure 7) and *Salmonella* spp. (Figure 8) positive colonies obtained during the study. Different bacterial strains may have the same morphological and biochemical characteristics on the agar plates that can lead to false-positive results when using selective media for identification (Pollock & Dahlgren, 1974; Wallace & Jones, 1996). To avoid incorrect interpretation of the colonies, the identity of these colonies was verified by multiplex PCR using *E. coli* O157:H7 and *Salmonella* spp. specific primers (Table 1). None of the samples tested using PCR were positive, and therefore, the colonies obtained were considered to be false-positives. The combined result of no *E. coli* coliforms and no pathogens indicates that there is no evidence of mishandling or contamination with pathogenic bacteria in the fresh produce samples tested.

CONCLUSION

In this study, we used several different methods to characterize the microbial quality of fresh lettuce and spinach samples obtained from the grocery stores and farmers' markets. Large variations in counts were found between produce types, sampling days, and purchase locations. In general, the lettuce from farmers' markets had more bacterial (mesophilic plate count) and yeast and mold contamination than the lettuce from grocery stores. The spinach from the grocery store had the highest number of bacteria of all samples tested. Despite the differences in the microbial populations found, all samples were "safe" in that no human pathogens were identified in any sample tested and no *E. coli* coliforms were found.

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