Prevalence of *Salmonella* and *E. coli* on Produce from Seattle Farmers Markets

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A thesis
submitted in partial fulfillment of the
requirements for the degree of

Master of Science

University of Washington
2013

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Program Authorized to Offer Degree:
Environmental Health

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1 INTRODUCTION

Farmers markets nationwide have grown drastically in numbers and popularity over the past decade. These markets are particularly integrated in the Seattle community, providing diverse communities with access to fresh produce from local farms. However, due to limited oversight and regulation for food safety among farmers markets, the extent of microbial contamination on farmers markets produce is unknown. CDC and Washington State foodborne outbreak records indicate that raw foods are associated with foodborne disease outbreaks, and that *Salmonella*, *E. coli,* and Norovirus (NoV) are etiologic pathogens associated with the consumption of raw produce. Lettuce and tomatoes in particular have been implicated as common vehicles in outbreaks of *Salmonella* and *E. coli*. In light of several recent multi-state foodborne outbreaks involving raw produce and the growing popularity of farmers markets, this study investigating the prevalence of *Salmonella* and *E. coli* on produce from Seattle farmers markets may aid in characterizing the risk of consuming farmers markets produce.

1.1 Significance of a Farmers Markets Study

Farmers markets have been growing in popularity in the past few decades. According to USDA data from 1994 to 2012, the numbers of farmers markets in directory listings have been increasing over the past two decades. Most recently, there was a 9.6% increase in the number of farmers markets from 2011 to 2012.[1] Figure 1, below, shows USDA data documenting farmers markets numbers

increasing over the years. Farmers markets provide communities with access to fresh produce, as well as a significant portion of income to local farmers. Seattle's farmers market consumers are diverse; since Seattle's farmers markets accept Electronic Benefit Transfer (EBT) and Senior Farmers Market Nutrition Program (SFMNP) vouchers, low-income shoppers, in addition to higher-income shoppers, are able to be regular consumers of farmers markets produce. In 2007, Seattle's Neighborhood Farmers Market alliance reported 250,000 shoppers – an increase of 20,000 shoppers from the previous year.[2] The increasing accessibility to farmers markets and growing numbers of markets and shoppers indicate higher exposure to potentially contaminated produce from farmers markets.

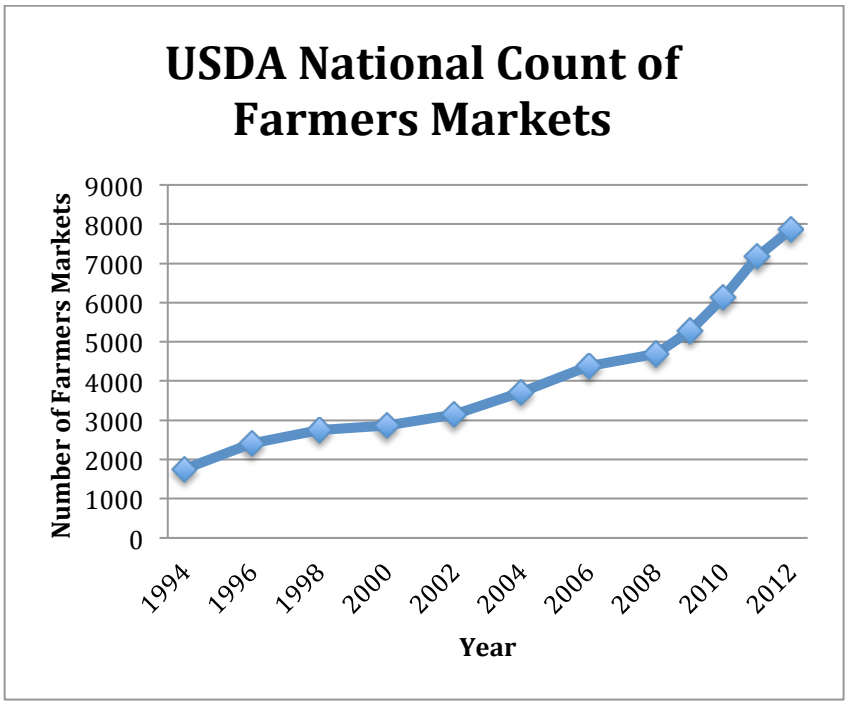

Figure 1 Growing Number of Farmers Markets, USDA 1994-2012

There are regulations in place for farmers markets in King County, WA. The updated 2011 Seattle/King County Department of Public Health (SKCDPH) requires

that Seattle Farmers Market Association produce vendors handing out samples must have on-site hand wash setups, protection guards, equipment to cut and display samples, washing of produce before sampling, and no bare hand contact with samples. Also required by the Seattle Farmers Market Association is that produce "must be fresh and have no residue that cannot be removed by normal washing." When there are complaints about quality and presentation of produce, however, market staff members who may not be food safety professionals are responsible for resolving complaints.[3] Aside from sampling procedures and qualitative produce presentation, there are no county level regulations in place regarding the sanitation of produce sold at farmers markets.

On the state level, farmers can sell most fresh produce without inspection by Washington State Department of Agriculture (WSDA). Currently, the only fruits and vegetables inspected are fresh apricots, Italian prunes, peaches, cherries, apples, pears, potatoes and asparagus. WSDA also refers to local county health departments to enforce their sampling regulations for vendors. However, WSDA recognizes that unprocessed fruits and vegetables "can be at risk for microbiological contamination during production and harvest"[4], and refers farmers to Good Agricultural Practices (GAPs) outlined by the USDA, FDA, and CDC. WSDA and other state departments of agriculture have collaborated with the USDA to establish the USDA's Good Agricultural/Good Handling Practices (GAP/GHP) Audit Program. GAPs are non-enforceable guidelines for farmers to minimize microbial hazards for fresh fruits and vegetables; having a GAP/GHP audit is voluntary and only performed by the request of a farmer. According to Kenneth Petersen, Section Head of Audit Programs

at the USDA Agricultural Marketing Services, an average of 200-250 growers in Washington are inspected each year. Most of these growers are large-scale producers who sell to grocery stores (i.e. Safeway, QFC) that require a third party auditor (such as the USDA) to inspect produce prior to purchasing directly from growers. The majority of these growers are producers of apples and potatoes (Personal Communication, Apr 17, 2013). With the review of regulations at the county, state, and federal level, it can be concluded that there is limited oversight on fresh produce sold at farmers markets. With the accessibility of farmers markets produce, growing number of shoppers, and overall limited regulatory measures for fresh produce sold at farmers markets, investigation of the extent of contamination on produce is warranted.

1.2 Foodborne Pathogens

1.2.1 *Salmonella*

Members of the *Salmonella* genus are gram-negative, rod-shaped enterobacteria with peritrichous flagella. There are two species, *S. enterica* and *S. bongori*, that can be divided into thousands of serogroups associated with human disease.[5] Based on dose-response models of *Salmonella* outbreak data, the ID_{50}–median infectious dose–may be as low as 7 cells for infection and 36 cells for illness among serotypes *S.* Enteritidis and *S.* Typhimurium.[6] Salmonellosis can be asymptomatic in some people, but for most people, common symptoms are gastroenteritis, including diarrhea and abdominal cramps. Additional symptoms that may occur include chills, headache, nausea, and vomiting. Typhoid and typhoid-like fever can develop from 8-72 hours after exposure to *Salmonella*.

Salmonellosis is usually self-limiting, and symptoms subside after 4-7 days.[5] Although complete recovery is expected for most people, long-term consequences include irregular bowel movement for several months and Reiter's syndrome: pain in the joints, irritation of eyes, and painful urination. This can last for years and cause chronic arthritis. Salmonellosis can be life-threatening for young, old, pregnant, and immuno-compromised people.[7]

According to the USDA and CDC FoodNet surveillance report for 2007, *Salmonella* bacteria are the most common cause of bacterial foodborne illness. 1.2 million cases of salmonellosis occur every year in the US, and about 400 deaths are attributed to salmonellosis each year.[8] These numbers may be a drastic underestimate. The CDC estimates that for every *Salmonella* case reported, there are 29 cases that are not reported or diagnosed.[9]

Since the 1990s, the CDC has considered raw produce a risky food to consume, serving as a vehicle for *Salmonella* and other pathogens. The first foods investigated by CDC in foodborne outbreaks of *Salmonella* are often raw produce.[10] There is particular concern about *Salmonella* on tomatoes. After recurring outbreaks of *Salmonella* on fresh tomatoes, the FDA developed the Tomato Safety Initiative in 2007. This safety initiative was modeled after the Lettuce Safety Initiative in the Produce Safety Action Plan in 2004. In April 2008, a national outbreak of *Salmonella enterica* serotype Saintpaul affecting 1,500 people was traced to a variety of produce that included tomatoes. In this study, *Salmonella spp.* presence will be evaluated among farmers markets lettuce and tomatoes samples.

1.2.2 Escherichia coli

E. coli are a species of gram-negative rod bacteria that are often found in raw food products, including ground beef, raw milk, and produce. Cattle, deer and sheep may carry *E. coli* in intestinal tracts and act as vehicles in the transmission of pathogenic *E. coli* to food products consumed by humans. Although most *E. coli* are not pathogenic, measuring the presence of *E. coli* will provide the best estimates of fecal contamination. The EPA recognizes that *E. coli* is the best available indicator for fecal contamination due to its specificity to fecal material from warm-blooded animals.[11] The species, *E. coli,* is a coliform bacteria; these bacteria have the ability to ferment lactose and produce acid and gas during incubation at elevated temperatures. Figure 2, below, illustrates the specificity scheme in coliform indicators used to indicate bacterial contamination. In this study, *E. coli* presence will be measured among samples of produce and will be used as an indicator organism for fecal contamination.

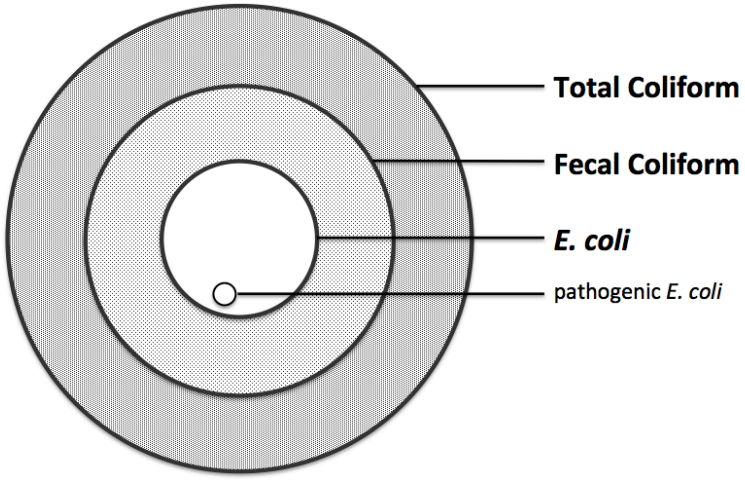

Figure 2 Coliform Bacteria Specificity Scheme, adapted from WA State Dept. of Health

Pathogenic *E. coli* causes gastroenteritis, including diarrhea and abdominal cramps. Although recovery occurs for most people within a week, HUS (hemolytic uremic syndrome), a type of kidney failure cause by the *E. coli* toxin, is estimated to develop in about 3-7% of those infected.[12] Kidney failure may then cause other organs to malfunction or fail, which may result in death. Sensitive populations include YOPIs: young, old, pregnant, and immuno-compromised people. HUS symptoms include fever, abdominal pain, fatigue, unexplained bruising and bleeding from the nose and mouth, problems in urination, and swelling of the face, hands, feet, or entire body.

E. coli O157:H7 and other Shiga toxin-producing *E. coli* can cause severe illness. Contamination can occur anytime from the initial harvesting of produce, to any processing that occurs, distribution, and to the final destination; this pathway is called the "farm to fork continuum".[13] In addition to the multiple routes of contamination in the farm to fork continuum, which includes contaminated irrigation water and infected produce handlers, Monaghan *et al.* demonstrated that contaminated soil can transfer *E. coli* onto healthy crops through rain-sized droplets and splashes.[14] The dangers of consuming raw produce contaminated with *E. coli* are evident in recent and historic outbreaks. In December 2011, the CDC documented a multi-state outbreak of *E. coli* O157:H7 linked to Romaine lettuce in 10 states. 60 people were infected, with 30 known hospitalizations and 2 cases of HUS. Just recently, another multistate outbreak of Shiga toxin-producing *E. coli* infections that affected 11 states (including Washington) was traced back to raw

clover sprouts at Jimmy John's restaurants. There were 29 cases of illness reported, and 7 cases that needed hospitalization.[15]

The CDC estimates that *E. coli* infections (primarily *E. coli* O157:H7) cause about 73,000 cases of illness and 61 deaths each year in the US.[16] After recurring outbreaks of *E. coli* on fresh lettuce, the FDA developed the Lettuce Safety Initiative – one of the three major initiatives under the Produce Safety Action Plan developed in 2004. Currently, three assessment-based safety initiatives exist under the Produce Safety Action Plan: one for leafy greens, one for lettuce, and another for tomatoes. Each one is targeted towards the evaluation of microbial hazards in the production process, as well as prevention of outbreaks.

1.2.3 Norovirus

A protocol was developed for NoV detection in this study; however, data analysis came to a halt when virus recovery levels were determined to be too low. There are standard methods to detect NoV in clinical settings with stool samples, but relatively few methods for detection of the virus in food samples.[17] An exception is for shellfish; filter-feeding bivalves concentrate pathogens from contaminated water, and methodologies for NoV detection have been well established.[18] NoV particles have a low infectious dose, so it is important for human health relevance that detection methods are sensitive enough to detect low numbers of virus particles on fresh produce. Environmental samples of NoV are expected to be far less concentrated than in clinical samples, so detection methods for food samples need to be particularly sensitive. Additionally, food samples need to be as large as

possible to take into consideration the limitation of detection methods; typical sample sizes are at least 10 to 25 grams.[19] The need for thorough investigation and sensitive methods for detecting NoV on foods like lettuce and tomatoes can partially explain the limited methodologies published in the literature on this subject.[20]

Norovirus (NoV) is the most common viral agent implicated in foodborne illnesses, affecting over 20 million people every year in the U.S. Almost one half of all foodborne illnesses are attributed to NoV. Gastroenteritis caused by NoV is characterized by sudden and severe symptoms that are usually self-limiting. NoV is a non-enveloped single-stranded RNA virus, and particles outside the human host are known to be very stable. As little as 10-100 particles can initiate infection, and people of all ages can be infected without acquiring lasting immunity. There are a limited number of studies that investigate NoV detection in non-clinical settings due to the lack of a tissue culture method for growing NoV in the laboratory setting.[19]

According to the CDC, foods like fresh produce that are usually eaten raw without heat treatment are considered high-risk for foodborne illnesses. Foods that are strongly associated with NoV outbreaks include lettuce, raspberries, strawberries, potato salad, coleslaw, fruit salad, and shellfish.[21] A 2010 study found the presence of NoV in 148 out of 275 packaged leafy green samples.[22] Among fresh produce, studies have investigated the high variation in distribution of NoV among different produce types and their growing conditions. In the production process of fresh produce, there are various stages pre- and post-harvest where NoV can contaminate fresh produce, including periods of growth where infectious virus

particles can propagate through water systems onto produce, processing, or handling from infected food workers.

1.3 Produce Types

Lettuce and tomatoes were chosen as the produce types to sample from farmers markets in Seattle due to the occurrence of outbreaks associated with these produce types. CDC databases indicate that *E. coli* has been associated with lettuce, while *Salmonella* has been associated with tomatoes. Table 1, below, shows recent *Salmonella*, *E. coli*, and NoV outbreaks involving Washington State that have had produce-related food vehicles.

Table 1 Subset of Foodborne Outbreaks involving Washington State

Genus /species	Serotype	Date	Location	Total Ill	Total Hospitalized	Total Dead	Food Vehicle
Salmonella enterica	Newport	May-10	WA	16	1	0	Tomatoes
	Typhimurium	Jul-09	Multistate	145	1	no report	Iceberg lettuce
	Saintpaul	Apr-08	Multistate	1500	308	2	Peppers, tomatoes
	Typhimurium	Sep-06	Multistate	192	24	0	Tomatoes
	Newport	Jun-06	Multistate	115	8	0	Tomatoes
E. coli STEC	O157:H7	May-08	WA	10	5	0	Lettuce
		Oct-05	WA	9	1	0	Caesar salad
		Jul-02	WA	55	no report	0	Caesar salad
NoV	no report	Mar-08	WA	20	0	0	Green salad
		Jan-07	WA	56	0	0	biscuits; lettuce based salads
		Jun-07	WA	128	no report	no report	Lettuce based salads

Source: CDC Foodborne Outbreak Online Database 1998-2010

In addition to federal recognition of the significance of lettuce and tomatoes in foodborne outbreaks of *Salmonella* and *E. coli* (see Pathogen section), CDC outbreak data analysis by Painter *et al.* revealed that the highest number of foodborne illnesses are attributable to produce–in particular, vegetables. Figure 3, below, summarizes annual foodborne illness attributions by food categories.[23]

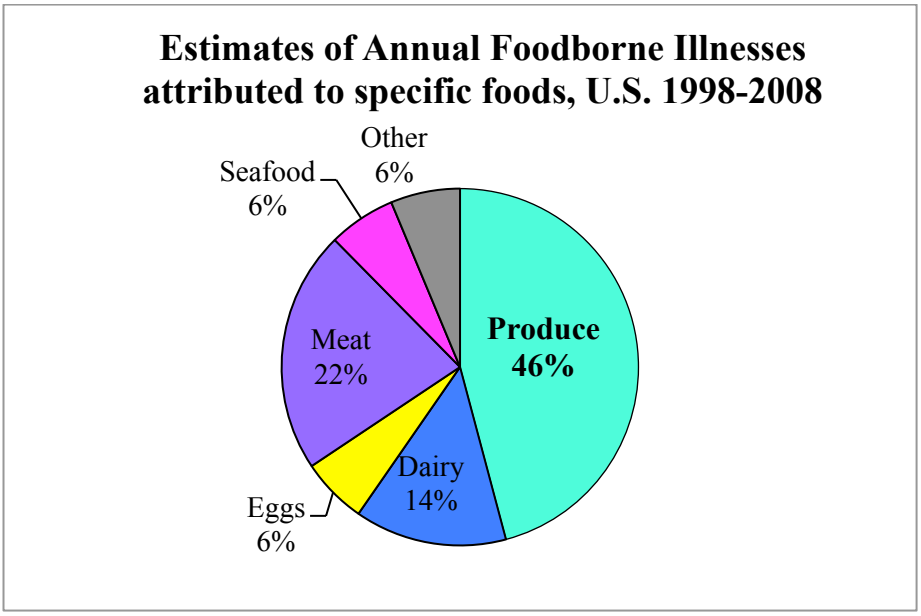

Figure 3 Produce causes most cases of foodborne illness [23]

A study evaluating microbial contamination on lettuce indicates that various factors affect attachment of *Salmonella* to lettuce. Kroupitski *et al.* found that *S.* Typhimurium has a higher affinity for older plants and regions close to the petiole (in comparison to outer leaf regions), and the abaxial (underside) side of the leaf compared to the adaxial side. Also, *Salmonella* was observed in tissue underneath stomata, suggesting that *Salmonella* can internalize lettuce.[24] Surface morphology of lettuce leaves makes it particularly vulnerable to contamination by enteric pathogens. Initial attachment rates of pathogens to surface areas of lettuce are expected to be higher than some other produce surfaces, such as tomato peel.

Variances in contamination of produce not only depend on permissible conditions, such as adequate moisture levels and elevated temperatures promoting the multiplication of bacteria, but also initial attachment levels that influence the affinity of produce for contaminants. Brandl and Amundson demonstrated that *E. coli* O157:H7 and *Salmonella enterica* can grow on lettuce leaves, and that cells inoculated onto lettuce grew at much faster rates (a 10-fold increased population size) on younger leaves compared to older leaves due to higher Nitrogen content in both exudates and tissue per gram of leaf.[25] In addition to atmospheric conditions and initial attachment rates onto lettuce, the age of leaves is another factor to consider in sampling procedures for the detection of enteric pathogens on lettuce leaves.

Lettuce cell wall materials have been examined for affinity to virus particles. As NoV infects humans through the carbohydrates of histo-blood group antigens, Esseili *et al.* investigated the affinity of NoV/GII.4 to the carbohydrates present in lettuce cell walls and found that virus particles used various carbohydrate moieties to bind to lettuce, particularly along cut edges, stomata, and minor veins.[26] Therefore, the properties of lettuce allows NoV to effectively bind to lettuce, and through its carbohydrate content, provide a surface for NoV to be resistant to decontamination procedures. An investigation in European produce by Yilmaz *et al.* found there was no association between NoV contamination on lettuce and tomatoes with levels of bacterial contamination on these produce.[27]

Contamination of tomatoes with enteric pathogens can be traced to contaminated fields, as with the recurrent multistate outbreaks of *Salmonella*

Newport. According to an epidemiological study by Greene *et al.*, the widely dispersed outbreaks of *Salmonella* suggest that contamination occurs early in the distribution chain while Cirelli *et al.* demonstrated that the highest levels of microbial contamination of fecal coliforms and *E. coli* on tomatoes were found on samples in contact with soil, in contrast with samples that did not contact soil.[28,29] In addition to contamination of tomatoes through contact with soil, Zheng *et al.* demonstrated that certain *Salmonella* serovars were associated with contamination of certain parts of the tomato plant and stages of growth. For examples, serovars Newport and Javiana were associated with soil, while serovars Montevideo and Newport were more prevalent on blossoms and leaves. *Salmonella* Typhimurium did not survive well in soils or plants, suggesting postharvest contamination with this serovar.[30]

The decision to sample lettuce and tomatoes for this project was also based on human consumption factors. Most of the U.S. population consumes lettuce according to the 2003-2006 National Health and Nutrition Examination Survey (NHANES). On average, 0.23 g/kg-day of lettuce is consumed per capita, or an average consumption rate of about 113 g/week for an average adult weighing 70 kg. Most of the U.S. population also consumes tomatoes; 0.72 g/kg-day of tomatoes is consumed per capita, or an average consumption rate of about 353 g/week among average adults weighing 70 kg. Lettuce and tomatoes are often consumed raw, which categorizes these produce types as "high-risk" foods.[31]

Accessibility of produce types to consumers shopping at farmers markets in Seattle was also evaluated. A preliminary survey conducted in September 2011

revealed that lettuce and tomatoes were sold at every farmers market visited in Seattle, and sold by at least two different vendors at each market.

The Pathogen-Produce Pair Attribution Risk Ranking Tool (P^3ARRT), a semi-quantitative risk ranking tool developed by Anderson *et al.* in 2011, used public health impact criterion and national outbreak data to rank impacts of pathogen-produce pairs in order to recognize and prioritize efforts in foodborne illness prevention and detection. The tool analyzed 53 pathogen-produce commodity pairs and assigned risk scores to each. The three highest ranked pairs were the following: 1) Enterohemorrhagic *E. coli* in leafy greens, followed by 2) *Salmonella* species in tomatoes, and 3) *Salmonella* in leafy greens.[32] Aside from *E. coli* and *Salmonella,* the authors recognized that NoV was commonly implicated in many foodborne produce-related outbreaks and had severe symptoms; however, due to the relatively low risk of hospitalization and death compared to other pathogens, NoV did not rank high in this risk analysis. This newly developed tool further confirms that tomatoes and lettuce are the most relevant produce types to survey for *Salmonella* and *E. coli* contamination.

With the variety of rationales – including produce physiology, consumption rates, accessibility of lettuce and tomatoes at farmers markets, and national public health foodborne risk assessments – sampling for bacterial contamination on lettuce and tomatoes would provide significant data relevant to public health.

1.4 Hypotheses and Specific Aims

Raw produce consumption has been associated with foodborne outbreaks of *Salmonella* and *E. coli*. Based on data analysis of national foodborne outbreaks, lettuce and tomatoes have been recognized as significant produce vehicles associated with *E. coli* and *Salmonella* outbreaks.[32] The number of farmers markets in the U.S. has been increasing over the past decade, and access to fresh produce has improved to include numerous communities across the nation. Farmers markets are particularly concentrated around larger metropolitan cities, such as Seattle. However, there is minimal oversight in the production and harvesting processes of raw produce sold at farmers markets. Previous to this study, raw produce sold at farmers markets in the U.S. has not been investigated for microbial contamination. The goal of this project is to survey the prevalence of *Salmonella* and *E. coli* contamination on lettuce and tomatoes from Seattle farmers markets, and to characterize the risk of encountering these foodborne pathogens on raw produce sold at farmers markets.

There are several hypotheses to further characterize potential causes of contamination. First, organic produce is more contaminated with microbes than conventional produce. In a systematic review by Smith-Spangler *et al.,* there was no difference in *E. coli* contamination risk on produce between organic and conventional production methods; however, limitations included publication bias and a limited number of studies.[33] Second, bacterial contamination of produce is associated with higher ambient temperatures.[34] The USDA Food Safety and Inspection Service recognizes that higher temperature and humidity during summer

months cause bacteria to reproduce at faster rates than during colder and drier months, which can explain annual peaks in foodborne illnesses during summer months. However, there have been no previous investigations on associating harvest time with microbial contamination on farmers markets produce. Third, lettuce will have a higher prevalence of microbial contamination than tomatoes. Studies analyzing produce surface contamination conclude that the surface characteristics of lettuce are prone to microbial attachment, while tomato surfaces are not as well characterized.[24,26]

As there may be other innate and environmental factors influencing microbial contamination load on different produce types, this study aims to investigate the difference in microbial contamination between lettuce and tomatoes. These hypotheses supplement this study's primary goal, which is to investigate the prevalence of *Salmonella* and *E. coli* on Seattle farmers markets lettuce and tomatoes. The specific aims, below, are designed to provide data on contamination prevalence on these produce types and to further characterize potential causes of microbial contamination based on prevalence data collected.

Aim 1. Determine the prevalence of *Salmonella* and *E. coli* on lettuce and tomatoes from Seattle farmers markets.

1A. Collect qualitative (presence/absence) data and quantitative data for *E. coli* using IDEXX Colilert® adapted for food samples.

1B. Collect qualitative data for *Salmonella* using enrichment protocol from FDA Bacteriological Analytical Manual; collect presumptive *Salmonella*

colonies grown on XLD agar, then confirm presence with qPCR for *Salmonella* invA gene and bioMérieux API® biochemical test.

Aim 2. Compare the prevalence of *Salmonella* and *E. coli* on lettuce and tomatoes from Seattle farmers markets between organic vendors and vendors not certified organic.

2A. Using the *E. coli* dataset collected from Aim 1, document the proportion of samples that have *E. coli* present among organic vendors and among vendors not certified organic. Document the levels of *E. coli* contamination in MPN/100mL sample eluent (from 50 g lettuce or 1 tomato) from each sample among organic vendors and among vendors not certified organic.

2B. Using the *Salmonella* dataset from Aim 1, document the proportion of samples that have confirmed *Salmonella* presence among organic vendors and among vendors not certified organic.

Aim 3. Compare the prevalence of *Salmonella* and *E. coli* on lettuce and tomatoes from Seattle farmers markets by harvest time.

3A. Using the *E. coli* dataset collected from Aim 1, document the proportion of samples that have presence of *E. coli* from each month of sampling. Document the levels of *E. coli* contamination in MPN/100mL sample eluent from each sample by month of sampling.

3B. Using the *Salmonella* dataset from Aim 1, document the proportion of samples that have confirmed *Salmonella* presence from each month of sampling.

Aim 4. Compare the prevalence of *Salmonella* and *E. coli* on lettuce and tomatoes from Seattle farmers markets between produce types.

4A. Using the *E. coli* dataset collected from Aim 1, document the proportion of lettuce and tomato samples that have *E. coli* present. Document the levels of *E. coli* contamination in MPN/100mL sample eluent for each produce type.

4B. Using the *Salmonella* dataset from Aim 1, document the proportion of lettuce and tomato samples that have confirmed *Salmonella* presence.

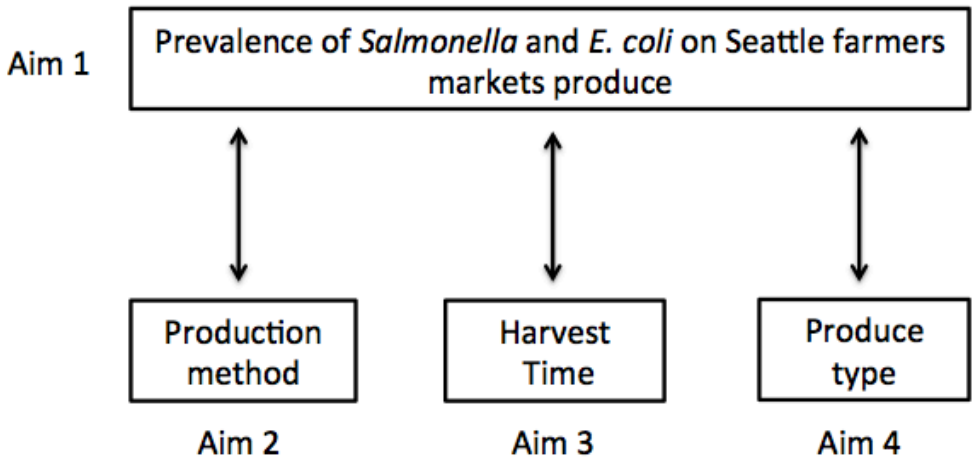

Figure 4 Schematic flowchart indicating specific aims. See text for details.

2 METHODS

From June to November 2012, 126 samples of lettuce and tomatoes were purchased from five Seattle farmers markets and analyzed for the presence of *Salmonella* and *E. coli* using laboratory protocols developed for detection on produce surfaces. A NoV detection protocol was developed but found to have insufficient virus recovery levels after sample collection; this protocol was abandoned after a subset of samples were processed. Similar amounts of produce, 50 g of lettuce leaves or one whole tomato, were allocated to each pathogen detection method. Individual samples were defined as one head of lettuce or one whole tomato. Most lettuce heads weighed significantly greater than 150 g, so there was enough lettuce from one lettuce sample to be distributed towards multiple detection protocols. All laboratory procedures involved the use of aseptic technique to ensure that there would be minimal contamination between samples and no introduction of contaminants into detection procedures.

Detection methods for *Salmonella*, *E. coli*, and NoV all began with a similar process: produce samples were distributed into sterile Whirl-Paks® and hand-massaged for 3-5 minutes in liquid media. A culture-based enrichment protocol for *Salmonella* was developed based on the FDA Bacteriological Analytical Manual (BAM) for isolation of presumptive *Salmonella* colonies. Stocks of isolated colonies were preserved at -80ºC and then confirmed via quantitative polymerase chain reaction (qPCR). The protocol developed for *E. coli* on produce surfaces is an adaptation of the IDEXX Colilert© water testing kit. The protocol for NoV detection uses PEG precipitation for virus concentration in produce eluent, QIAamp Viral RNA

Mini Kit for RNA extraction, and then confirmation of NoV presence via quantitative reverse transcription polymerase chain reaction (RT-qPCR). Field sampling methods, sample preparation, and laboratory detection methods will be described in more detail in this chapter.

2.1 Collection of Samples in the Field

Five major farmers markets within Seattle were identified as sampling sites for this project. Geographic distribution across Seattle and size of markets based on number of vendors present were determining factors in choosing which markets to sample from; markets that spanned approximately equidistant across Seattle and had more than five produce vendors present were chosen. Additionally, market operation times had to coincide with the availability of the field sampler. Therefore, convenience sampling was implemented in field sampling for this project. Figure 4, below, is a map showing the names and geographic distribution of the five farmers markets visited.

Figure 5 Seattle-area map labeled with farmers markets sampling sites

Field sampling was conducted on a weekly basis between June 24, 2012 and November 18, 2012. No sampling occurred in September. An even distribution of lettuce and tomato samples from the five markets identified above was targeted for this study, as well as a wide variety of vendors among farmers markets. Field sampling involved shopping like a farmers market customer, with the exception of avoiding bare hand contact with samples and preventing samples from one vendor from contacting samples from another vendor or produce type. To collect a sample, a clear produce bag supplied by the vendor was inverted then used to grab a lettuce head or tomato. Samples were stored in a designated Field Lab refrigerator (Meschke Lab, Dept. of Environmental & Occupational Health Sciences) within 3 hours of purchase, and sample bags were labeled with a sharpie indicating the date of purchase, vendor, and farmers market the sample was purchased from.

2.2 Sample Preparation

Refrigerated farmers market samples were prepared for pathogen detection protocols in the Field Lab. A single sterile 5'' x 12'' Whirl-Pak® (Product B00990WA) was used to hold each produce sample. More lettuce by weight was processed in this study than outlined in the FDA BAM based on recommendations from Eyob Mazengia, food safety professional. More grams of lettuce processed for each detection method increases detection levels and increases the chance of encountering target organisms, such as *Salmonella spp.* cells (Personal Communication, March 2012).

Lettuce leaves were placed on large weigh boats on an electronic balance and 50 ± 2 g of leaves, constituting one sample, were allocated to each Whirl-Pak® bag.

Whole tomatoes weighing 45–135 g per sample were also placed into Whirl-Pak® bags. Figure 6, below, is a snapshot of the preparation process for packing produce samples into bags. Equal numbers of samples were allocated to each detection method for *Salmonella, E. coli,* and NoV. Therefore, tomatoes were purchased in multiples of three from each vendor, and lettuce heads were split into three 50 ± 2 g samples.

Figure 6 Aseptic Preparation of Produce Samples into Whirl-Pak® Bags

2.3 *Salmonella* Detection Method

2.3.1 Pre-enrichment

The detection method for *Salmonella* was adapted from the FDA BAM protocol for whole tomatoes and leafy greens.[35] The FDA recommends pre-enrichment of food samples with Universal Preenrichment (UP) broth. UP Broth is used by the FDA on a variety of foods including whole tomatoes. For leafy greens, however, the FDA indicated that lactose broth be used for pre-enrichment. Hammack *et al.* investigated the recovery of *Salmonella* serovars using various

isolation methods and broths, and demonstrated on orange juice that the CDC method, which included UP Broth initial enrichment, followed by selective enrichment, and then selective plating, resulted in significantly higher recovery of *Salmonella* compared to FDA methods using lactose broth.[36]

UP Broth is highly buffered, while lactose broth is weakly buffered. The pH of lactose broth may change too quickly for injured *Salmonella* cells to fully recover.[36] With an approximate quadrupling of lettuce grams processed per liquid media volume in this current study compared to the FDA BAM *Salmonella* detection protocol, a change in pH using lactose broth was probable. The addition of sodium pyruvate in media such as UP Broth aids in the recovery of sub-lethally injured *Salmonella* cells.[36,37] Lactose broth lacks sodium pyruvate. Therefore, UP Broth was used for the initial, non-selective enrichment of all produce samples.

Produce samples were placed into sterile Whirl-Pak® bags. 150 mL of UP Broth was poured into bags containing lettuce, and enough UP Broth for tomatoes to float was poured into bags containing tomatoes.[35] Before incubating bags of produce containing UP Broth, produce samples were manually rubbed/massaged inside Whirl-Pak® bags for 3-5 minutes for the liquid broth to have contact with all parts of the produce samples, and to move surface-attached particles into the surrounding liquid.[30,38,39]

2.3.2 Selective Enrichment

Following sample enrichment in UP Broth for 24 ± 2 hours at 35ºC, 1 mL of broth was transferred to selective enrichment broth for *Salmonella*. Tetrathionate (TT) Broth is an ideal broth for selectively promoting growth of *Salmonella* cells that

may be competing for nutrients with other intestinal organisms. A modification of the FDA BAM *Salmonella* protocol was to use only TT Broth instead of both Rappaport-Vassiliadis (RV) and TT Broths. According to studies investigating the effectiveness of TT and RV Broths as selective medium for *Salmonella spp.,* there are no significant differences between TT and RV Broths. Foods tested included poultry, dairy, spices, and egg products.[40,41] However, TT Broth was found to be more effective in isolation of *Salmonella* from artificially contaminated tropical fruit mamey.[42] TT Broth was used as the selective enrichment media for samples instead of both TT Broth and RV Broth due to similar performance of the two selective media. TT Broth was prepared with Difco™ Tetrathionate broth base with addition of I_2-KI (iodine-iodide solution) and Brilliant Green solution.[35]

2.3.3 Streaking for Isolation on Selective Media

Following selective sample enrichment in TT Broth for 24 ± 2 hours at 35ºC, one loopful (10 μL) was streaked for isolation onto Difco™ Xylose Lysine Desoxycholate (XLD) agar. According to Brian Hiatt, Director of Public Health Microbiology at Washington State Department of Health, there is no standard streaking technique for the isolation of bacteria. Streaking two to four quadrants on an agar plate is usually sufficient for isolation of bacterial colonies, with potentially more quadrants needed for heavily contaminated environmental samples (Personal Communication, May 10, 2013). With unpredictable and variable bacterial loads from each sample, at least two XLD agar plates were allocated to each sample and streaked in a consistent manner among all samples from each weekly batch of samples. Agar plates were streaked with a minimum of three quadrants, and up to

five quadrants per plate. After incubation for 24 ± 2 hours at 35ºC, presumptive colonies were picked with a loop for another round of streaking and isolation on XLD agar. Presumptive *Salmonella* colonies were identified by examining plates for red colony formation with black precipitate, as described in the Difco & BBL Manual.[43] A third round of streaking occurred on XLD agar to produce a lawn of presumptive *Salmonella*. Overall, three rounds of XLD agar platings was the minimum number of platings to ensure isolation of colonies and fresh growth of presumptive *Salmonella* colonies before freezer storage.

2.3.4 Storage of Presumptive *Salmonella*

Lawns of presumptive *Salmonella* were grown on XLD agar. Lawns were scraped with a loop within 24 hours of incubation, and suspended in a 1 mL solution of tryptic soy broth (TSB) and 20% glycerol. TSB with 15-20% glycerol is an effective cyroprotectant medium for storing stocks of *Salmonella spp.* in freezers, and is an internationally recognized standard medium for long-term freezer storage of bacteria.[44] The presumptive *Salmonella* stocks in TSB and glycerol solution were vortexed for 30 seconds in a 1.5 mL microcentrifuge tube, labeled with sample identification numbers/letters, and stored at -80ºC.

2.3.5 Confirmation of Presumptive *Salmonella*

Quantitative polymerase-chain reaction (qPCR) on a 116-base pair fragment of the *Salmonella invA* gene was used to confirm presence of *Salmonella spp.* from presumptive *Salmonella* freezer stocks. Almost all *Salmonella* serotypes contain the *invA* gene, which codes for protein necessary for epithelial cell invasion.[45] In

addition to its ubiquitous presence among *Salmonella spp.,* it is a highly conserved gene.[46] Zhang *et al.* tested these primers on 81 different strains of *Salmonella,* including strains in subspecies *Salmonella enterica* and *Salmonella bongori.* All 81 strains were detected by qPCR of the *invA* gene. The set of primers used were from studies by Zhang *et al.* 2011 and González-Escalona *et al.* 2009.[39,47] Both studies were conducted at the Center for Food Safety and Applied Nutrition at the FDA. Before ordering primers, primer sequences were inputted into NCBI Blast and shown to effectively detect *Salmonella spp.* and did not appear to cross amplify other bacterial species. Primers were ordered from Integrated DNA Technologies (Coralville, IA). Table 2 shows the forward and reverse primer sequences used to detect the *Salmonella invA* gene.

Table 2 Primers used for *Salmonella* Detection Method

Primers	**Sequence**	**Gene Position**
invA_176F	CAACGTTTCCTGCGGTACTGT	176
invA_291R	CCCGAACGTGGCGATAATT	291

The qPCR confirmation step followed the Zhang *et al.* protocol with several modifications after preliminary experimentation with primers and lab-strain *Salmonella* as a positive control: 1) final volume of reaction mixtures was changed to 25 μL instead of 20 μL, 2) no probe was ordered because SYBR Green reagents were available and demonstrated to be effective for *invA* detection, 3) the number of qPCR cycles was increased from 35 to 40, and 4) no MgCl was added to the reaction

mixture due to inhibitory effects–demonstrated with higher cycle threshold (Ct) values–in MgCl-containing mixtures compared to mixtures without MgCl.

All presumptive *Salmonella* samples were heat-released for 5 minutes (105ºC lid, 98ºC tube) in a thermal cycler to release DNA from bacterial cells. Heat-released tubes all contained 20 μL of each sample, of which 2 μL were pipetted into each qPCR reaction tube. qPCR cycling was set at the following conditions: 2 min of incubation at 50ºC, 2 min of Taq polymerase activation at 95ºC, 15 sec of denaturation at 95ºC, and 30 sec of primer annealing and extension at 60ºC. A total of 40 cycles was completed, and SYBR Green fluorescence was read after each cycle by Opticon Monitor™ Software Informer version 3.1 in sync with MJ Research Real-Time PCR Detection System.

Postive qPCR results, if any, were confirmed with amplification for the *invA* gene and melting curve matching the *Salmonella* positive control. If no amplification occurred, isolated presumptive *Salmonella* specimens from freezer tubes were plated on XLD agar to produce fresh colony growth for additional biochemical confirmation. API® 20e strips (bioMérieux) were used to identify bacterial growth not confirmed by qPCR. These commercially available test strips are considered a gold standard and are used in many clinical microbiology labs around the world.[48]

2.4 *Escherichia coli* Detection Method

2.4.1 Adaptation of IDEXX Colilert® for Rapid Detection of *E. coli*

A novel approach to rapid and sensitive detection of *E. coli* on produce surfaces was developed using EPA-approved water quality testing kit IDEXX Colilert® paired with Quanti-Tray®/2000. Following sample preparation of

produce in Whirl-Pak® bags, autoclaved 1X Phosphate Buffered Saline (PBS) was used as an eluent to wash surface particles off from produce; 100 ± 2 mL eluent was then mixed with IDEXX Colilert® reagent and sealed in Quanti-Tray®/2000. IDEXX Colilert® is an EPA-approved water quality test kit approved for use in over 90% of U.S. State laboratories, and has a limit of detection of 1 MPN/100 mL. Initial method validation experiments using produce samples confirmed this level of detection with use on food products. Hands-on time with the kit is minimal (< 1 min), and results confirming *E. coli* and total coliform presence are obtained in 24-28 hours.[49] This method of detection is more rapid and sensitive compared to the FDA BAM 3-tube MPN method, which takes a minimum of 120 hours, or 5 days, for confirming the presence of *E. coli.* An estimate of the limit of detection for the FDA BAM 3-tube MPN method is <36 MPN/100 mL, which is the reported limit of detection for the Presumptive Test for the Coliform MPN method.[50] A rapid and sensitive method for detection of fecal coliforms is advantageous for food safety and public health. IDEXX Colilert®, as a highly sensitive and rapid method for water quality testing, was identified for its qualities and adapted for use on food surfaces.

IDEXX Colilert® uses two nutrient-indicators, ONPG and MUG, for coliforms to metabolize. Coliforms use the enzyme β-galactosidase to metabolize ONPG, changing the nutrient indicator to a yellow color. Most *E. coli* (97% of the *Escherichia* genus) have an additional enzyme β-glucuronidase,[51] which metabolizes MUG to produce fluorescence visible under a 365 nm, long-wave UV lamp as seen in Figure 7. Thus, IDEXX detects both total coliforms and *E. coli* simultaneously with

yellow color change for total coliforms, and yellow color change and fluorescence for *E. coli.*

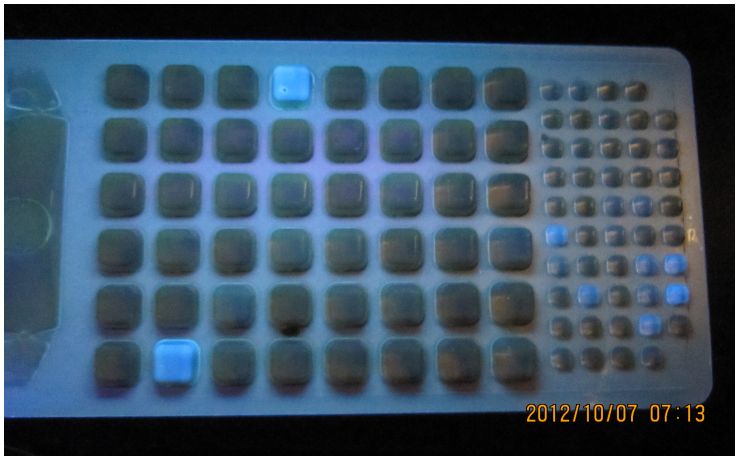

Figure 7 IDEXX Colilert® *E. coli* Fluorescence in Quanti-Tray®/2000 under UV Lamp

MUG nutrient indicator for the detection of *E. coli* on food has been used in the past. In 1996, Venkateswaran *et al.* compared various kits with standard 3-tube MPN methods, and evaluated the use of a Colilert ONPG-MUG kit (Aska Diagnostics, Inc., Tokyo, Japan) using 25 g retail food samples homogenized with Phosphate Buffered Saline (PBS). Another commercial kit compared with standard methods was LST-MUG. Venkateswaren *et al.* reported better performance and higher levels of detection for both the Colilert and LST-MUG tube kits (Central Kagaku) in comparison to the FDA method and Japanese standard method.[52]

2.4.2 Produce Sample Elution and Incubation

A study by Liao *et al.* demonstrated with 148 strains of bacteria (including *E. coli* O157:H7) that sterile water and PBS are equally capable of preserving viable bacteria for years at room temperature, with the exception Gram-positive bacteria dying off more rapidly in water after 30 weeks.[53] PBS (pH 7.4) was the chosen media

to use in this study not only because of its ability to preserve bacteria, but also for its pH buffering effects as an eluent for food produce. 100 mL aliquots of PBS were poured into tomato sample bags, and 110 mL aliquots into lettuce sample bags. An additional 10 mL was necessary for lettuce samples so that enough eluent, 100 ± 2 mL, could be poured out of the bag after lettuce elution. According to IDEXX technical support, 100 ± 2 mL is the permissible range of liquid that can be poured into trays, with up to two entire wells on each tray lacking liquid, in order for MPN estimates to be reportable and acceptable by EPA standards (Personal Communication, April 2012).

Samples of produce with PBS inside Whirl-Pak® bags were manually rubbed, or massaged, for 3-5 minutes.[30,38,39] Sterile 120 mL screw-cap vessels marked with a 100 mL line were filled to the line with the resulting produce wash/eluent. The wire openings on Whirl-Pak® bags served as a spout for eluent to be poured from the bag into the vessel while minimizing large particles, particularly lettuce leaves, from flowing into the vessel. IDEXX Colilert® reagent was added to the sample in the vessel. The vessel was closed and gently shaken for about 30 seconds until reagent dissolved. The liquid in the vessel was then poured in through the top of a Quanti-Tray®/2000 and sealed with Quanti-Tray® Sealer. Trays were incubated at 35ºC for 24-28 hours.

2.4.3 Reading Quanti-Tray®/2000 after Incubation

Results obtained from Quanti-Tray®/2000 need no further confirmation step. The numbers of wells that changed color, from colorless to yellow, was recorded for each tray and for each size of well (small and large). Afterwards, the

tray was brought to a dark room and examined with a 365 nm, long-wave UV lamp. The lamp was held approximately 15 cm from the surface of the tray. Among the wells that changed to a yellow color, the number of wells that fluoresced were recorded for small and large wells. An MPN table, supplied by IDEXX, was used to obtain MPN/100 mL results from each tray's combination of fluorescing small and large wells.

2.5 Norovirus Detection Method

The method for the detection of NoV in this project was modeled primarily after Allwood *et al.*, with deviations on temperatures and centrifuge speeds incorporated from Summa *et al.,* Sanchez *et al.,* and Guevremont *et al.* Briefly, samples placed into sterile bags were eluted with 110 mL of sterile 0.5 M Glycine and 3% beef extract solution. Bags were manually massaged for 3-5 minutes, and the resulting eluent was centrifuged at 1,000 x g for 10 minutes at 4 ºC to pellet plant material and other debris. Supernatant was poured into a second conical tube, and 8% PEG weight per volume was added. Conical tubes were left overnight (12-18 hours) on an orbital shaker at 4-8ºC to allow precipitate to form. The conical tubes were then centrifuged at 10,000 x g for 30 minutes at room temperature. Supernatant was discarded and the pellet was resuspended with 1 mL of PBS. Approximately 1.3 mL of the resuspended pellet was stored in a microcentrifuge tube and frozen at 80ºC.[38,54–56]

RNA extractions were performed on previously frozen PEG-pelleted samples with QIAamp Viral RNA Mini Kit (QIAGEN). Both spin protocol and vacuum protocols were used depending on pellet consistency; most pellets from lettuce

samples required the spin protocol. A modification to the QIAamp standard protocol was that three times the recommended sample volume to be lysed was used. After RNA extraction, RT-qPCR screened extractions for presence of NoV GI and GII, the most common groups affecting humans.[19] Positive controls used were NoV GI and GII RNA extractions previously extracted from stool samples (Personal Communication, Nicola K. Beck, April 10, 2013). Primers used were from Kageyama *et al.*[57]

2.6 Quality Control and Quality Assurance

2.6.1 Sample Collection

Sample collection involved minimal contact of produce with bare hands. A plastic bag, supplied by vendor, was used to pick up produce. Bags supplied by vendors separated discrete lettuce heads and tomato samples by vendor. Each vendor was asked if produce was locally produced, and whether it was organic or conventional. Identifying sample information was recorded onto each bag of produce directly after purchasing samples to avoid accidental mixing of samples. Samples were handled with care, packed into a secure tote bag, and shipped to the Field Lab refrigerator within 3 hours from purchase and remained stored at 4ºC until time of processing. All samples were processed within 5 days from purchase date.

2.6.2 Sample and Media Preparation

During sample preparation, all samples were handled with clean gloves. Between samples, gloves were sprayed with ethanol and wiped dry on clean paper, or replaced with new gloves between samples. All media was made in a media room

where separate lab coats and clean gloves are required at all times. XLD agar was made before farmers markets produce samples were processed to test the growth medium. *Salmonella* Typhimurium LT2 strain was used for initial plating on XLD agar. Expected typical *Salmonella* morphologies grew on the agar, confirming that XLD agar was in good condition for use.

2.6.3 Method Validation - *Salmonella*

An experiment using *Salmonella* Typhimurium LT2 strain investigated the recovery of spiked produce samples with the modified FDA BAM method, as described in Section 2.3. *Salmonella* was grown overnight in TSB, and 100 μL of varying dilutions were plated onto XLD agar for quantitation and examination of colonies. Simultaneously, 100 μL of varying dilutions was spiked onto 50 g samples of previously washed lettuce inside sterile Whirl-Pak® bags. The spiked lettuce was allowed to sit for an hour, and the *Salmonella* detection method protocol was followed. Briefly, produce samples were manually rubbed with 150 mL of UP Broth and entire Whirl-Pak® bags were placed in a large sturdy bin for 24 hours of incubation at 35ºC. After incubation, 1 mL of liquid from the bag was transferred into 10 mL of TT broth and incubated for another 24 hours at 35ºC. After incubation, a loopful (10 μL) was streaked for isolation on XLD agar. Agar plates were then incubated for 24 hours at 35ºC. Resulting colonies were observed on XLD agar and compared to initial *Salmonella* dilutions plated XLD agar from the beginning of method validation.

All plates recovered typical *Salmonella* morphology, with the exception of the produce spiked with the lowest dilution of Salmonella. Based on initial XLD agar

plating of this dilution, the calculated average concentration of *Salmonella* cells was 5 cfu/mL. As 100 μL of this concentration was initially spiked onto produce, having no *Salmonella* growth at the end of this method was expected for this dilution. All produce spiked with higher dilutions of *Salmonella* had observable recovery of typical *Salmonella* at the end of this method validation experiment.

2.6.4 qPCR for *Salmonella invA* gene

Prior to processing samples for qPCR, primers were tested in an experimental run on a positive control, *Salmonella* Typhimurium LT2 strain, and a negative control, *E. coli* F-amp. 20 μL from each stock of bacteria was heat-released and 2 μL of supernatant containing DNA was used for qPCR. Amplification of only the positive control, along with a standard melting curve, was observed after initial qPCR. These experiments were run in duplicate PCR wells. The qPCR run on the positive control and negative control confirmed that primers would effectively and specifically amplify *Salmonella spp.*

2.6.5 Sample Replication - *Salmonella*

14% of samples (n=18) were processed in duplicates. Most samples were lettuce (n=17). Heads of lettuce large enough to be split into more than three 50 g sections were used for duplicate analysis of samples. The tomato sample processed in duplicate were individual tomatoes noted to be in contact with each other before the time of purchase. Other tomatoes in this study were processed as discrete samples. In addition to duplicate of field samples, two XLD agar plates in laboratory analysis were used for all initial plating from incubated TT Broth. Duplicate XLD

agar plates were used in further plating of presumptive *Salmonella* colonies identified from previously plated XLD agar.

2.6.6 Method Validation - *E. coli*

Limit of detection was investigated for this novel approach of using IDEXX Colilert®. *E. coli* B, an *E. coli* strain known to fluoresce from incubation with IDEXX Colilert® reagent, was first grown in an overnight suspension of nutrient broth. Nutrient agar plates were plated in 10^{-6} to 10^{-8} dilutions of overnight growth made in PBS to estimate the concentration of overnight *E. coli* B suspension. Simultaneously, two 50 g samples of previously washed lettuce were aseptically placed in sterile Whirl-Pak® bags and then spiked with 100 μL of the overnight growth suspension of varying dilutions of *E. coli* B, ranging from 10^{-1} to 10^{-5}. Approximately 10 droplets from the 100 μL of *E. coli* B were dispersed onto lettuce surfaces and allowed to sit for one hour before following the *E. coli* method as described in Section 2.4, *E. coli* Detection Method.

Briefly, 110 mL of PBS was added to the Whirl-Pak® bags containing the spiked samples, samples were massaged, 100 mL eluent was poured into vessels with IDEXX Colilert® reagent, and then transferred to Quanti-Tray®/2000 for incubation. After 24 hours, trays were read under a UV light for fluorescence. At the dilution of 10^{-8}, 1 large well fluoresced resulting in an MPN of 1. Based on the initial estimate of *E. coli* concentration used to spike the lettuce samples, about 2 cells were presumed to be on the produce spiked with 10^{-8} dilution. Assuming that all *E. coli* cells were collected off the surface of lettuce and into the 110 mL of eluent, the limit of detection was for this novel IDEXX Colilert® process was calculated to be <2

MPN/100 mL, which is in concordance with IDEXX's claimed limit of detection of 1 MPN/100 mL.[49]

2.6.7 Interpretation of *E. coli* Fluorescence

A comparator tray supplied by IDEXX was examined in a dark room alongside each set of samples as a standard for fluorescence. In addition to the comparator tray supplied by IDEXX, additional trays using 100 mL of PBS, Colilert® reagent, and 10 μL *E. coli* B (equivalent to one loopful) were made every few weeks throughout the study for the quality assurance of Colilert® reagent. Fluorescing wells after 24 hours of incubation at 35ºC indicated that MUG, the nutrient indicator for *E. coli,* was not impaired. As required by IDEXX, Colilert® reagent was always stored in a cardboard box at room temperature away from direct sunlight.

2.6.8 Quantification of *E. coli*

For over 90% of samples, two or more people were involved in identifying fluorescing wells for the accuracy of recorded well numbers. IDEXX expects that fluorescing wells should be clearly identifiable under a UV lamp. With the addition of some plant material, however, some wells may fluoresce less brightly compared to other wells. To assess the accuracy of recording "partially" fluorescing wells at wells positive for *E. coli*, wells in question were punctured aseptically and a loopful of liquid from the well was streaked for isolation on nutrient agar. After 24 hours of incubation, nutrient agar plates were examined for colony morphology resembling *E. coli.* This additional quality assurance procedure validated partially fluorescing wells as positive for *E. coli*.

2.6.9 Sample Replication - *E. coli*

14% of samples (n=18) were processed in duplicate (triplicate and quadruplicate were processed from one sample). Most duplicates were lettuce(n=17); heads of lettuce weighing at least 300 g were used for duplicate analysis. One head of lettuce constituted one sample from which multiple 50 g portions can be taken, whereas each individual tomato was one sample that could be processed only once. The tomato samples processed in duplicate were separate tomatoes noted to be in contact with each other before the time of purchase. All other tomatoes were processed as discrete samples.

2.6.10 Norovirus Method Validation/Conclusion

Experiments on method percent recovery using MS2 bacteriophage as a surrogate for NoV indicated that recovery levels were too low in comparison to other studies; RT-qPCR was halted for PEG-pelleted samples prepared for NoV detection. Preparation for recovery experimentation involved making virus stocks MS2 bacteriophage, a surrogate for NoV. MS2 bacteriophage is an RNA coliphage in the family Leviviridae, and is commonly used as a surrogate for NoV due its similarity to NoV; it is a positive sense single-stranded RNA viruses in the range of 26 nm in diameter, has icosahedral symmetry, and is adapted to the intestinal tract. With a bacterial host of *E. coli,* MS2 bacteriophage can be readily cultured in a laboratory setting, unlike NoV.[58] An overview of the NoV method validation and recovery experiment scheme is included in Figure 10 of the Appendix. Percent recovery experimentation revealed that recover levels of produce samples spiked with MS2 ranged from 0.4% to 3.0%. This is significantly lower than 3%–25%, the

typical range of reported recoveries of MS2 bacteriophage on fruits and vegetables.[54,59] A study by Scherer *et al.* examined the recovery of MS2 bacteriophage on lettuce, specifically, and reported a 23% average recovery.[60]

Upon examination of other NoV detection methods previously used to concentrate virus particles, an experimental error was identified. PEG concentration in produce eluent requires additional activation with NaCl in order for PEG to effectively bind to virus particles. No NaCl was added, which explained the low recoveries of MS2 bacteriophage. An additional recovery experiment between PEG-only and PEG with 0.4 M NaCl pelleted eluent showed that recovery levels would have been approximately 4-5 times higher if 0.4 M NaCl were added to eluent containing PEG. This approximation was based off of double-agar layer (DAL) plates used to detect MS2 bacteriophage plaque-formation on a lawn of *E. coli.*

Although methodological error was identified, 20 random samples from those extracted and stored throughout this study were amplified for both NoV GI and GII. Expectedly, there was no amplification by RT-qPCR for either group of NoV. NoV GI and GII RNA extractions previously extracted from stool samples served as positive controls for RT-qPCR (Personal Communication, Nicola K. Beck, April 10, 2013). The need for sensitive detection methods is especially for NoV detection on foods, which may carry NoV virus particles in low numbers.[54,59,60] Without a sensitive detection method for NoV, RT-qPCR results would likely include false-negatives and be irrelevant to food safety. Thus, further sampling and analysis was abandoned for NoV detection in this study.

3 RESULTS

3.1 *Salmonella*

One hundred and twenty-six produce samples were evaluated for the presence of *Salmonella.* Among these samples, there were 63 lettuce samples and 63 tomato samples. Presumptive *Salmonella* colonies with typical *Salmonella* morphology were isolated from two samples of lettuce, both collected on the same day from one vendor. Stocks of presumptive *Salmonella* isolates were made and stored at -80ºC for further qPCR analysis. There was, however, no amplification of the *Salmonella invA* gene upon qPCR analysis. Presumptive *Salmonella* from stocks were then plated onto XLD agar and API® 20e strips (bioMérieux) were used to confirm the identity of newly grown presumptive *Salmonella* colonies. Figure 8, below, shows an API® 20e strip after completion of the API® protocol. The bioMérieux database identified both presumptive *Salmonella* samples as *Citrobacter youngae*, a common false-positive for *Salmonella.*[61,62]

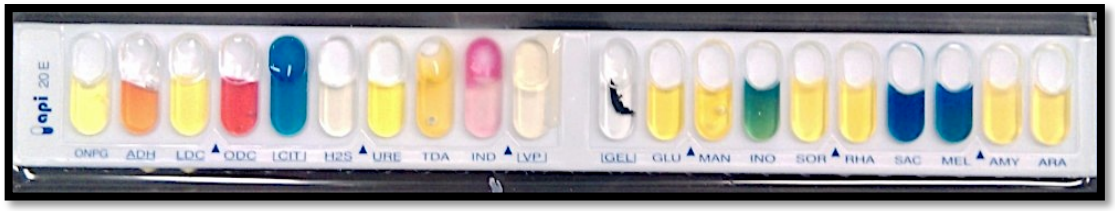

Figure 8 API® 20e strip with confirmed *Citrobacter* identification

3.2 *Escherichia coli*

A detailed spreadsheet of results is included in the Appendix, Table 6.

3.2.1 Qualitative Results

It was hypothesized that lettuce would have a higher prevalence of microbial contamination than tomatoes due to surface morphology.[24,26] The surface characteristics of lettuce are prone to attachment with microbial particles, while tomato surfaces have not been well characterized. Among lettuce samples, *E. coli* was found on 39 out of 63 of samples, or 62%. Among tomatoes, *E. coli* was found on 4 out of 63 samples, or 6%. There was a significant difference in presence of *E. coli* between produce types; significantly more lettuce samples were contaminated with *E. coli* than tomatoes (*STATISTICA* Mann-Whitney U Test, $p < 0.05$).

It was hypothesized that *E. coli* contamination would occur on more organic produce than conventional produce with the limited data presented by Spangler *et al.*[33] There were 68 organic produce samples collected, and 58 conventional produce samples. Visualizations of these data comparing organic/conventional produce contamination and contamination by month can be found in the Appendix (Figure 11, Figure 12). Although 0% of organic tomatoes (n=26) had *E. coli* contamination, there was no significant differences in *E. coli* contamination between organic or conventional produce sorted by produce type (*STATISTICA* Mann-Whitney U Test, $p >> 0.05$). Overall, no significant differences were found in presence of *E. coli* between organic and conventional produce (*STATISTICA* Mann-Whitney U Test, $p >> 0.05$).

Based on the annual peaks in foodborne illnesses during summer months, and USDA recognition that higher temperature and humidity cause bacteria to reproduce at faster rates than during colder and drier months,[34] it was hypothesized that there may be significant differences in contamination by harvest time. However, no significant differences were detected between presence of contamination and sampling dates ranging from June 24 to November 18, 2012 (*STATISTICA* Mann-Whitney U Test, $p >> 0.05$).

3.2.2 Quantitative Results

MPN values per 100 mL read from the IDEXX MPN Table ranged from 0 to >2419.6. Table 3, below, is a summary table of MPN values for each produce type.

Table 3 MPN values per 100 mL per Produce Sample - Summary Results

	Lettuce (n=63)	**Tomatoes (n=63)**
Average	**82**	**0.425**
Min	0	0
25th Pct	0	0
Median	**1**	**0**
75th Pct	6.65	0
Max	>2419.6	23.3

As previously stated, it was hypothesized that lettuce would have a higher prevalence of microbial contamination than tomatoes. The surface characteristics of lettuce are prone to attachment with microbial particles, while tomato surfaces have not been well characterized;[24,26] other innate and environmental factors may also influence microbial contamination. MPN values were assessed by produce type, and there was no significant difference in the levels of *E. coli* between produce types (*STATISTICA* Mann-Whitney U Test, $p >> 0.05$).

Based on a limited systematic review conducted by Smith-Spangler *et al.,* it was hypothesized that *E. coli* contamination would occur on more organic produce than conventional produce.[33] There were 68 organic produce samples collected, and 58 conventional produce samples. There was no significant difference in levels of *E. coli* contamination between organic or conventional produce sorted by produce type (*STATISTICA* Mann-Whitney U Test, $p >> 0.05$). Overall, no significant differences were found in *E. coli* levels between organic and conventional produce (*STATISTICA* Mann-Whitney U Test, $p >> 0.05$).

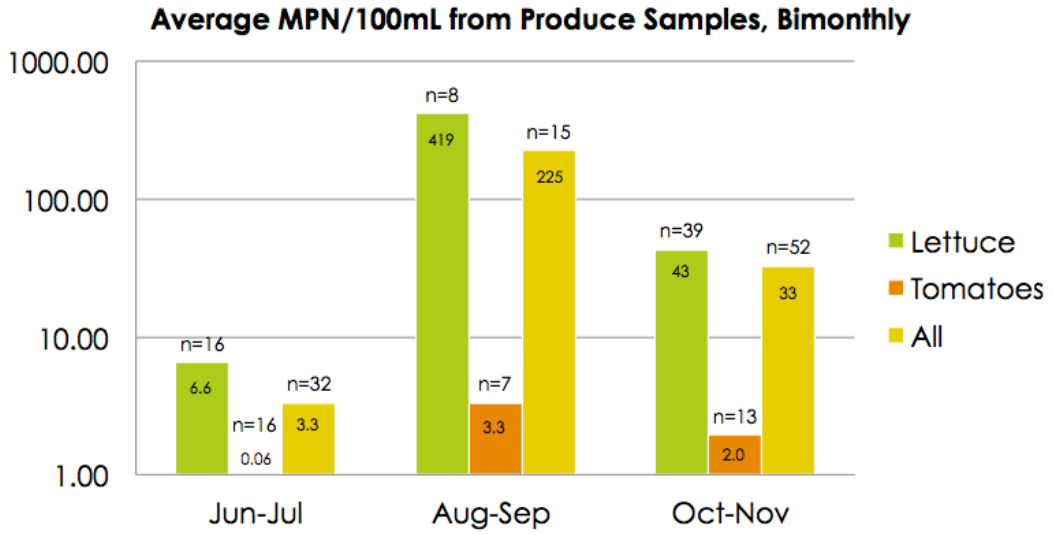

Figure 9 *E. coli* MPN Trend with 2012 Bimonthly Harvest Time grouping

Based on the annual peaks in foodborne illnesses during summer months and USDA recognition that higher temperature and humidity cause bacteria to reproduce at faster rates than during colder and drier months,[34] it was hypothesized that there may be significant differences in contamination levels by time of harvest. A trend was observed with bimonthly groupings of MPN values for both lettuce and tomatoes (Figure 9). The rise in MPN values was correlated with a rise in average

monthly Seattle temperatures in August and September 2012. However, no significant differences were detected between levels of contamination and sampling dates ranging from June 24 to November 18, 2012 (*STATISTICA* Mann-Whitney U Test, $p >> 0.05$).

3.2.3 Quality Assurance/Quality Control

A minimum of 10% of produce samples were duplicated for all detection methods. Table 4 shows resulting MPN values and presence/absence data from duplicates analyzed for the *E. coli* dataset. Mean differences between pairs and standard deviation between pairs were calculated to assess variance between samples.

Table 4 Summary of Duplicate Analysis for *E. coli* Detection

Sample ID	Date	Market	Vendor	Organic*	Produce	MPN1/ 100 mL	MPN2/ 100 mL
PP-K-L-0624	2012/6/24	Pike Place	K	0	Lettuce	2	1
UD-AA-L-0630	2012/6/30	U District	AA	0	Lettuce	3	0
Bal-H-L-0701	2012/7/1	Ballard	H	1	Lettuce	0	0
PP-K-L-0701	2012/7/1	Pike Place	K	0	Lettuce	3	0
UD-M-T-0714	2012/7/14	U District	M	0	Tomatoes	0	0
Bal-EE-L-0715	2012/7/15	Ballard	EE	1	Lettuce	43.1	34.7
Bro-P-L-0715	2012/7/15	Broadway	P	0	Lettuce	1	0
UD-DD-L-0721	2012/7/21	U District	DD	1	Lettuce	26.4	23.3
PP-A-L-0722A	2012/7/22	Pike Place	A	0	Lettuce	17.1	8
PP-A-L-0722B	2012/7/22	Pike Place	A	0	Lettuce	5	4
Bal-EE-L-0722	2012/7/22	Ballard	EE	1	Lettuce	0	0
PP-U-L-0728	2012/7/28	Pike Place	U	0	Lettuce	1	0
Bal-S-L-0729B	2012/7/29	Ballard	S	1	Lettuce	1	0
LC-D-L-0802B	2012/8/2	Lake City	D	0	Lettuce	501.2	58.2
LC-A-L-1015**	2012/10/15	Lake City	A	0	Lettuce	4	1
UD-DD-L-1015A	2012/10/15	U District	DD	1	Lettuce	0	0
UD-DD-L-1015B	2012/10/15	U District	DD	1	Lettuce	0	0
LC-GG-L-1015	2012/10/15	Lake City	GG	1	Lettuce	0	0

* "0" = Conventional; "1" = Organic

**triplicate and quadruplicate from this sample (both 0 MPN) were excluded from this table

The percent agreement between duplicates for presence/absence data is 66%, as 12 out of 18 duplicate samples were in agreement. Between samples that had differing results, however, differences between levels of *E. coli* detected is <2.2 MPN/100 mL. Table 5, below, shows the calculated mean differences and standard deviations among all duplicates, and also among duplicates without presence/absence agreement. Among quantitative MPN data gathered, one sample was identified with significantly different levels of *E. coli* between duplicates (Sample LC-D-L-0802B as shown in Table 4). This sample was excluded from summary statistics to avoid skewing the summary statistics.

Table 5 Summary Statistics among Duplicates for *E. coli* Detection*

	All Duplicates	Duplicates with P/A Difference
Mean Difference	2.0 MPN	2.2 MPN
Standard Dev	2.8 MPN	1.3 MPN

* Excludes sample LC-D-L-0802B, an outlier; P/A = Presence/Absence.

Duplicate results indicate that there is minimal variation in MPN values within a sample. This indicates that samples are expected to have minimal MPN variation within one sample, or among different leaves from one head of lettuce. The outlier in this dataset suggests that some leaves from a head of lettuce may be significantly more contaminated than others. This occurred in 1 out of the 18 duplicate samples. In general, it can be concluded that MPN data collected using this *E. coli* detection method is precise in reflecting the MPN value of an entire sample.

4 DISCUSSION

This study surveyed the extent of microbial contamination on lettuce and tomatoes purchased from farmers markets, an increasingly popular source of local produce. Foodborne outbreaks in the U.S. have implicated *Salmonella* and *E. coli* as etiologic agents associated with raw produce-related illness; lettuce and tomatoes in particular have been implicated as common vehicles for *Salmonella* and *E. coli*. Over 120 samples of lettuce and tomatoes were collected from 5 major farmers markets in the Seattle metropolitan area throughout the 2012 summer/fall harvest season. Samples were analyzed for surface contamination of *Salmonella spp.* and generic *E. coli* as an indicator for fecal contamination. A novel approach using Colilert® tests, paired with Quanti-Tray®/2000 (IDEXX), was developed for qualitative and quantitative detection of generic *E. coli* on produce surfaces.

Two presumptive *Salmonella* stocks were collected from two heads of lettuce originating from an organic farm known to raise pigs as an integral part of their crop's nutrient cycle. It was suspected that farming practices involving the integration of such livestock with the production of crops would contribute to the presence of *Salmonella* on produce harvested from these farms. However, no *Salmonella spp.* were detected from the presumptive *Salmonella* stocks collected. API® later confirmed that the presumptive Salmonella isolates were actually *Citrobacter*. *Escherichia coli,* indicating fecal contamination, was present on 62% of lettuce and 6.4% of tomatoes samples. No significant differences were found in *E. coli* contamination by sampling date or between organic and conventional

production methods. There were significantly more lettuce samples contaminated with *E. coli* than tomatoes ($p < .05$).

4.1 Comparison to Similar Studies

The absence of *Salmonella* from these 126 produce samples suggests a low exposure to *Salmonella* from Seattle farmers markets produce. No information is available regarding microbial prevalence on farmers markets produce in the United States. However, a farmers markets study in Alberta, Canada by Bohaychuck *et al.* 2009 provides a source of comparison for this study. In this Canadian study, no *Salmonella* was isolated from any produce samples, which included 128 samples of lettuce and 120 samples of whole tomatoes. Additionally, *E. coli* was found on 18% of lettuce samples and 0% of tomato samples. The method used for detecting *Salmonella* followed the Health Canada procedure MFLP-29, "Qualicon BAX® System Method for the Detection of *Salmonella* in a Variety of Food and Environmental Samples". The method involves a similar process as outlined in the FDA BAM protocol for *Salmonella* detection. The methods are similar through the steps of selective enrichment. After selective enrichment, the Canadian method uses a commercially available BAX® *Salmonella* test kit. The method used for generic *E. coli* detection by Bohaychuck *et al.* was a 3-tube MPN method, Health Canada procedure MFHPB-19 "Enumeration of coliforms, Fecal Coliforms and *E. coli* in Foods using the MPN Method". There was no significant difference between contamination and organic or conventional produce.[63]

The results of this study by Bohaychuck *et al.* are similar to the results of this current study. There was no *Salmonella* found, and no significant differences found

between organic or conventional produce and presence of contamination. However, a difference exists between results for *E. coli.* Over 60% of lettuce samples from Seattle farmers markets had *E. coli*, while only 18% of lettuce from Alberta, Canada had *E. coli*. No tomatoes had *E. coli* from Alberta; about 6% of tomatoes from Seattle had *E. coli*. One possible explanation for this difference could be a difference in detection limits between the 3-tube MPN method used in Canada and the highly sensitive IDEXX Colilert® reagent. Sample sizes of 50 g of lettuce in this current study are also approximately double the amount used in other studies. Increased amounts of produce used in laboratory analysis potentially contribute to increased numbers of positive samples.

Another study for comparison of results was a survey of selected Ontario-grown fresh fruits and vegetables by Arthur *et al.* in 2004. This large-scale study on "farm-fresh" produce included leaf lettuce (n=263), organic leaf lettuce (n=112), head lettuce (n=155), and tomatoes (n=141), analyzed for *Salmonella* and generic *E. coli*. One sample of tomato and one sample of organic leaf lettuce was positive for *Salmonella* from the Arthur *et al.* study.[64] Prevalence of *Salmonella,* therefore, was approximately 0.3% among all lettuce and tomato samples in this study (n=271). Results from this study additionally suggest that there may be a very low baseline level of *Salmonella* on produce fresh from local farms.

Generic *E. coli* prevalence on lettuce and tomato samples from Ontario was detected on 11.6% of organic leaf lettuce and 6.5% of leaf lettuce, with none detected on other types of lettuce or tomato samples.[64] Again, possible explanations

for this difference in *E. coli*-positive sample proportions could be a difference in the sensitivity of methods and the different amounts of lettuce analyzed.

4.2 Future Directions

A novel approach was developed for sensitive and rapid detection of generic *E. coli* on produce surfaces using IDEXX Colilert® paired with Quanti-Tray®/2000. Although it is widely recognized that *E. coli* is the best available indicator for fecal contamination[11] and that IDEXX Colilert® is a highly sensitive and rapid reagent for detecting *E. coli,* no information on the pathogenicity of detected *E. coli* was obtained. For human health relevance, one idea to further develop this detection method is to incorporate multiplex qPCR on *E. coli*-positive/fluorescing wells for the identification of pathogenic *E. coli.* In 2010, Omar *et al.* sampled various surface waters in South Africa and identified significant levels of pathogenic *E. coli* with additional steps after incubating sample waters with IDEXX Colilert® paired with Quanti-Tray®/2000. Liquid was removed from fluorescing wells with a sterile syringe, DNA was extracted from the liquid, and then multiplex PCR was carried out and visualized on agarose gel.[65] Although 97% of the *Escherichia* genus have the enzyme (β-glucuronidase) necessary for fluorescence with IDEXX Colilert® reagent,[51] it should be noted that *E. coli* O157:H7 and other *E. coli* lacking β-glucuronidase were not detected. Using the method by Omar *et al.*, similar results could be obtained from the elution of food surfaces. Modifications to the Omar *et al.* method could include substitution of DNA extraction with heat-release followed by multiplex qPCR for more rapid detection of pathogenic *E. coli*, and an additional detection protocol for pathogenic *E. coli* lacking β-glucuronidase.

Results obtained from the 126 samples in this study resulted in few statistically significant results. A significant difference in *E. coli* presence on lettuce and tomatoes was detected; significantly more lettuce samples were contaminated with *E. coli* than tomato samples. Additionally, a trend in MPN values grouped bimonthly was observed for both lettuce and tomatoes (Figure 9). However, no significant differences were found between contamination and harvest time or organic/conventional production method. Based on these results, a larger sample size should be incorporated in future produce-related sampling studies in order to increase statistical power and pinpoint associations.

4.3 Limitations

This study aids in establishing a baseline for microbial contamination on locally grown produce in Washington. Although results from this study were similar to the few farmers markets-related studies published, there were fewer samples processed for this study in comparison to others. With an increase in the number of samples, more statistically significant associations may arise to help understand and identify factors influencing increased microbial contamination on produce. Additionally, there may be factors that were not accounted for in this study such as farm characteristics, geographic origin of produce, and harvesting and shipping methods. In addition to organic/conventional produce, sampling date, produce type, and vendor, other factors may influence produce contamination and should be further investigated.

5 CONCLUSION

Seattle farmers markets provide diverse communities with access to fresh produce. In light of recent outbreaks associated with produce and minimal regulations existing for sanitation of farmers markets produce, this study aims to establish a baseline for occurrence of fecal contamination and *Salmonella* prevalence on farmers markets produce. This is the first study investigating the extent of microbial contamination on farmers markets produce in the United States. *Salmonella spp.* and *E. coli* on lettuce and tomatoes are the most relevant organisms and produce types to analyze based on Washington State data, national outbreak data and public health impact criterion.

No *Salmonella spp.* were found on lettuce and tomato samples from Seattle farmers markets (n=126). However, the prevalence of generic *E. coli* on the majority of lettuce samples and on 34% of total produce samples indicate that there is room for improvement in Good Agricultural Practices (GAP) and other USDA-recommended produce handling practices. Currently, GAP audits are completely voluntary and are typically performed at larger farm operations.

The application of IDEXX Colilert® paired with Quanti-Tray®/2000 is a rapid and sensitive option for detecting *E. coli* on food surfaces. Further development of pathogen detection methods on foods should be geared towards rapid and sensitive methods for food safety applications. Future investigation of pathogenic organisms other than *Salmonella spp.* on produce surfaces should also be incorporated into detection methods for human health relevance.

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7 APPENDIX

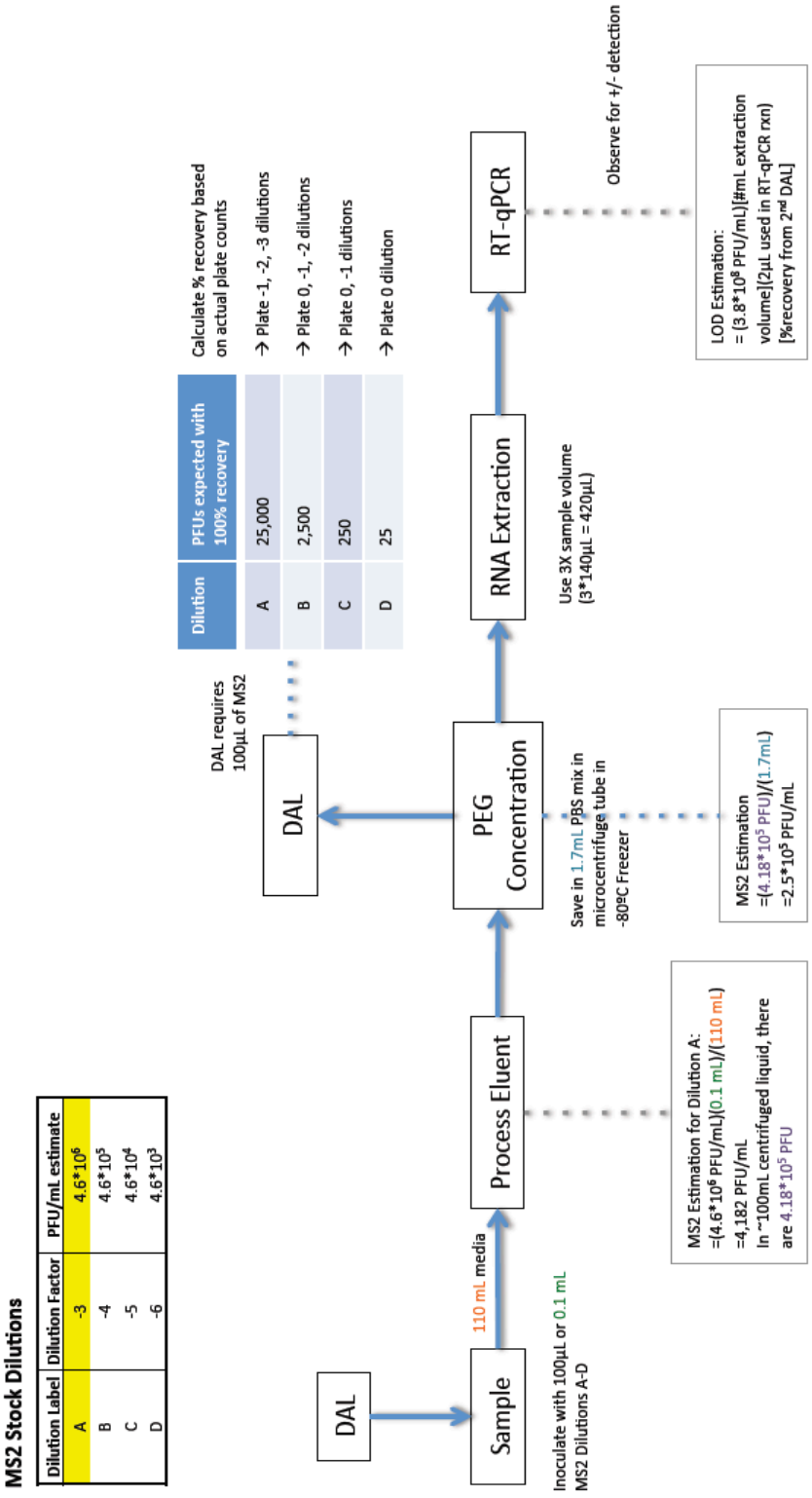

Figure 10 NoV Method Validation Scheme

Table 6 Raw Data for *E. coli* Presence/Absence and Quantification (MPN/100mL)

ID	Date	Market	Vndr	Organic	Produce	MPN1	MPN2
Bal-H-L-0701	2012/7/1	Ballard	H	1	Lettuce	0	0
Bal-R-T-0701	2012/7/1	Ballard	R	0	Tomatoes	0	
Bal-EE-L-0715	2012/7/15	Ballard	EE	1	Lettuce	43.1	34.7
Bal-R-T-0715	2012/7/15	Ballard	R	0	Tomatoes	0	
Bal-A-T-0722	2012/7/22	Ballard	A	0	Tomatoes	0	
Bal-EE-L-0722	2012/7/22	Ballard	EE	1	Lettuce	0	0
Bal-A-T-0729	2012/7/29	Ballard	A	0	Tomatoes	0	
Bal-C-T-0729	2012/7/29	Ballard	C	1	Tomatoes	0	
Bal-S-L-0729A	2012/7/29	Ballard	S	1	Lettuce	1	
Bal-S-L-0729B	2012/7/29	Ballard	S	1	Lettuce	1	0
Bal-A-T-1022	2012/10/22	Ballard	A	0	Tomatoes	0	
Bal-B-T-1022	2012/10/22	Ballard	B	1	Tomatoes	0	
Bal-C-T-1022	2012/10/22	Ballard	C	1	Tomatoes	0	
Bal-DD-L-1022	2012/10/22	Ballard	DD	1	Lettuce	1	
Bal-DD-T-1022	2012/10/22	Ballard	DD	1	Tomatoes	0	
Bal-E-T-1022	2012/10/22	Ballard	E	1	Tomatoes	0	
Bal-EE-L-1022	2012/10/22	Ballard	EE	1	Lettuce	0	
Bal-Q-T-1022	2012/10/22	Ballard	Q	0	Tomatoes	0	
Bal-T-T-1022	2012/10/22	Ballard	T	1	Tomatoes	0	
Bal-V-L-1022A	2012/10/22	Ballard	V	1	Lettuce	0	
Bal-V-L-1022B	2012/10/22	Ballard	V	1	Lettuce	0	
Bal-V-L-1118A	2012/11/18	Ballard	V	1	Lettuce	1299.7	
Bal-V-L-1118B	2012/11/18	Ballard	V	1	Lettuce	105	
Bal-V-L-1118C	2012/11/18	Ballard	V	1	Lettuce	87.8	
Bal-V-L-1118D	2012/11/18	Ballard	V	1	Lettuce	62.4	
Bal-V-L-1118E	2012/11/18	Ballard	V	1	Lettuce	61.3	
Bal-V-L-1118F	2012/11/18	Ballard	V	1	Lettuce	19.9	
Bal-V-L-1118G	2012/11/18	Ballard	V	1	Lettuce	13.2	
Bal-V-L-1118H	2012/11/18	Ballard	V	1	Lettuce	4.1	
Bro-J-L-0701	2012/7/1	Broadway	J	0	Lettuce	0	
Bro-M-T-0715	2012/7/15	Broadway	M	0	Tomatoes	0	
Bro-P-L-0715	2012/7/15	Broadway	P	0	Lettuce	1	0
Bro-A-T-1030	2012/10/30	Broadway	A	0	Tomatoes	0	
Bro-B-T-1030	2012/10/30	Broadway	B	1	Tomatoes	0	
Bro-GG-L-1030	2012/10/30	Broadway	GG	1	Lettuce	0	
Bro-GG-T-1030	2012/10/30	Broadway	GG	1	Tomatoes	0	
Bro-M-T-1030	2012/10/30	Broadway	M	0	Tomatoes	0	
Bro-P-L-1030A	2012/10/30	Broadway	P	0	Lettuce	0	
Bro-P-L-1030B	2012/10/30	Broadway	P	0	Lettuce	0	
Bro-P-T-1030	2012/10/30	Broadway	P	0	Tomatoes	0	

Bro-M-T-1118A	2012/11/18	Broadway	M	0	Tomatoes	0	
Bro-M-T-1118B	2012/11/18	Broadway	M	0	Tomatoes	0	
Bro-A-T-0701	2012/7/1	Broadway	A	0	Tomatoes	0	
LC-B-T-0802	2012/8/2	Lake City	B	1	Tomatoes	0	
LC-D-L-0802A	2012/8/2	Lake City	D	0	Lettuce	629.4	
LC-D-L-0802B	2012/8/2	Lake City	D	0	Lettuce	501.2	58.2
LC-II-L-0802A	2012/8/2	Lake City	II	0	Lettuce	2	
LC-II-L-0802B	2012/8/2	Lake City	II	0	Lettuce	0	
LC-M-T-0802A	2012/8/2	Lake City	M	0	Tomatoes	23.3	
LC-M-T-0802B	2012/8/2	Lake City	M	0	Tomatoes	0	
LC-A-L-1004A	2012/10/4	Lake City	A	0	Lettuce	8.1	
LC-A-L-1004B	2012/10/4	Lake City	A	0	Lettuce	1	
LC-A-T-1004A	2012/10/4	Lake City	A	0	Tomatoes	0	
LC-A-T-1004B	2012/10/4	Lake City	A	0	Tomatoes	0	
LC-A-T-1004C	2012/10/4	Lake City	A	0	Tomatoes	0	
LC-B-T-1004A	2012/10/4	Lake City	B	1	Tomatoes	0	
LC-B-T-1004B	2012/10/4	Lake City	B	1	Tomatoes	0	
LC-B-T-1004C	2012/10/4	Lake City	B	1	Tomatoes	0	
LC-B-T-1004D	2012/10/4	Lake City	B	1	Tomatoes	0	
LC-B-T-1004E	2012/10/4	Lake City	B	1	Tomatoes	0	
LC-B-T-1004F	2012/10/4	Lake City	B	1	Tomatoes	0	
LC-FF-T-1004A	2012/10/4	Lake City	FF	1	Tomatoes	0	
LC-FF-T-1004B	2012/10/4	Lake City	FF	1	Tomatoes	0	
LC-FF-T-1004C	2012/10/4	Lake City	FF	1	Tomatoes	0	
LC-GG-L-1004A	2012/10/4	Lake City	GG	1	Lettuce	0	
LC-GG-L-1004B	2012/10/4	Lake City	GG	1	Lettuce	0	
LC-M-T-1004A	2012/10/4	Lake City	M	0	Tomatoes	0	
LC-M-T-1004B	2012/10/4	Lake City	M	0	Tomatoes	0	
LC-A-L-1015	2012/10/15	Lake City	A	0	Lettuce	4	1
LC-A-T-1015	2012/10/15	Lake City	A	0	Tomatoes	0	
LC-GG-L-1015	2012/10/15	Lake City	GG	1	Lettuce	0	0
LC-M-T-1015	2012/10/15	Lake City	M	0	Tomatoes	0	
PP-BB-T-0624	2012/6/24	Pike Place	BB	0	Tomatoes	0	
PP-K-L-0624	2012/6/24	Pike Place	K	0	Lettuce	2	1
PP-K-L-0701	2012/7/1	Pike Place	K	0	Lettuce	3	0
PP-R-T-0701	2012/7/1	Pike Place	R	0	Tomatoes	0	
PP-A-L-0722A	2012/7/22	Pike Place	A	0	Lettuce	17.1	8
PP-A-L-0722B	2012/7/22	Pike Place	A	0	Lettuce	5	4
PP-R-T-0722A	2012/7/22	Pike Place	R	0	Tomatoes	0	
PP-R-T-0722B	2012/7/22	Pike Place	R	0	Tomatoes	0	
PP-A-T-0728	2012/7/28	Pike Place	A	0	Tomatoes	0	
PP-U-L-0728	2012/7/28	Pike Place	U	0	Lettuce	1	0
PP-BB-L-1030A	2012/10/30	Pike Place	BB	0	Lettuce	5.2	

PP-BB-L-1030B	2012/10/30	Pike Place	BB	0	Lettuce	4.1	
PP-BB-L-1030C	2012/10/30	Pike Place	BB	0	Lettuce	4.1	
PP-BB-L-1030D	2012/10/30	Pike Place	BB	0	Lettuce	1	
PP-BB-L-1030E	2012/10/30	Pike Place	BB	0	Lettuce	0	
PP-G-L-1030A	2012/10/30	Pike Place	G	0	Lettuce	0	
PP-G-L-1030B	2012/10/30	Pike Place	G	0	Lettuce	0	
PP-W-L-1030A	2012/10/30	Pike Place	W	0	Lettuce	1	
PP-W-L-1030B	2012/10/30	Pike Place	W	0	Lettuce	0	
UD-AA-L-0630	2012/6/30	U District	AA	0	Lettuce	3	0
UD-M-T-0630	2012/6/30	U District	M	0	Tomatoes	0	
UD-M-T-0714	2012/7/14	U District	M	0	Tomatoes	0	0
UD-X-L-0714	2012/7/14	U District	X	1	Lettuce	0	
UD-DD-L-0721	2012/7/21	U District	DD	1	Lettuce	26.4	23.3
UD-M-T-0721	2012/7/21	U District	M	0	Tomatoes	0	
UD-D-L-0728	2012/7/28	U District	D	0	Lettuce	17.1	
UD-M-T-0728	2012/7/28	U District	M	0	Tomatoes	1	
UD-FF-T-0804	2012/8/4	U District	FF	1	Tomatoes	0	
UD-O-L-0804	2012/8/4	U District	O	1	Lettuce	14.6	
UD-CC-L-0818	2012/8/18	U District	CC	0	Lettuce	2419.6	
UD-FF-T-0818	2012/8/18	U District	FF	1	Tomatoes	0	
UD-I-L-0818	2012/8/18	U District	I	1	Lettuce	4.1	
UD-L-T-0818	2012/8/18	U District	L	1	Tomatoes	0	
UD-N-L-0818	2012/8/18	U District	N	0	Lettuce	3.1	
UD-Y-T-0818	2012/8/18	U District	Y	1	Tomatoes	0	
UD-D-L-1015A	2012/10/15	U District	D	0	Lettuce	0	
UD-D-L-1015B	2012/10/15	U District	D	0	Lettuce	0	
UD-DD-L-1015A	2012/10/15	U District	DD	1	Lettuce	0	0
UD-DD-L-1015B	2012/10/15	U District	DD	1	Lettuce	0	0
UD-F-T-1015A	2012/10/15	U District	F	1	Tomatoes	0	
UD-F-T-1015B	2012/10/15	U District	F	1	Tomatoes	0	
UD-HH-T-1015A	2012/10/15	U District	HH	0	Tomatoes	0	
UD-HH-T-1015B	2012/10/15	U District	HH	0	Tomatoes	0	
UD-Z-T-1015A	2012/10/15	U District	Z	0	Tomatoes	1	
UD-Z-T-1015B	2012/10/15	U District	Z	0	Tomatoes	0	
UD-A-T-1103	2012/11/3	U District	A	0	Tomatoes	0	
UD-DD-L-1103A	2012/11/3	U District	DD	1	Lettuce	1	
UD-DD-L-1103B	2012/11/3	U District	DD	1	Lettuce	0	
UD-DD-L-1103C	2012/11/3	U District	DD	1	Lettuce	0	
UD-GG-L-1103A	2012/11/3	U District	GG	1	Lettuce	3.1	
UD-GG-L-1103B	2012/11/3	U District	GG	1	Lettuce	2	
UD-GG-T-1103	2012/11/3	U District	GG	1	Tomatoes	0	
UD-L-T-1103	2012/11/3	U District	L	1	Tomatoes	0	
UD-M-T-1103	2012/11/3	U District	M	0	Tomatoes	2	

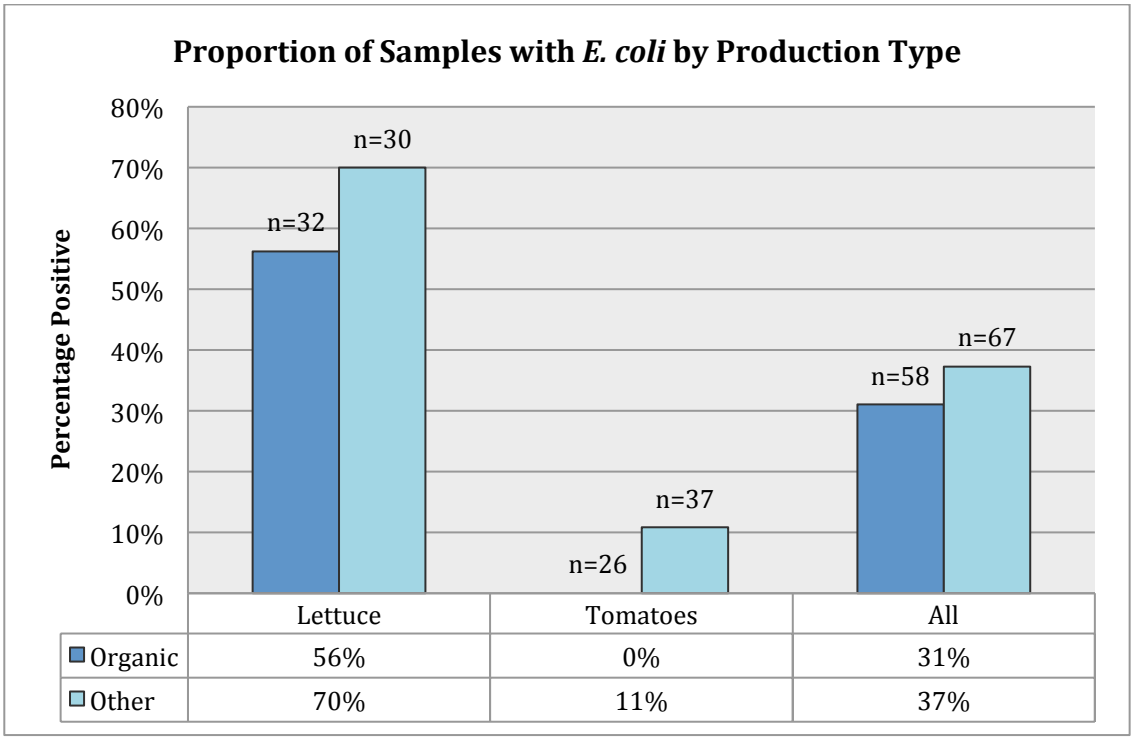

Figure 11 *E. coli* Presence and Production Type

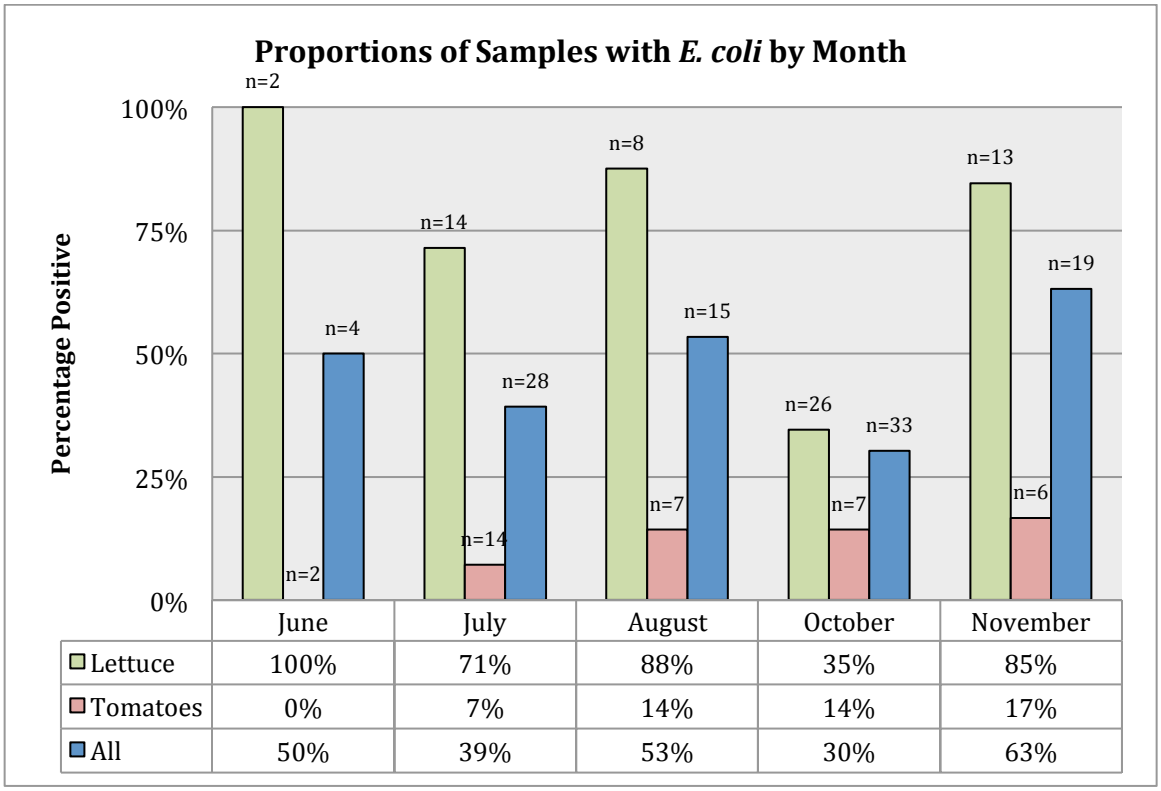

Figure 12 *E. coli* Presence and Month of Sampling