SPATIO-TEMPORAL DISTRIBUTION AND ECOLOGY OF *LISTERIA*, *SALMONELLA*, AND SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN NEW YORK STATE

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ABSTRACT

Agricultural lands are often interspersed with areas of natural land cover (e.g., forests, wetlands) that may serve as environmental reservoirs of pathogens. However, the potential for natural areas to serve as reservoirs of food-associated pathogens is poorly understood. As well, *Listeria* species have been isolated from diverse environments, often at considerable prevalence, and are known to persist in food processing facilities. The presence of *Listeria* spp. has been suggested to be an indicator of *L. monocytogenes* contamination.

In the studies presented here, we investigated the distribution of *Listeria monocytogenes*, *Listeria* spp., *Salmonella*, and Shiga toxin-producing *Escherichia coli* (STEC) in five study sites representing natural environments across New York State (NYS). Data from an additional study was also used to compare the prevalence and diversity of *Listeria* spp. between produce production and natural environments. Geographical and meteorological factors that affect the prevalence of *L. monocytogenes* and *Listeria* spp. in these environments were also explored.

We found that (i) *L. monocytogenes* is found at considerably higher prevalence than *Salmonella* and STEC in natural areas of NYS, (ii) the prevalence of *L. monocytogenes* shows considerable variation associated with season, geospatial, and meteorological factors, and (iii) the effects of proximity to water and pasture lands on *L. monocytogenes* prevalence differ between locations. Differences in baseline values of *L. monocytogenes* prevalence among the five study sites suggested that different sets of ecosystem dynamics influence the presence of *L. monocytogenes* in different study sites.

We also found that the prevalence of *Listeria* spp. was approximately 34% and 33% for samples obtained from produce production (201/588) and natural environments (245/734), respectively. The co-isolation of *L. monocytogenes* and at least one other species of *Listeria* from

a sample was 9% in produce production environments, compared to 3% in natural environments. Soil moisture and proximity to water and pastures were identified as important factors for detection of *Listeria* spp. in produce production environments, while elevation, study site and proximity to pastures were identified as important predictors for detection of *Listeria* spp. in natural environments, as determined by randomForest models. Our data show (i) that *Listeria* spp. were prevalent in both agricultural and non-agricultural environments and (ii) that geographical and meteorological factors associated with *Listeria* spp. detection were considerably different between the two environments.

Educational modules were also developed, inspired in part by the research presented here. Career and educational opportunities in food science and food safety are under-recognized by K-12 students and educators. Additionally, misperceptions regarding nature of science understanding continue to persist in K-12 students. In an effort to increase awareness concerning career and educational opportunities in food science and food safety and to improve nature of science understanding among K-12 students, a series of problem-based learning modules was developed and pilot tested with a total of 61 K-12 students. Evaluations and assessments indicated that (i) interest in science and food safety increased and (ii) content knowledge related to the nature of science, food science, and food safety was improved. We further suggest that these modules provide opportunities for educators in traditional as well as extracurricular settings to demonstrate important concepts contained in the newly released Next Generation Science Standards.

BIOGRAPHICAL SKETCH

Travis Kyle Chapin was born and raised in North Texas as the first of two children to Mark and Wanda Chapin. Travis graduated from Texas Tech University magna cum laude with a Bachelors of Science in Food Science and Technology. Travis then joined the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases (a.k.a., the Food Safety Lab) under the direction of Dr. Martin Wiedmann at Cornell.

Travis' research interests center on the ecology of *Listeria* in New York State, with a special focus on natural areas such as forests, wetlands, and natural areas. Food safety education is another subject of interest to Travis. During his time at Cornell, Travis served as a teaching assistant and played a key role in the development of a case study-based course in food safety for graduate students. Additionally, Travis worked to improve ongoing K-12 food safety outreach efforts and to develop new efforts.

After graduation, Travis plans to begin work at the University of Florida and extend his research to understanding the ecology of other foodborne pathogens in agricultural lands of Florida.

To Dr. Ronald “Max” Miller

for encouraging me to seek out new opportunities in unfamiliar places.

I am infinitely wiser for it.

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Lastly, I thank Brewster for late night jogs through snow and rain, waking me in the mornings, and always welcoming me home.

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PREFACE

The bulk of the work presented here comprises my research into the ecology of food-associated pathogens (i.e., *E. coli* O157:H7 and other Shiga toxin-producing *E. coli*, *Listeria monocytogenes*, and *Salmonella*) and also *Listeria* species. The results of this work are presented in the second and third chapters of this thesis (the first chapter provides an introduction). In addition to researching the ecology of the aforementioned organisms, I was given the unique opportunity to devote portions of my time at Cornell to K-12 food science and food safety outreach efforts. While seemingly unrelated to my primary research, the modules that I co-developed for these efforts were often inspired by my own research experiences and findings; this work is presented in the fourth chapter.

CHAPTER 1

INTRODUCTION

Foodborne illness represents a significant public health burden in the United States. Each year there are an estimated 9.4 million illnesses, 55,961 hospitalizations and 1,351 deaths due to domestically acquired foodborne pathogens (1). *Listeria monocytogenes*, *Salmonella*, and Shiga-toxin producing *Escherichia coli* (STEC; including both *E. coli* O157 and non-O157 STEC) are key foodborne pathogens that, combined, are estimated to represent a considerable proportion of domestically acquired foodborne illnesses (28%), hospitalizations (71%), and deaths (76%) attributed to known bacterial pathogens in the United States each year (1). These pathogens have also been associated with environmental sources and transmission via wildlife.

L. monocytogenes, *Salmonella*, and STEC have been implicated in various outbreaks linked to environmental sources. For example, a listeriosis outbreak linked to contaminated cabbage was likely caused by contamination of the cabbage in the field, possibly linked to manure, from sheep with listeriosis, that was used to fertilize this field (2). Sources of *Salmonella* Newport appear to be responsible for repeat contamination of tomatoes grown on the eastern shore of Virginia (3). Wild ruminants were identified as the likely source of STEC that was linked to an outbreak involving the consumption of apple cider (12). STEC was also linked to direct transmission from wildlife fecal material to humans when an outbreak of *E. coli* O157 infections was associated with exposure of children to contaminated elk feces on a sports field (4). Previous work has demonstrated survival of these pathogens in the environment for extended periods of time (5-11).

A previous study by our group suggested that specific geographical factors influence the distribution of *Listeria* spp. in the natural environment (12). Our first goal was to further

elucidate the role of these geographical factors specifically for *L. monocytogenes*-positive samples, and additionally to establish a baseline prevalence of *Salmonella* and STEC in natural environments of New York State (NYS). An improved understanding concerning the roles of geographical factors on pathogen distribution, as well as environmental prevalence data, will play an important role in the development of science-based pre-harvest risk reduction strategies (e.g., developing appropriate buffer zones between agricultural fields and other land covers, such as forests, pastures, and bodies of water).

The next phase of our study examined the geographical factors important to other *Listeria* sp., in comparison to *L. monocytogenes*. *Listeria* spp. detection has often been used to identify conditions that may indicate the presence of *L. monocytogenes*. *Listeria* spp. are often described as ubiquitous in nature and are considered widely distributed in a variety of environmental habitats around the world including food processing plants, sewage outfalls, silage, soil, vegetation, and water (7). *L. monocytogenes* in particular has been shown to exist on every continent except Antarctica (13). However, other *Listeria* sp. appear to have considerably narrower, and/or possibly unique, host ranges as illustrated by the newly reported *L. marthii* species having only been detected in a forest in central NYS (14) and interestingly, the newly reported *L. fleischmannii* subsp. *coloradensis* genome encodes a mosquitocidal toxin, possibly suggesting adaptation to an insect host (15). Considerable data support species- and lineage-specific associations with different sources (e.g., (16-19).

The third component of this thesis presents the results of K-12 food science and food safety outreach efforts. The overarching goals of these efforts were three-fold: (i) to stimulate interest and expose students to career opportunities in food science, food safety and science in general, (ii) to simultaneously increase the scientific literacy and nature of science understanding

of the participants in agreement with current science education reform efforts, and (iii) to create a food science/safety curriculum that K-12 educators can use in a variety of traditional or extracurricular settings. The discipline of food science as a career is not well recognized among high school students and the number of food science graduates is insufficient to supply the demand in the workforce (20-22). Regardless of whether students remain in the food science or food safety pipeline, scientific literacy and nature of science understanding are crucial to success in other educational and career fields (23, 24). The educational modules developed here as part of these efforts should also be of interest to K-12 educators who may need to adapt their curriculum in recognition of the recently released Next Generation Science Standards (NGSS).

Additional studies are needed to further elucidate pathogen ecology in the environment. As well, novel educational modules such as those presented here require continuous refinements and validation to align with current science teaching reform efforts. Overall, the work presented here builds on the body of knowledge concerning *Listeria* ecology and provides important baseline data regarding the prevalence of two important food-associated pathogens, *Salmonella* and STEC, in understudied environments. The development of food science and food safety-based K-12 educational modules may prove useful to educators for conveying important concepts contained in the NGSS, and the continued distribution and use of these modules in traditional and extracurricular educational settings may eventually contribute to increasing the number of food science graduates.

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CHAPTER 2

SPATIO-TEMPORAL DISTRIBUTION OF *LISTERIA MONOCYTOGENES*, *SALMONELLA*, AND SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN NATURAL ENVIRONMENTS OF NEW YORK STATE*

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ABSTRACT

Agricultural lands are often interspersed with areas of natural land cover (e.g., forests, wetlands) that may serve as environmental reservoirs of pathogens. However, the potential for natural areas to serve as reservoirs of food-associated pathogens is poorly understood. A study was conducted to determine the prevalence and distribution of *Listeria monocytogenes*, *Salmonella*, and Shiga toxin-producing *Escherichia coli* (STEC) in five study sites representing wildlife refuges and management areas, national forests, and forest preserves across New York State (NYS). A total of 734 samples (e.g., wildlife fecal, soil, water) were geographically referenced and analyzed for the presence of the target pathogens. The overall prevalence was 8% (59/734) for *L. monocytogenes*, 1% (8/734) for *Salmonella*, and 1% (6/734) for STEC. Classification tree (CT) models and randomForest (RF) analysis were used to analyze the influence of season, geospatial, and meteorological factors on the distribution of *L. monocytogenes*. Differences in baseline values of *L. monocytogenes* prevalence among the five study sites suggested that different sets of ecosystem dynamics influence the presence of *L. monocytogenes* in different study sites. Our data indicate that (i) *L. monocytogenes* is found at considerably higher prevalence than *Salmonella* and STEC in natural areas of NYS, (ii) the prevalence of *L. monocytogenes* shows considerable variation associated with season, geospatial, and meteorological factors, and (iii) the effects of proximity to water and pasture lands on *L. monocytogenes* prevalence differ between locations.

INTRODUCTION

Listeria monocytogenes, *Salmonella*, and Shiga-toxin producing *Escherichia coli* (STEC), including both *E. coli* O157 and non-O157 STEC, are key foodborne pathogens that have been associated with environmental sources and transmission via wildlife. Combined, these pathogens are estimated to represent a considerable proportion of domestically acquired foodborne illnesses (28%), hospitalizations (71%), and deaths (76%) attributed to known bacterial pathogens in the United States each year (1). *L. monocytogenes* has been isolated from domestic ruminants (e.g., cattle and sheep) as well as a variety of environmental sources (e.g., food processing plants, sewage outfalls, and silage) (2). While *L. monocytogenes* contamination of foods is often associated with environmental sources in food processing plants (3), a listeriosis outbreak linked to contaminated cabbage was likely caused by contamination of the cabbage in the field, possibly linked to manure, from sheep with listeriosis, that was used to fertilize this field (4). Along with recent concerns about listeriosis outbreaks and recalls linked to raw produce, this illustrates the importance of environmental sources in transmission of *L. monocytogenes*. While *Salmonella* is a zoonotic pathogen that causes gastroenteritis in a variety of animal hosts and is often associated with livestock (e.g., poultry and cattle), environmental sources and wildlife have also been well documented to be important sources of this pathogen. For example, environmental sources of *Salmonella* Newport appear to be responsible for repeat contamination of tomatoes grown on the eastern shore of Virginia in the US (5). While *E. coli* O157 contamination has been linked to both domestic and wild ruminants, sources of non-O157 STEC are much less well understood. For *E. coli* O157, wildlife has not only been linked to contamination of food in pre-harvest environments, such as contamination by wild ruminants of apples used to produce apple cider (12), but also has been linked to direct transmission from wildlife fecal material to humans. For example, in Colorado, an outbreak of *E. coli* O157 infections was linked to exposure of children to contaminated elk feces on a sports field (6).

Environmental sources of the target pathogens studied here (i.e., *L. monocytogenes*, *Salmonella*, and STEC) have the potential to contribute to human (and possible animal) disease burden. While previous work has suggested that these pathogens can survive in the environment, potentially for extended periods of time (2, 7-9), our understanding of the ecology of these organisms in natural areas is limited. For example, little is known about the role that natural areas may have in harboring pathogens for potential transmission to food production areas including produce fields. Agricultural lands in New York State (NYS) and other agricultural producing regions are widely interspersed with areas of natural land cover (e.g., forests, wetlands), which may serve as environmental reservoirs of foodborne pathogens such as *L. monocytogenes*, *Salmonella*, and STEC. We thus studied five natural areas in NYS as a model to gain a more complete understanding of (i) the ecology of *L. monocytogenes*, *Salmonella*, and STEC and (ii) the role these areas may play as reservoirs for these pathogens. We conducted a two-year study in 5 distinct study sites across NYS to elucidate the prevalence of these pathogens in natural areas and to identify geographical predictors for pathogen detection.

METHODS

Study sites. Five natural areas (i.e., undeveloped areas that provide refuge to wildlife and show minimal human disturbance) were selected as study sites from across NYS. The study sites were Adirondack Forest Preserve (ADK), Catskill Forest Preserve (CATSK), Connecticut Hill Wildlife Management Area (CHWMA), Finger Lakes National Forest (FLNF), and Montezuma National Wildlife Refuge (MNWR; see Table 2.1 and Fig. 2.1). Based on their location, these study sites were also grouped into "eastern study sites" (ADK, CATSK) and "western study sites" (CHWMA, FLNF, MNWR). Within each of the 5 study sites, three different sampling areas were selected for sample collection on each sampling date; each sampling area was approx. 10,000 $m^{2.}$. Sampling areas exhibited evidence of recent wildlife activity as supported by visual identification of bedding areas, tracks, and wildlife scat. Sampling was conducted three times a year (spring, summer and fall) in each 2009 and 2010 with new sampling areas visited on each

field excursion. Overall, samples were collected from a total of 90 different sampling areas (5 study sites x 3 sampling areas/site x 6 sampling events/site).

TABLE 2.1. Study Sites

Park	Management	Approx. Size (ha)	Land uses
Connecticut Hill Wildlife Management Area (CHWMA)	NYS DEC[a]	4.5×10^3	game refuge, recreation
Catskill Forest Preserve (CATSK)	NYS DEC[a]	1.2×10^5	timber preserve, recreation, plant and wildlife habitat
Adirondack Forest Preserve (ADK)	NYS DEC[a]	1.0×10^6	timber preserve, recreation, plant and wildlife habitat
Montezuma National Wildlife Refuge (MNWR)	USFWS[b]	2.9×10^3	refuge/breeding ground for migratory birds and other wildlife
Finger Lakes National Forest (FLNF)	USFS[c]	6.6×10^3	timber, recreation, wildlife habitat, livestock grazing

[a]New York State Department of Environmental Conservation
[b]United States Fish and Wildlife Service
[c]United States Forest Service

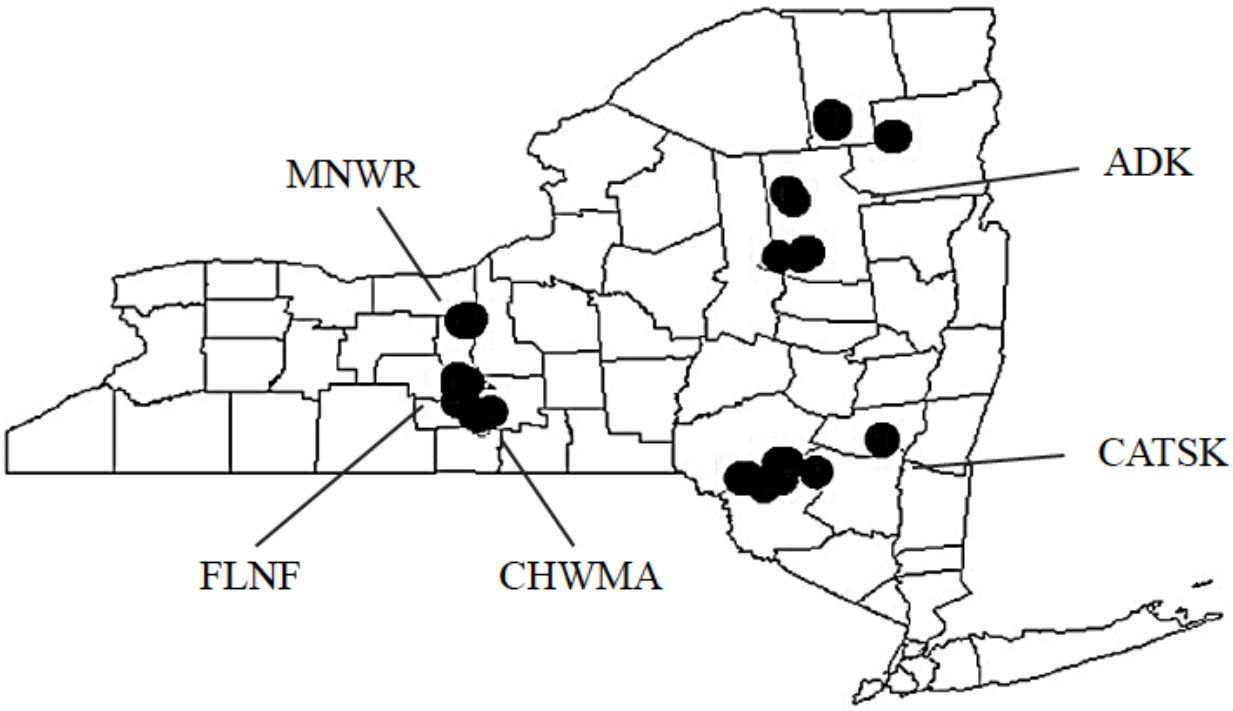

FIG. 2.1. Study sites in natural areas of New York State included areas within the Adirondack Forest Preserve (ADK), Catskill Forest Preserve (CATSK), Connecticut Hill Wildlife Management Area (CHWMA), Finger Lakes National Forest (FLNF), and Montezuma National Wildlife Refuge (MNWR).

Sample collection. At each sampling area, five soil samples, five water samples and a single surface drag swab were collected. An effort was made to obtain a total of 15 wildlife fecal samples across the sampling areas at each study site at each sampling event. Over the 30 total sampling events, 15 fecal samples were collected on 28 occasions, and 13 and 16 fecal samples were collected on one occasion each, yielding a total of 449 wildlife fecal samples. The animal origin of wildlife scat was determined using the National Audubon Society Field Guide to Mammals (10). Soil samples were taken from the surface to an approximate depth of 5 to 8 cm. Fecal (up to 25 g, if available) and soil samples (at least 15 g) were collected into sterile 532 ml (18 oz) Whirl-Pak bags (Nasco, Fort Atkinson, WI). Water samples (approx. 250 ml) were collected directly into whirl-pak bags from the surface water sources in closest proximity to the wildlife congregation areas. Drag swabs were prepared as described by Uesugi et al. (7) with minor modifications. Briefly, a 50 ml volume of tryptic soy broth with phosphate buffer (TSB-PO_4) (11) was added to each drag swab and swabs were stored frozen until use. Using clean, disposable latex gloves, the drag swabs were dragged along the ground, for approximately 10 min throughout the sampling area. GPS coordinates were recorded for each sample location; coordinates for drag swabs were taken at the end of dragging. Samples were stored on ice during transit to the laboratory and processed within 24 h of collection.

Sample processing. Each sample was used as inoculum in enrichment cultures to detect four target organisms (i.e., *L. monocytogenes*, *E. coli* O157:H7, non-O157 STEC, and *Salmonella*). Drag swabs and fecal samples were tested individually, while soil and water samples were pooled prior to testing. Briefly, two 25 g pooled soil samples were created by combining five grams of soil from each of five samples collected per sampling area. One fecal sample aliquot (1/2 of a given fecal sample, representing 10 g if available) or one 25 g pooled soil sample were used for *L. monocytogenes* isolation as detailed below. The second fecal sample portion and another 25 g pooled soil sample were used for an initial 2 h recovery step (as detailed below), which was followed by separate procedures for isolation of (i) *E. coli* O157 and (ii) *Salmonella*

and non-O157 STEC. For water samples and drag swabs, three sample aliquots were prepared and used for (i) *L. monocytogenes* isolation; (ii) *E. coli* O157 isolation, and (iii) *Salmonella* and non-O157 STEC isolation, which shared a common enrichment step (11, 12), as detailed below. Water samples from each sampling area were pooled by passing 50 ml of each of the five water subsamples (250 ml total) through a single 0.45 μm pore-size filter unit (Nalgene, Rochester, N.Y.); samples were vigorously shaken before application to the filter. Each filter was aseptically cut into thirds for subsequent enrichment. Similarly, drag swabs were hand massaged in Whirl-Pak™ bags for 1 min and three (10 ml) aliquots of broth were squeezed from the swab and used for the three separate enrichments.

***L. monocytogenes* detection.** *L. monocytogenes* detection was performed using procedures adapted from the FDA Bacteriological Analytical Manual (13) and Nightingale et al. (14). Briefly, Buffered *Listeria* Enrichment Broth (BLEB) was added to a given sample aliquot to achieve a 1:10 dilution, followed by manual homogenization until solid matter was completely suspended. After an initial incubation at 30°C for 4 h, the selective antimicrobial supplement (Oxoid, Cambridge, UK) containing acriflavine hydrochloride, cycloheximide, and nalidixic acid was added to each sample, followed by continued incubation at 30°C. After incubation for a total of 24 and 48 h, separate 50 μl aliquots of enrichments were streaked onto each Oxford medium (Oxoid) and *Listeria monocytogenes* Chromogenic Plating Medium (LMPM; R&F Laboratories, Downers Grove, Illinois), followed by incubation for 48 h at 30°C (Oxford) or 35°C (LMPM). PCR amplification and sequencing of *sigB* (15) was used to confirm up to 4 presumptive *L. monocytogenes* colonies from LMPM. If no colonies that resembled *L. monocytogenes* were obtained from LMPM, up to 4 *Listeria* colonies from Oxford were substreaked onto LMPM and confirmed if they showed typical *L. monocytogenes* morphology.

Detection of *E. coli* O157, non-O157 STEC and *Salmonella*. Enrichment for *Salmonella* and STEC was performed as reported by Barkocy-Gallagher et al. (11, 12) with some modifications. During year 1, samples were initially incubated in TSB-PO_4 at room temperature for 2 h, followed by incubated for 6 h at (i) 35°C, for isolation of *Salmonella* and non-O157 STEC and

(ii) 42°C, for the isolation of *E. coli* O157. During year 2, the incubation period for the enrichments at 35°C and 42°C was increased to 24 h to allow for improved recovery of *Salmonella.*

Isolation of *E. coli* O157:H7 was performed as described by Barkocy-Gallagher et al. (11, 12) and Carlson et al. (16). Briefly, after enrichment in TSB-PO_4 at 42°C, immunomagnetic bead separation (IMS) was performed using anti-O157 immunomagnetic beads, followed by plating on modified sorbitol MacConkey agar (MSMAC) and CHROM-O157 (CHROMagar, Paris, France) as previously described (16, 17). For the isolation of non-O157 STEC, *E. coli* broth (Neogen, Lansing, MI) was inoculated with TSB-PO_4 enrichments that had been incubated at 35°C. After incubation with aeration (shaking) for 24 h at 37°C, a 50 µl enrichment aliquot was plated on washed sheep blood agar containing Ca^{2+} ions and mitomycin C (WBMA) (18). Up to 20 presumptive STEC colonies on WBMA were substreaked to sorbitol MacConkey agar (SMAC). Presumptive *E. coli* isolates from both *E. coli* O157 and non-O157 enrichments were screened for the presence of six genes (*stxI*, *stxII*, *eaeA*, *hlyE*, $fliC_{H7}$, *rfbE*) by a multiplex PCR (16, 19). Isolates were classified as STEC if this assay revealed the presence of either *stxI* or *stxII*.

Methods for the isolation of *Salmonella* were adapted from the FDA Bacteriological Analytical Manual (20). Rappaport-Vassiliadis (RV) broth and tetrathionate (TT) broth were inoculated with TSB-PO_4 enrichments (at 1:100 and 1:10, respectively) that had been incubated at 35°C. RV and TT broth were incubated at 42°C in a shaking water bath for 24 h, followed by plating on xylose lysine deoxycholate agar (XLD, Neogen) and CHROM-*Salmonella* (CHROMagar). Up to 20 presumptive *Salmonella* colonies were confirmed by a PCR assay that targets *invA* (21).

Isolate storage and characterization. All confirmed *L. monocytogenes*, *Salmonella*, and STEC isolates were preserved in BHI with 15% glycerol at -80°C. Confirmed STEC and *Salmonella* isolates were sent for serotyping to, respectively, the *E. coli* Reference Center at Pennsylvania

State University (State College, PA) or the NYS Department of Health (Albany, NY) as detailed previously (22).

Univariate statistical analysis. Categorical analyses, using the statistical package R version 2.13.0 (R Foundation for Statistical Computing [www.r-project.org/]), were conducted to determine associations between study sites, seasons, and sample types. Statistical associations were evaluated using chi-square tests; Fisher's exact tests were applied if more than 20% of expected values were less than five.

Input data for classification tree analysis. Classification trees (CTs) were generated to identify seasonal, geospatial, and meteorological factors that were determinants of *L. monocytogenes* prevalence; this approach was adapted from Ivanek et al. (23) and Strawn et al. (22) as detailed below. Of the 734 samples collected, 49 samples were excluded due to missing data. Geospatial data (e.g., soil characteristics, elevation, proximity to relevant landscape features such as water; see Table 2.5) were obtained for each sample site essentially as detailed by Strawn et al. (22) using the Geographical Resources Analysis Support System (GRASS) GIS (Geographic Information Systems) environment (version 6.4.1; Open Source Geospatial Foundation [http://grass.osgeo.org]).

Meteorological variables for each sample collection date were obtained, essentially as detailed in Strawn et al. (22), from the weather station in the National Oceanic and Atmospheric Administration (NOAA) National Climate Data Center (NCDC) Surface Daily Observation Database (http://gis.ncdc.noaa.gov/map/cdo/) that is the nearest to each sampling area. In total, 56 different meteorological factors were obtained for CT model development, including temperature (maximum and minimum) and precipitation amounts (Table 2.5). Measures of temperature and precipitation were acquired for the day of sampling and three days antecedent. The average temperature and precipitation amount was calculated for each time period ranging from 1 to 10 days prior to sample collection.

As large numbers of geospatial and meteorological variables were included in our classification analysis (Table 2.5), there was a high potential for covariation among geospatial

and meteorological predictors of pathogen presence. Detrending and principal components analysis (PCA) techniques were applied to account for the linear covariation among predictors, using the approaches described by Strawn et al. (22). Temperature and precipitation were detrended for seasonal effects by performing linear regressions and retaining the residuals from these regressions to represent variation of temperature and precipitation within seasons. Similarly, soil properties and elevation were detrended against the five study sites using linear regressions. As substantial covariation among meteorological and geospatial data remained after detrending, detrended residuals were standardized and used as input for two separate PCAs to synthesize variation among meteorological and geospatial data, respectively, into eigenvectors representing the characteristic behavior of these variables. PCA on meteorological variables yielded an eigenvector that represented 56.9% of the total variation and corresponded well to all temperature variables. The same PCA yielded a second eigenvector representing 22.0% of the total variation that corresponded well to all precipitation variables except precipitation on the third day antecedent to sampling. The second PCA showed that geospatial data were less likely to be heavily loaded on eigenvectors and demonstrated less covariation among landscape data. This PCA did not yield any useful eigenvectors. Soil and topographic data were thus retained as independent predictors of pathogen presence.

CT model development. CT modeling was used to determine rules, based on geospatial and meteorological variables, which classified sampled sites by pathogen presence or absence (see Table 2.5 for all variables). CTs were built using the rpart package in R 2.13.1 (version 3.1-55; Recursive Partitioning, Therneau, Atkinson, and Ripley [http://cran.r-project.org/web/packages/rpart/index.html]) as previously described (22). Cross-validation and weighting of the response variable (i.e., pathogen presence or absence) to reflect probabilities of false negatives were also performed as previously described (22, 23).

randomForest analysis. Multiple iterations of CT model fitting on the *L. monocytogenes* data yielded consistent split rules, but the size of the trees, in number of splits, was not reproducible and ranged from 4 to 14 splits. In this case, it was difficult to determine which split rules had the greatest predictive value for environmental prevelance of *L. monocytogenes* from a single CT. We used randomForest (RF) analysis to evaluate the importance of the different predictor variables for the distribution of *L. monocytogenes*; this technique has been described extensively in other recent publications (24). RF generates consensus classification schemes by summarizing multiple unpruned CTs across bootstrapped samples of presence/absence data. The technique withholds a subset of "out-of-bag" (OOB) samples during growth of each CT. The withheld samples are then added back into each CT, and the ability of the CT to properly classify the OOB samples is measured. The importance of predictor variables is scored for each CT in the RF by comparing the classification error for OOB samples across all CTs against the decrease in classification accuracy for OOB samples when a predictor variable is randomly permuted before adding OOB samples back into the CTs. In this work, RF was run for 10,000 CTs with four randomly selected predictor variables tested at each split. A simulation of 10,000 bootstrap samples (with replacement) of the *L. monocytogenes* presence/absence observations indicated that, on average, 63% of the unique observations were utilized as in-bag samples in each bootstrap sample (range=59% to 68%). Based on this simulation, we expect that approx. 255 samples were withheld from each CT.

Pasture density analysis. A 10-m-resolution map of New York State from the National Land Cover Database was projected into the UTM coordinate system with the North American Datum of 1983 (NAD83) ellipsoid. This map was used to estimate the density of pasture-class land cover near sample sites. Pasture-class land cover was isolated into a binary map and a moving window neighborhood analysis was used to sum the amount of pasture-class pixel coverage within a 2 km diameter circle around every point on the map. This sum was then divided by the total possible window area (30,787 10-m square pixels) to produce the percentage of pasture-class land cover within a 1 km radius of each sample location. Mean and variance in land cover

were calculated on the total population of pasture-class land-cover values associated with samples within each study site.

RESULTS

***Salmonella* and STEC were found at 1% prevalence in environmental samples from New York State natural areas.** Among a total of 734 samples tested (449 fecal, 15 pond sediment, 90 drag swab and 90 each of pooled soil and water samples), none tested positive for multiple pathogen species, although one sample yielded two STEC isolates with different *stx* profiles (Table 2.2). *Salmonella* was isolated from 1% of samples (8/734; Tables 2.3 and 2.4); the eight *Salmonella* isolates further characterized (one per positive sample) were identified as serovars Hartford (2 isolates), Holcomb (2 isolates), Newport, Thompson, Typhimurium var. O:5-, and IV 40:z4,z32:0- (Table 2.3). Six of the *Salmonella*-positive samples were wildlife fecal samples identified as raccoon feces (3 positive samples among 15 total samples) as well as deer, coyote, and goose feces (one positive each among 210, 45, and 130 samples, respectively). Overall, six *Salmonella*-positive samples were collected from MNWR and *Salmonella*-positive sample(s) were detected during four out of six MNWR sampling events. MNWR was the only location that yielded *Salmonella* isolates from samples other than wildlife fecal samples (i.e., soil and water; Table 2.3).

STEC were isolated from 1% of samples (6/734; Tables 2 and 4). The seven STEC isolates serotyped (1 per sample, plus 1 additional isolate for a particular sample that yielded isolates with different Shiga-toxin gene profiles) were identified as *E. coli* O157:H7 (5 isolates), *E. coli* O8:H19, and *E. coli* O91:H49 (Table 2.2). The STEC-positive samples included 2 wildlife fecal samples (both from white-tailed deer), 2 pooled soil samples, and 2 drag swab samples. All STEC-positive samples were collected during the spring (Table 2.3).

TABLE 2.2. *Salmonella* isolated from the natural environment

Isolate ID	Study site	Season	Sample	Serotype
FSL C7-142	MNWR	Summer	Fecal, raccoon	*Salmonella* Hartford
FSL C7-144	MNWR	Summer	Fecal, raccoon	*Salmonella* Hartford
FSL C7-150	MNWR	Summer	Fecal, deer	*Salmonella* Thompson
FSL C7-325	FLNF	Fall	Fecal, coyote	*Salmonella* Newport
FSL C7-523	MNWR	Spring	Water	*Salmonella* IV 40:z4,z32:-
FSL C7-650	MNWR	Summer	Soil	*Salmonella* Holcomb
FSL C7-1028	MNWR	Fall	Fecal, raccoon	*Salmonella* Holcomb
FSL C7-1193	ADK	Fall	Fecal, goose	*Salmonella* Typhimurium var. O:5-

TABLE 2.3. STEC isolated from the natural environment

Isolate ID	Study site	Season	Sample	Serotype and Shiga toxin gene profile[b]
FSL C7-029	CHWMA	Spring	Soil	O8:H19; *stx*II
FSL C7-528	MNWR	Spring	Soil	O157:H7; *stx*I/*stx*II
FSL C7-616	FLNF	Spring	Fecal, deer	O157:H7; *stx*I/*stx*II
FSL C7-618[a]	FLNF	Spring	Drag swab	O157:H7; *stx*I/*stx*II
FSL C7-619[a]	FLNF	Spring	Drag swab	O157:H7; *stx*I
FSL C7-620	FLNF	Spring	Drag swab	O157:H7; *stx*I/*stx* II
FSL C7-636	CATSK	Spring	Fecal, deer	O91:H49; *stx*I

TABLE 2.4. Frequency of *L. monocytogenes, Salmonella* and STEC in Natural Areas of New York State

		Frequency (percent)[a]		
	No. samples	*L. monocytogenes*	*Salmonella*	STEC
Study site				
CHWMA	152	9 (5.9%)	0 (0.0)	1 (0.7)
FLNF	145	7 (4.8)	1 (0.7)	3 (2.1)
MNWR	146	7 (4.8)	6 (4.1)	1 (0.7)
CATSK	148	20 (13.5)	0 (0.0)	0 (0.0)
ADK	143	16 (11.2)	1 (0.7)	0 (0.0)
Season				
spring	241	5 (2.1)^{x}	1 (0.4)	5 (2.1)
summer	248	31 (12.5)^{y}	4 (1.6)	0 (0.0)
fall	245	23 (9.4)^{y}	3 (1.2)	0 (0.0)
Sample Type				
fecal	449	45 (10.0)^{x}	6 (1.3)	1 (0.2)
soil (pooled)	90	7 (7.8)^{xy}	1 (1.1)	2 (2.2)
drag swab	90	5 (5.6)^{xy}	0 (0.0)	2 (2.2)
water (pooled)	90	1 (1.1)^{y}	1 (1.1)	0 (0.0)
sediment	15	1 (6.7)^{xy}	0 (0.0)	0 (0.0)

[a] Different letter (x, y) indicate a significant difference between factors (determined by 95% confidence interval). Overall chi square analyses showed that the number of *L. monocytogenes* positive samples was not randomly distributed ($P<0.016$) for season (3 x 2 chi square) and sample type (5 x 2 chi square).

***L. monocytogenes* was found at 8% prevalence in environmental samples from New York State natural areas (Table 4).** While the highest prevalence of *L. monocytogenes* was observed in CATSK (14%) and ADK (11%), there was no significant difference in prevalence among the five study sites. Observed prevalence did differ significantly by season though, with the lowest prevalence observed in the spring (2.1%; see Table 2.4). *L. monocytogenes* prevalence also differed significantly by sample type with highest prevalence in fecal samples (10.0%) and lowest in pooled water samples (1.1%). CATSK and ADK had a higher percentage of *L. monocytogenes*-positive wildlife fecal samples (17% and 12%, respectively) as compared to CHWMA (8%), MNWR (7%) and FLNF (7%).

Classification trees divided sites by season, study site, and impervious surface proximity according to the presence or absence of *L. monocytogenes*. Among the six split rules in the CT (Fig. 2.2), four were based on spatial variables, and one each was based on seasonality and precipitation (Fig. 2.2). The first three split rules (which showed the highest improvement scores) were based on season, study site, and proximity to impervious surfaces (i.e., roads, urban development). In order to identify alternative splitting criteria, competitor rules, which produce splits with similar importance scores but use alternative variables, and surrogate rules, which produce splits that correlate with the primary rules, were also generated; these rules are described below along with the primary rules.

Figure 2.2 Caption. Classification tree (CT) categorizing *L. monocytogenes* positive and negative samples into homogenous groups ("nodes") based on key landscape and meteorological factors. Data used represent observed prevalence for 685 sampling locations (Table 5 provides a description of all variables used in CT). The CT shown is that with the lowest overall cross validation error among CTs created with different weights for negative samples and was pruned to minimize the cross validation error. The tree shown divides sites by season, study sites, and proximity to impervious surfaces (i.e., roads). Each box shows the primary rule (in bold) as well as competing and surrogate rules that partition samples to the left-hand daughter node; each split rule increases homogeneity in the daughter nodes by enriching for negative samples in the left-hand daughter nodes and for positive samples in the right-hand daughter nodes; percentages indicate *L. monocytogenes* prevalence for a given node (L and R indicate the number of samples partitioning in the left and right daughter node). For terminal modes, "+" and "-" indicate the number of cultured positive and negative samples. Primary rules are those used to make the split shown, while competing rules produce similar splits as the primary rules but use alternative variables and surrogate rules produce different splits than the primary rules. Figure 4 provides a full summary of the CT. Briefly, the competing rule for split 1 ("Preciptation.day3. residuals $\geq$ -.6120742") is interpreted as less-than-average precipitation at day 3 prior to sample collection. The competing rule for split 2 ("Temperature < 1.594907") is the eigenvector for temperature variables (climate.loadings.1) and is interpreted as greater-than-average. The primary rule for split 4 ("Water Storage.residuals (100cm) $\geq$ 0.6813527") is interpreted as greater-than-average. The primary rule for split 5 ("Slope $\geq$ 3.1%") is interpreted as land that is not flat. The primary rule for split 6 (Precipitation < -0.5214732) is the eigenvector for precipitation variables (climate.loadings.2) and is interpreted as greater-than-average. The competing (Loam soil = No) and primary rule (Precipitation < -0.5214732) for split 6 had the same improvement score.

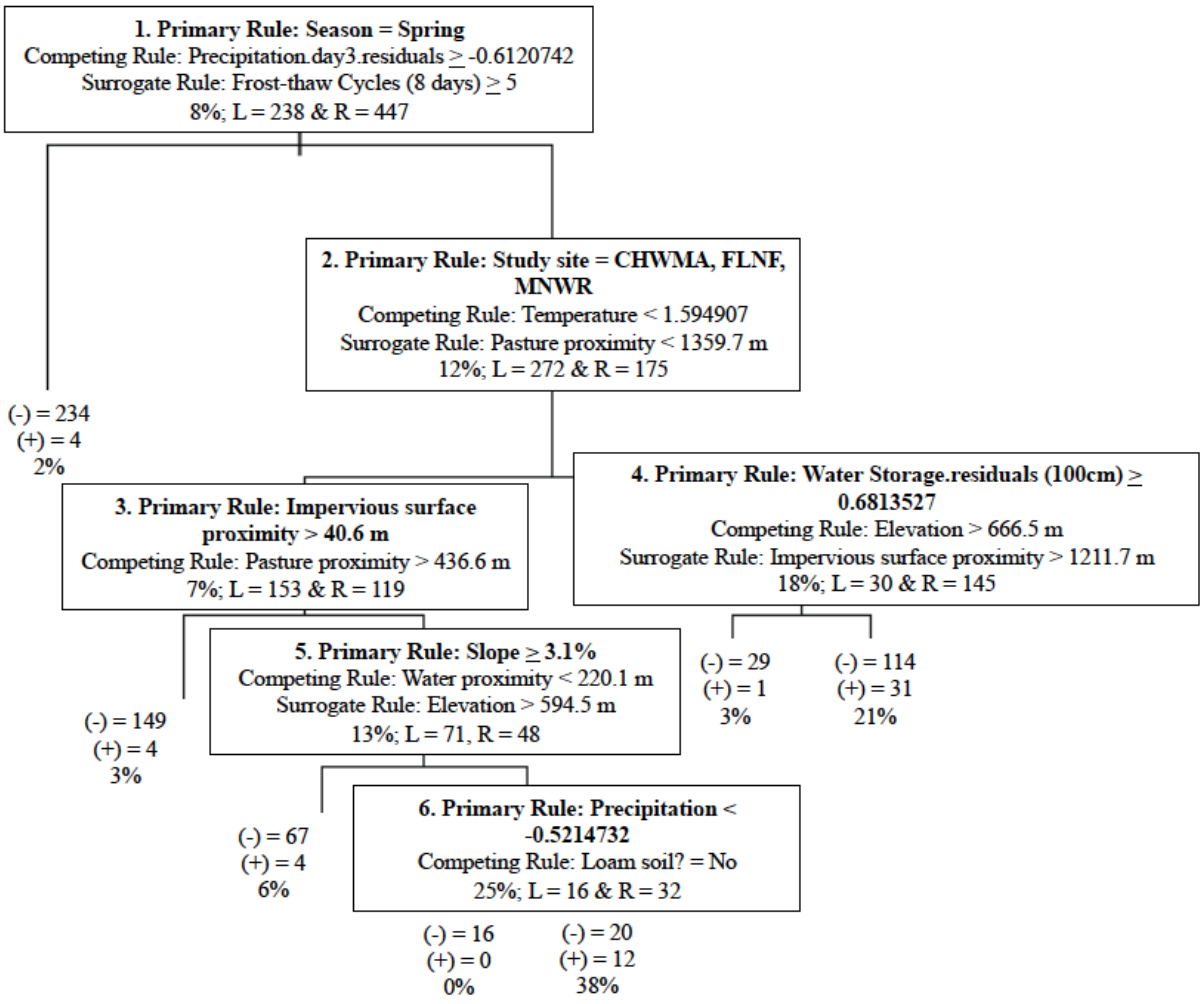
1. Primary Rule: Season = Spring
Competing Rule: Precipitation.day3.residuals ≥ -0.6120742
Surrogate Rule: Frost-thaw Cycles (8 days) ≥ 5
8%; L = 238 & R = 447
(-) = 234
(+) = 4
2%
2. Primary Rule: Study site = CHWMA, FLNF, MNWR
Competing Rule: Temperature < 1.594907
Surrogate Rule: Pasture proximity < 1359.7 m
12%; L = 272 & R = 175
3. Primary Rule: Impervious surface proximity > 40.6 m
Competing Rule: Pasture proximity > 436.6 m
7%; L = 153 & R = 119
4. Primary Rule: Water Storage.residuals (100cm) ≥ 0.6813527
Competing Rule: Elevation > 666.5 m
Surrogate Rule: Impervious surface proximity > 1211.7 m
18%; L = 30 & R = 145
(-) = 29
(+) = 1
3%
(-) = 114
(+) = 31
21%
(-) = 149
(+) = 4
3%
5. Primary Rule: Slope ≥ 3.1%
Competing Rule: Water proximity < 220.1 m
Surrogate Rule: Elevation > 594.5 m
13%; L = 71, R = 48
(-) = 67
(+) = 4
6%
6. Primary Rule: Precipitation < -0.5214732
Competing Rule: Loam soil? = No
25%; L = 16 & R = 32
(-) = 16
(+) = 0
0%
(-) = 20
(+) = 12
38%

Figure 2.2.

Table 2.5. Names, Descriptions, and Units of Measurement (where applicable) of the Variables Used in the Classification Trees and randomForest Analysis (Table adapted from Strawn and Ivanek (23, 25)

Variable name	Description	Unit
Soil.loadings.1	Eigenvector generated for spatially derived properties	
ElevLog10.residuals	Residuals of log transformed vertical elevation	
SLOPE.residuals	Residuals of difference in elevation between two points, expressed as percentage between two points	
LN_AWS025WTA.residuals	Residuals of natural log transformed volume of water that the soil (to 25 cm) can store that is available to plants and expressed as the weighted average of all components	
LN_AWS050WTA.residuals	As for AWS025WTA, but 0-50 cm	
LN_AWS0100WTA.residuals	As for AWS025WTA, but 0-100cm	
URBAN_PROX_LOG10.residuals	Residuals of log transformed distance to nearest impervious surface	
WATER_PROX_LOG10.residuals	Residuals of log transformed distance to nearest open water	
Pasture_prox_LOG10.residuals	Residuals of log transformed distance to nearest pasture land cover	
climate.loadings.1	Eigenvector generated for temporally derived properties including temperature max and min	
TMIN.0	Minimum temperature on the specified day, t0	°C
TMIN.1	As for TMIN.0, but 1 day before day, t1	°C
TMIN.2	As for TMIN.0, but 2 days before day, t2	°C
TMIN.3	As for TMIN.0, but 3 days before day, t3	°C
TMIN.0_1	Average minimum temperature for the specified time period, t0-t1	°C
TMIN.0_2	As for TMIN.0_1, but t0-t2	°C
TMIN.0_3	As for TMIN.0_1, but t0-t3	°C
TMIN.0_4	As for TMIN.0_1, but t0-t4	°C
TMIN.0_5	As for TMIN.0_1, but t0-t5	°C
TMIN.0_6	As for TMIN.0_1, but t0-t6	°C
TMIN.0_7	As for TMIN.0_1, but t0-t7	°C
TMIN.0_8	As for TMIN.0_1, but t0-t8	°C
TMIN.0_9	As for TMIN.0_1, but t0-t9	°C
TMIN.0_10	As for TMIN.0_1, but t0-t10	°C
TMAX.0	Maximum temperature on the specified day, t0	°C
TMAX.1	As for TMAX.0, but 1 day before day, t1	°C
TMAX.2	As for TMAX.0, but 2 days before day, t2	°C

Table 2.5 (Continued).

TMAX.3	As for TMAX.0, but 3 days before day, t3	°C
TMAX.0_1	Average maximum temperature for the specified time period, t0-t1	°C
TMAX.0_2	As for TMAX.0_1, but t0-t2	°C
TMAX.0_3	As for TMAX.0_1, but t0-t3	°C
TMAX.0_4	As for TMAX.0_1, but t0-t4	°C
TMAX.0_5	As for TMAX.0_1, but t0-t5	°C
TMAX.0_6	As for TMAX.0_1, but t0-t6	°C
TMAX.0_7	As for TMAX.0_1, but t0-t7	°C
TMAX.0_8	As for TMAX.0_1, but t0-t8	°C
TMAX.0_9	As for TMAX.0_1, but t0-t9	°C
TMAX.0_10	As for TMAX.0_1, but t0-t10	°C
climate.loadings.2	Eigenvector generated for temporally derived properties including precipitation	
PRCP.1	Amount of rain 1 day before day of collection, t1	mm
PRCP.2	As PRCP.1, but 2 days before, t2	mm
PRCP.3	As PRCP.1, but 3 days before, t3	mm
PRCP.0_1	Average precipitation for the specified time period, t0-t1	mm
PRCP.0_2	As for PRCP.0_1, but t0-t2	mm
PRCP.0_3	As for PRCP.0_2, but t0-t3	mm
PRCP.0_4	As for PRCP.0_2, but t0-t4	mm
PRCP.0_5	As for PRCP.0_2, but t0-t5	mm
PRCP.0_6	As for PRCP.0_2, but t0-t6	mm
PRCP.0_7	As for PRCP.0_2, but t0-t7	mm
PRCP.0_8	As for PRCP.0_2, but t0-t8	mm
PRCP.0_9	As for PRCP.0_2, but t0-t9	mm
PRCP.0_10	As for PRCP.0_2, but t0-t10	mm
Independent	Variables added to classification tree step not as an eigenvector	
AWS025WTA.residuals	The volume of water that the soil to the specified depth can store that is available to plants and expressed as the weighted average of all components	NA
AWS050WTA.residuals	As for aws025wta, but 0-50 cm	NA
AWS0100WTA.residuals	As for aws025wta, but 0-100 cm	NA
AWS0150WTA.residuals	As for aws025wta, but 0-150 cm	NA
URBAN_PROX	Distance to nearest impervious surface	m
WATER_PROX	Distance to nearest open water	m
pasture_prox	Distance to nearest pasture land cover	m

Table 2.5 (Continued).

LNAUSE	NLCD land cover classification	NA
LOAM	Loam soil (yes/no)	NA
ELEV	Vertical elevation	m
SLOPE	Difference in elevation between two points, expressed as a percentage between two points	%
WTDEPANNMI	The shallowest depth to a wet soil layer (water table), annual minimum	cm
DRCLASSDCD	The natural drainage condition of the soil (referring to the frequency and duration of wet periods) of the dominant drainage class	NA
park	Natural area (i.e., ADK, CATSK, CHWMA, FLNF, MNWR)	NA
SEASON	Season when samples were collected (i.e., spring, summer and fall)	NA
PRCP.3mm.residuals	Daily precipitation 3 days before day of collection, t3	NA
FT_0	No. of Freeze/thaw cycles on the specified day, t0	NA
FT_1	As for FT_0, but 1 day before day, t1	NA
FT_2	As for FT_0, but 2 days before day, t2	NA
FT_3	As for FT_0, but 3 days before day, t3	NA
FT_0_1	No. Freeze/thaw cycles for the specified time period, t0-t1	NA
FT_0_2	As for FT_0_1, but t0-t2	NA
FT_0_3	As for FT_0_1, but t0-t3	NA
FT_0_4	As for FT_0_1, but t0-t4	NA
FT_0_5	As for FT_0_1, but t0-t5	NA
FT_0_6	As for FT_0_1, but t0-t6	NA
FT_0_7	As for FT_0_1, but t0-t7	NA
FT_0_8	As for FT_0_1, but t0-t8	NA
FT_0_9	As for FT_0_1, but t0-t9	NA
FT_0_10	As for FT_0_1, but t0-t10	NA

[a] The day of sample collection is denoted at t0, the day before is t1, and so on until 10 days before collection (t10). NA is not applicable.

In agreement with the univariate analysis, season was a strong predictor of *L. monocytogenes* detection. The first primary split rule (designated split 1) separated samples collected in the spring (March, April, May; n=238; 2% *L. monocytogenes*) from those collected in the fall or summer (n=447; 12% *L. monocytogenes*). The competing rule divided the sites by precipitation on the third day prior to sample collection. Among the 129 samples in the node with less-than-average-precipitation, 27 were collected during the summer 2010 CATSK sampling event which had a high amount of precipitation on the day of sample collection (36.1 mm), the previous day (59.4 mm), and two days prior (17.3 mm) but had no recorded precipitation on the third day prior to sample collection. Therefore, this site would not have been included in this node based on a split rule from any other precipitation variable from this data set. The surrogate rule for this split divided sites by the total number of frost-thaw cycles (i.e., $\geq$ 5 or < 5) for the day of sample collection and the 8 days prior. The node with < 5 frost-thaw cycles favored *L. monocytogenes*-positive samples with 54/59 *L. monocytogenes*-positive samples occurring in this node.

Split 2 (Fig. 2.2) divided the samples collected in summer and fall into nodes according to study site with (i) a node that contains CHWMA, FLNF, and MNWR sites (7% *L. monocytogenes* prevalence) and (ii) a node that contains the ADK and CATSK sites (18% *L. monocytogenes* prevalence). The competing rule, based on the temperature eigenvector, divided the samples into a node with 7% *L. monocytogenes* prevalence and higher average temperatures and a node with 17% *L. monocytogenes* prevalence and lower average temperatures. Because the eigenvector of the competing split rule is not easily interpretable, the average of the daily maximum temperatures reported for the day of sample collection and five days antecedent (TMAX.0_5; Table 2.5) was used as a representative of the temperature eigenvector. *L. monocytogenes*-positive samples were enriched for in the node with an average temperature of 15.3 °C (TMAX.0_5) while *L. monocytogenes*-negative samples were enriched for in the node with an average temperature of 23.7 °C (TMAX.0_5). This temperature-based rule created a split

similar to the primary rule; 222 of 233 sites in the high temperature node were from CHWMA, FLNF, or MNWR while 164 of 214 sites in the low temperature node were from ADK or CATSK. The surrogate rule for split 2 divided the sites by proximity to pastures into a node of samples within 1359.7 m of pastures that was enriched for *L. monocytogenes*-negative sites (9% positives) and a node with greater distance from pastures that favored *L. monocytogenes*-positive samples (18% positives). In the node with close proximity to pastures, the average distance to pastures was 460 m. In the node with greater distance to pastures, the average distance to pastures was 1800 m. Like the competing (temperature-based) rule, this surrogate (pasture proximity-based) rule made a similar split as the primary (study site-based) rule; 264 of 313 sites in the node nearer to pastures were from CHWMA, FLNF, or MNWR while 126 of 132 sites in the node farther from pastures were from ADK or CATSK. The density of pasture-class land cover within 1 km of sampling sites demonstrated that ADK and CATSK had considerably lower pasture density (0 and 1.1%, respectively) than FLNF and MNWR (18.7, and 10.8%, respectively).

Split 3 divided the CHWMA, FLNF, and MNWR node by proximity ($\geq$ 40.6 m or < 40.6 m) to impervious surfaces (e.g., roads, urban development) into a node with greater distance to impervious surfaces (mean = 342 m) that was enriched for *L. monocytogenes*-negative samples and a node with close proximity to impervious surfaces (mean = 14 m) that favored *L. monocytogenes*-positive samples. A competitor rule was proximity to pastures (greater than, or less than, 436.6 m), which divided the samples into a node enriched for *L. monocytogenes*-negative samples (average distance of 856 m to pastures) and a node that favored *L. monocytogenes*-positive samples (average distance of 198 m to pastures).

randomForest analysis. RandomForest (RF) analysis tested the importance of geospatial and meteorological predictor data in accurately predicting the presence or absence of *L. monocytogenes* across 10,000 CT runs. RF results indicated that quantitative geospatial and meteorological variables were universally more accurate than their categorical counterparts (Fig. 2.3). Slope, proximity to pasture class land, proximity to surface water, mean temperature in 0-5

d prior to sampling and precipitation 3 d prior to sampling were the most important predictor variables. Random permutation of these predictors reduced the accuracy of *L. monocytogenes* presence/absence results by > 2 standard deviations below the mean prediction accuracy for all CTs in the RF. This suggests that interpretation of the competing rules from the CT (Fig 2.2) should be given more weight than the primary rule in most nodes. However, it should be noted that there is broad agreement between the finer grained quantitative geospatial and meteorological variables and the categorical variables like season and study site.

Figure 2.3 Caption. Importance of predictor variables used in the RF analysis model. The predictive power of the RF analysis was improved when specific topographic and spatial variables were used rather than broad sample categories like park or season. The mean decrease in accuracy shows the loss of predictive power that occurs when randomly permuted predictor values were used for model validation. Values are standardized to the mean classification accuracy for OOB samples across all CTs in the RF. For example, a decrease in accuracy of -3.2 for slope indicates that, randomly permuting slope values amongst the OOB samples, decreases the classification accuracy of *L. monocytogenes* positive samples in the RF by an average of 3.2 standard deviations over 10,000 CTs.

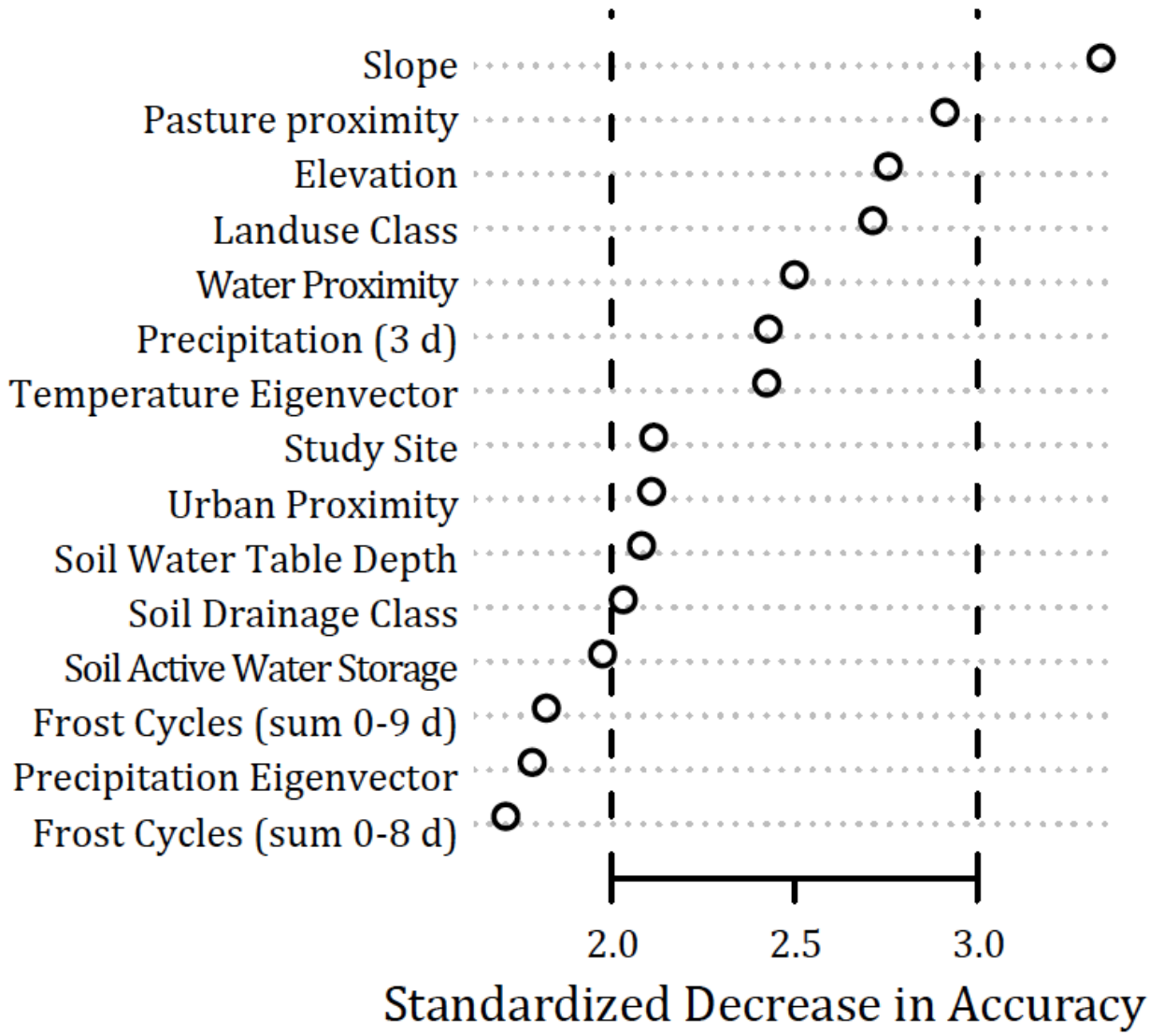
Slope
Pasture proximity
Elevation
Landuse Class
Water Proximity
Precipitation (3 d)
Temperature Eigenvector
Study Site
Urban Proximity
Soil Water Table Depth
Soil Drainage Class
Soil Active Water Storage
Frost Cycles (sum 0-9 d)
Precipitation Eigenvector
Frost Cycles (sum 0-8 d)
2.0
2.5
3.0
Standardized Decrease in Accuracy

Figure 2.3.

Classification tree and randomForest analyses provide consensus rules that allow for the prediction of sites with high prevalence of *L. monocytogenes*. Both RF (Fig. 2.3) and CT (Fig. 2.2) analysis indicated that the study sites in eastern NYS exhibited different patterns of presence/absence of *L. monocytogenes* from samples collected in the Finger Lakes Region (FLNF, MNWR and CHWMA). Therefore, the results of these two analyses were jointly used to determine the consensus rules that are the most accurate predictors of *L. monocytogenes* in these study sites. These consensus rules may be useful to find conditions under which *L. monocytogenes* isolation is expected to be most (or least) likely. For both the eastern and Finger Lakes study sites, more positive samples were observed at lower elevations and flatter slopes. While partial dependence plots exhibited substantial noise, samples collected at 0 to 2% slopes had between a 39% to 120% greater likelihood of being positive than samples collected at greater slopes. Median elevation values for positive and negative samples were (i) 521 and 537 m, respectively, for ADK and CATSK and (ii) 485 and 509 m, respectively, for FLNF, CHWMA and MNWR.

There were a number of differences with regard to the factors that appear to affect the likelihood of *L. monocytogenes* isolation in the eastern and Finger Lakes study sites. In ADK and CATSK study sites, samples obtained close to surface water were more likely to be positive for *L. monocytogenes*, but for the FLNF, MNWR and CHWMA study sites samples obtained close to pasture-class land cover were more likely to be positive. The dependence on proximity to pasture class land cover in the western study sites was possibly influenced by the fact that local pasture density was higher at two of the eastern sites (FLNF and MNWR) than the western study sites. For the eastern study sites, samples obtained in close proximity to roads and urban development also were more likely to be positive for *L. monocytogenes*. In the ADK and CATSK sites, the median distance to surface water was 90 and 108 m for positive and negative samples, respectively; for the same study sites the median distance to pasture class land cover was approx. 1,700 m and 2,600 m for positive and negative samples, respectively. In general,

samples collected within 110 m of surface water were approximately 5 to 27% more likely to be positive than samples collected at greater distances. By contrast, for the FLNF, MNWR and CHWMA study sites, the median distance to surface water was approximately 320 m and 230 m for positive and negative samples and the median distance to pasture-class land cover was 330 m and 430 m for positives and negatives, respectively. In these study sites, samples collected within 100 m of pasture-class land cover were approximately 28 to 75% more likely to be positive than samples collected at greater distances.

DISCUSSION

Testing of 734 samples, from five natural areas (e.g., wildlife management areas and forest preserves), for the presence of *L. monocytogenes*, STEC, and *Salmonella* allowed us to assess the prevalence of these pathogens in environments that may serve as pathogen reservoirs that can lead to contamination of preharvest environments. Subsequent categorical and GIS based analyses allowed us to identify and rank season as well as geospatial and meteorological factors that may be predictors of *L. monocytogenes* presence. Overall, our data indicate that (i) *L. monocytogenes* is found at considerably higher prevalence than STEC and *Salmonella* in natural areas in NYS; (ii) the prevalence of *L. monocytogenes* shows considerable variation associated with geospatial and meteorological factors; and (iii) the effects, of proximity to open water sources and pasture lands, on *L. monocytogenes* prevalence differ between locations.

***L. monocytogenes* is found at considerably higher prevalence than STEC and *Salmonella* in natural environments of NYS.** Our data indicate that natural environments can harbor STEC and *Salmonella,* two key pathogens for which contamination originating from environmental sources may play an important role in their transmission. While presence in preharvest environments may appear to be less of a factor in the foodborne transmission of *L. monocytogenes*, frequent isolation of *L. monocytogenes* from natural environments suggests that preharvest environments cannot be ignored as potential sources of this pathogen. It may be that *L. monocytogenes* in the pre-harvest environment and surrounding lands is dispersed to

packaging or processing operations where it can contaminate these facilities (26, 27). While we found an overall *L. monocytogenes* prevalence of 8%, in our study sites, a number of previous studies reported lower prevalences, including a previous study in NYS (1.4% prevalence; 907 samples) (28), a study of wilderness areas in Colorado, USA (0.5% prevalence, 572 samples) (29) and several studies outside the US that reported *L. monocytogenes* prevalences ranging from 0 to 5% in natural areas and wildlife feces (30-32). The fact that we intentionally targeted wildlife congregation areas as study sites may have contributed to the elevated prevalence found here. On the other hand, similar or higher *L. monocytogenes* prevalences were observed in urban environments of NYS (7.5%) (28), dairy farms in NYS (24.4% and 43% in two separate studies) (14, 33), produce fields in NYS (15%) (25) and a Canadian watershed (19%) (34). While *L. monocytogenes* appears to be commonly found in a variety of environments, a considerable range of prevalences has been reported, which suggests that site characteristics and sampling time may have considerable influence on observed *L. monocytogenes* prevalence.

While previous studies have demonstrated that wildlife can transmit STEC (35-37), STEC prevalence in our study was low (1%) with 5 samples positive for O157 and two positive for other STEC. The non-O157 STEC isolates detected here were identified as *E. coli* O8:H19 and *E. coli* O91:H49, both of which are among the more common serogroups associated with human illnesses (38), suggesting that wildlife and from natural areas may occasionally act as a source of human disease associated STEC. A similar study (29) conducted in natural areas of Colorado also reported a STEC prevalence of 1% (572 samples); isolates from all five positive samples were non-O157 STEC. In our study reported here, both wildlife fecal samples that were positive for STEC (one O157 and one non-O157 STEC) were from white-tailed deer, consistent with previous reports that wildlife, with the occasional exception of deer, are rarely associated with *E. coli* O157:H7 (reviewed in (39) and (40)). Consistent with the low STEC prevalence in white-tailed deer reported here, other studies reported *E. coli* O157:H7 prevalences in deer feces ranging from 0% to 2.4% (40), even though several outbreak investigations (41, 42) have reported higher *E. coli* O157:H7 prevalences in deer fecal samples. By comparison, studies

outside the U.S. reported STEC prevalences in wild ruminants ranging from 2% to as high as 53% (32, 43-49); in studies that distinguished between *E. coli* O157:H7 and non-O157 STEC, the prevalence of *E. coli* O157:H7 has regularly been reported at <2% in wild deer (43-48). While there is considerable evidence that wildlife and deer in particular are infrequent carriers of *E. coli* O157:H7, it is possible that carriage of *E. coli* O157:H7 among wildlife may be increased under certain conditions (e.g., if close contact with domestic animals occurs, as previously suggested (43, 50)). Consistent with this hypothesis, five out of seven STEC-positive samples reported here were collected in study sites with the highest pasture density.

The low *Salmonella* prevalence reported here (1%) was also comparable to the low *Salmonella* prevalence reported in a similar study conducted in natural areas of Colorado (29) (<1% with 2/572 samples positive for *Salmonella*). The majority (6/8) of our *Salmonella*-positive samples were fecal samples from wildlife, including raccoons, deer, coyotes, and geese. Consistent with our finding that 3 of 15 raccoon fecal samples tested positive for *Salmonella*, previous studies have reported *Salmonella* prevalences of 7, 28, and 47% for raccoon fecal samples (51-53); by comparison previously reported *Salmonella* prevalences in deer fecal samples ranged from 0 to 2% (32, 54-57), similar to the <1% prevalence that we observed in white-tailed deer fecal samples. Both our results and previous studies showed a low *Salmonella* prevalence in Canada geese (56-59). While Thomason et al. (60) reported a high *Salmonella* prevalence (>10%) in soil and vegetation samples from two separate urban parks, other studies typically reported lower prevalences in soil samples, including prevalences of 2 and 3% in soil samples from produce growing areas in California and NYS (22, 57). Similar to our data for STEC, seven of eight *Salmonella*-positive samples were collected in natural areas with the highest nearby pasture density (i.e., FLNF and MNWR). Combined, these data suggest that, where environmental contamination with the pathogens occurs, wildlife may facilitate transfer of both of these pathogens from natural areas to other areas, potentially including preharvest produce environments.

***L. monocytogenes* prevalence shows considerable variation associated with season as well as geospatial, and meteorological factors.** The relatively high prevalence of *L. monocytogenes* allowed us to use both categorical analyses and classification trees to further explore the effects of study site, season, and finer resolution geospatial and meteorological factors on *L. monocytogenes* prevalence. Initial categorical analyses showed that *L. monocytogenes* prevalence differed significantly by season with a significantly higher prevalence in summer and fall as compared to spring. Previous studies have also documented seasonal trends with regard to *L. monocytogenes* prevalence, even though the seasons that showed highest prevalence appear to differ among studies. For example, the highest *L. monocytogenes* prevalence has been observed in spring for both a Canadian watershed and urban environments in NYS (28, 34) and in both winter and summer for NYS dairy cattle farms and NYS produce fields (25, 33). As effects of season on prevalence could be due to a number of different factors that were not relevant to our study, such as seasonal manure applications to cropland (34), we used CT and RF analyses to further probe for geographical variables that may affect seasonal *L. monocytogenes* prevalence. Interestingly, we found that precipitation and frost/thaw variables may be able to explain much of the seasonal variation in *L. monocytogenes* prevalence we observed. Our findings are consistent with previous observations (23) that higher *Listeria* spp. prevalences were linked to increased precipitation and reduced number of frost-thaw cycles in the days preceding sampling. In conjunction with data reported by Strawn et al. (22), who found an increased *Salmonella* prevalence in produce fields after precipitation events, these data may suggest a possible broad link between recent precipitation events and an increased pathogen contamination risk.

Interestingly, our CT also identified a number of geospatial factors that appear to affect *L. monocytogenes* prevalence. While there was numerically, but not statistically significant, higher prevalence in the eastern sites (ADK and CATSK) as compared to the western sites, the second primary rule in the CTs separated the eastern and western sites, suggesting differences in the baseline prevalences. While there are a number of possible differences between the eastern and western sites that may contribute to the different prevalences (e.g., typically lower temperatures

in the eastern sites, which may increase survival of *L. monocytogenes* (61), our RF analyses indicated that a number of geospatial factors appear more important factors than temperature related parameters. Key factors identified by the RF as contributing to CT accuracy included percent slope, pasture proximity, elevation, land cover class, and water proximity. While several previous studies have determined that steep slopes increase the prevalence of *Listeria* spp. and fecal coliforms in water samples (23, 62), our results indicated that *L. monocytogenes* was more likely to be detected from relatively flat landscapes. In our study, only one pooled water sample (out of 90 pooled water samples) was positive for *L. monocytogenes*, so the association between flat landscapes and *L. monocytogenes* prevalence largely reflect the likelihood of detecting *L. monocytogenes* from soil and fecal samples. Taken together these data suggest that the temporal and spatial trends often observed in *L. monocytogenes* prevalence are influenced by complex, site-specific interactions and additional work is needed to identify global and/or site specific predictors of *L. monocytogenes* presence in the environment and to validate predictors identified here. In this study, sample sites were selected by convenience of access to sample areas; in future studies, use of a systematic approach for *a priori* selection of sampling sites, along with formal sample size calculations will further strengthen the study design. The results reported here provide a basis for hypotheses to inform sample area selection and a quantitative basis for sample size calculations.

The effects of proximity to water and pasture lands on *L. monocytogenes* prevalence differ between sites. Our data indicate that there are different sets of predictors for *L. monocytogenes* detection at the eastern versus western sites. We observed that the eastern sites had a somewhat higher prevalence of *L. monocytogenes* in close proximity to water, whereas the western sites had a somewhat higher prevalence of *L. monocytogenes* in closer proximity to pasture land cover. One possible explanation for this difference is that the western sites, on average, had a considerably higher pasture density within 1 km of study sites. Previous studies have demonstrated that cattle amplify *L. monocytogenes* contamination in farm environments (14, 33) and it has been shown that proximity to pastures or farms may contribute to an increased

prevalence of *L. monocytogenes* in non-farm environments (28, 63, 64) and produce fields (25). Hay grass fields (included in the pasture land cover class) also serve as forage for wildlife and it is plausible that higher numbers of wildlife congregate near pasture land cover and this could also contribute to higher levels of *L. monocytogenes* near these areas even in the absence of domesticated ruminants. With the low pasture density near the eastern sample areas, it is conceivable that other factors, which may not have been apparent in an area where sampling sites are close to pasture-class land cover, become apparent in an analyses of the factors that affect *L. monocytogenes* detection. Specifically, the eastern sites showed greater prevalence of *L. monocytogenes* near open water sources. Similar to pastures, surface water features may be frequented by wildlife. As well, factors related to high soil moisture, which may be observed near open water sources, have previously been implicated in increased prevalence of *L. monocytogenes* (23, 25, 65).

Conclusions. While the frequency of *Salmonella* and STEC in NYS natural environments is low, our study offers further evidence of the potential for these pathogens to exist in natural, non-food associated environments and our data provide an initial estimate of *Salmonella*, *E. coli* O157:H7 and non-O157 STEC prevalence in natural areas of NYS. As well, these data contribute to the understanding of the roles of various geographical factors that influence the spatial and temporal distribution of *L. monocytogenes*. In particular, our data suggest that the detection of *L. monocytogenes* is more common in the lower elevations of a landscape with flatter slopes, and that, in certain landscapes, proximity to pasture-class land cover or open water may increase the detection of *L. monocytogenes*. The high prevalence of *L. monocytogenes* suggests that this pathogen can be introduced from natural areas to food-associated environments, and our data may be used to gain further insight into the environmental reservoirs of *L. monocytogenes*.

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CHAPTER 3

GEOGRAPHICAL AND METEOROLOGICAL FACTORS PREDICTING THE PREVALENCE OF *LISTERIA* SPECIES IN NEW YORK STATE PRODUCE PRODUCTION AND NATURAL ENVIRONMENTS*

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ABSTRACT

Listeria species have been isolated from diverse environments, often at considerable prevalence, and are known to persist in food processing facilities. The presence of *Listeria* spp. has been suggested to be a marker for *L. monocytogenes* contamination. Therefore, a study was conducted to (i) determine the prevalence and diversity of *Listeria* spp. in produce production and natural environments and (ii) identify geographical and meteorological factors that affect the prevalence of *Listeria* spp. in these environments. These data were also used to evaluate *Listeria* spp. as index organisms for *L. monocytogenes* in produce production environments. Environmental samples were collected from produce production (n=588) and natural (n=734) environments in New York State (NYS) and microbiologically analyzed to detect and isolate *Listeria* spp. The prevalence of *Listeria* spp. was 34% and 33% for samples obtained from produce production (201/588) and natural environments (245/734), respectively. The co-isolation of *L. monocytogenes* and at least one other species of *Listeria* from a sample was 9% in produce production environments, compared to 3% in natural environments. Soil moisture and proximity to water and pastures were identified as important factors for detection of *Listeria* spp. in produce production environments, while elevation, study site and proximity to pastures were identified as important predictors for detection of *Listeria* spp. in natural environments, as determined by randomForest models. Our data show that *Listeria* spp. were prevalent in both agricultural and non-agricultural environments and that geographical and meteorological factors associated with *Listeria* spp. detection were considerably different between the two environments.

INTRODUCTION

The genus *Listeria* is comprised of 15 species, including *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua*, *L. marthii*, *L. grayi*, *L. rocourtiae*, *L. weihenstephanensis*, *L. fleishmannii*, *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia* and *L. grandensis* (1). *L. monocytogenes* is a foodborne pathogen that represents a significant burden to public health and accounts for an estimated 1,591 cases of listeriosis, 1,455 hospitalizations, and 255 deaths annually in the United States (2). There is an increased concern about *L. monocytogenes* in produce, as a consequence of *L. monocytogenes* outbreaks (3, 4) and recalls (5). In 2011, there were 147 illnesses, 33 deaths, and 1 miscarriage due to a *L. monocytogenes* outbreak in cantaloupe (5). The remaining species of *Listeria* are generally not considered a concern to public health. For instance, *L. ivanovii* is primarily associated with listeriosis in animals (e.g., sheep) and rarely causes human disease (6). Here, we use the term *Listeria* spp. to refer to all species of *Listeria* (e.g., *L. monocytogenes*, *L. innocua*), and it is explicitly stated when *Listeria* spp. excludes *L. monocytogenes* for our analyses and discussion.

Listeria spp. have been detected in a wide variety of environments, from wilderness areas to retail food establishments (7-13). The range of *Listeria* spp. prevalence in non-agricultural and agricultural environments has been estimated from 3.7-81% (7, 13-16) and 5.7-51% (12, 17-19), respectively. It has been suggested that specific environmental factors (e.g., soil moisture and precipitation) may influence the prevalence of *Listeria* spp. (14, 20-23). For example, Ivanek *et al*. (2009) observed that *Listeria* spp. were more prevalent in soil samples when it rained two days prior to sample collection (21). Additional studies (22, 24-26) have observed *L. monocytogenes* to be more prevalent in soil and vegetation samples when soil is moist. Specifically in one study (26), it was predicted that soils in produce production environments

with an available water storage of greater than 4 cm (in 0-25 cm depth) had a three-fold higher prevalence of *L. monocytogenes*, compared to less moist soils. Moisture has also been shown to influence the presence of *Listeria* spp. in food processing plants. Slade (1992) showed *Listeria* spp. were found more often in processing plant locations where moisture levels were high (27). These findings demonstrate the importance of moisture and precipitation on the occurrence of *Listeria* spp. Yet, there is no quantitative data to elucidate whether the same or different factors influence the detection of certain species of *Listeria* spp. (excluding *L. monocytogenes*) in produce production environments.

Listeria spp. detection has often been used to identify conditions that may indicate the presence of *L. monocytogenes*. There has been some confusion over the use of indictor, index and surrogate organisms as food safety measures. Indicator organisms are commonly defined as markers whose presence relate to the general microbiological condition of the food or environment (i.e., hygienic quality), while index organisms are commonly defined as markers whose presence relates to the possible occurrence of ecologically similar pathogens (28, 29). Surrogate organisms are commonly defined as non-pathogenic organisms that correlate with the behavior (e.g., growth and survival) of specific pathogens (10, 28, 30). According to Kornacki (29) the term indicator organism has often been used to refer to (i) index organisms, (ii) indicators of hygiene and sanitation on equipment and surfaces, (iii) process controls of spoilage or potential spoilage, and also (iv) surrogate organisms in the context of critical control point validation. As a result, the terms have been used interchangeably throughout the literature. Here, we use the term index organism for our discussion as we examine the relationship between the occurrence of non-pathogenic *Listeria* spp. and *L. monocytogenes*.

Few studies (7, 13, 21) have focused on *Listeria* spp. prevalence and its association to geographical and meteorological factors in natural environments, and there have been no studies to our knowledge that have focused on the ecology and prevalence of *Listeria* spp. (excluding *L. monocytogenes*) in produce production environments. The purpose of this study was to gain a more complete understanding of the ecology of *Listeria* spp. in the produce production and natural environment. Specifically, the objectives of this study were to (i) determine the prevalence and diversity of *Listeria* spp. in produce production and natural environments, (ii) identify geographical and meteorological predictors (e.g., soil moisture, precipitation) of each species of *Listeria* detected in produce production and natural environments, and (iii) evaluate the application of *Listeria* spp. as index organisms for *L. monocytogenes* in the produce production environment.

MATERIALS AND METHODS

Description of Study Data. Data were assembled for this study using two field study datasets, one published (26) and one unpublished. These two datasets had been collected to determine the prevalence of *Salmonella*, Shiga toxin producing *Escherichia coli* (STEC) and *L. monocytogenes* in produce and natural environments. In addition, these two datasets were also used to identify geographical and/or meteorological factors that influenced pathogen prevalence in the produce production (26) or natural environment. In the study reported here, we used these previously collected samples; in addition to previously retrieved geographical and meteorological data to examine the (i) prevalence of *Listeria* spp. and (ii) geographical and/or meteorological factors that affect *Listeria* spp. prevalence in produce production and natural environments. Data on *Listeria* spp. (excluding *L. monocytogenes*) have not been published prior.

A total of 1,322 samples were collected between the two studies; global positioning system (GPS) coordinates for each sample location (i.e., geo-referenced samples) were recorded. Briefly, from produce production environments, 588 geo-referenced samples (178 soil, 175 drag swab, 174 water, and 61 wildlife and domesticated animal fecal samples) were collected over a two year period (summer, fall, winter and spring 2009-2011) from five produce farms across New York State (NYS). Samples were obtained from four fields within each of the five farms. From natural environments, 734 geo-referenced samples (90 soil, 90 drag swab, 90 water, and 449 wildlife fecal samples) were collected over a two-year period (spring, summer and fall 2009-2010) from five natural areas across NYS. Natural areas were defined as undeveloped locations, with minimal human presence that provided prime habitat for wildlife (e.g., national forests, wildlife refuges). Samples were obtained from three sites within each of the five natural areas.

Sample Collection and Preparation. Samples obtained in both studies were collected using sample collection protocols previously described (26). Briefly, latex gloves (Nasco, Fort Atkinson, WI) were worn and changed between each sample site. Five soil samples (per field/site) were collected approximately 15.2 cm below the top-soil surface using sterile scoops (Fisher Scientific, Hampton, NH) and deposited into sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI). One drag swab was collected at each site or field. Swabs were squeezed, tied to sterile string and dragged across the sample area for 10 min. Water samples were collected (250 ml directly into sterile Whirl-Pak bag or jar) from surface (e.g., creek, pond) and engineered (e.g., well, municipal) water sources, when available, closest to each field and site. Fecal samples were collected when observed in produce fields, and an effort was made to collect 15 fecal samples at each natural site. All samples were transported on ice, stored at 4±2°C and processed within 24 h.

Five grams from each of the five soil samples collected in a given area were pooled to form one 25 g composite sample per field/site in a sterile filter Whirl-Pak bag. Each drag swab was hand massaged and then squeezed to remove the liquid from the swab. A 10 ml aliquot of the liquid was then aseptically transferred to a new sterile filter Whirl-Pak bag. Water samples were analyzed using the Environmental Protection Agency (EPA) standard methods (31, 32). Water samples were passed through a 0.45 μm filter unit (Nalgene, Rochester, NY) and a third of the filter was aseptically transferred to a sterile Whirl-Pak bag for *Listeria* enrichment. Each fecal sample was weighed, and 10 g of each fecal sample was deposited into a sterile Whirl-Pak filter bag.

***Listeria* spp. Detection and Confirmation.** *Listeria* spp. detection and isolation was performed using a modified version of the Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM) as previously described (8-10, 16). Controls were processed in parallel with environmental samples. *L. monocytogenes* (FSL R3-001; (33)) and uninoculated enrichment media were used as the positive and negative control, respectively. Environmental samples were mixed with buffered *Listeria* enrichment broth (BLEB; Becton Dickinson, Franklin Lakes, NJ) (225 ml for soil samples and 90 ml for drag swab/water/fecal samples) to enrich for the presence of *Listeria* spp. Enrichments were incubated at 30±2°C for 48 h, with addition of the selective supplement (Oxoid, Cambridge, UK) at 4 h. At 24 and 48 h, 50 μl of each enrichment was streaked onto Oxford agar (OX, Becton Dickinson, Franklin Lakes, NJ), which was subsequently incubated for 48 h at 30±2°C. Up to four presumptive *Listeria* spp. colonies were sub-streaked to *L. monocytogenes* plating medium (LMPM, Biosynth International, Itasca, IL) and incubated for 48 h at 35±2°C. White and blue colonies on LMPM plates (white colonies representing presumptive non-pathogenic *Listeria*; blue colonies representing presumptive pathogenic

Listeria) were sub-streaked to brain heart infusion agar (BHI; Becton Dickinson, Franklin Lakes, NJ), which was subsequently incubated at 37±2°C for 24 h. Presumptive *Listeria* spp. colonies (maximum four colonies per sample) were confirmed by amplification of the *sigB* gene by polymerase chain reaction (PCR). *Listeria* spp. identification was performed by sequencing *sigB* (Sanger sequencing performed by the Cornell University Life Sciences Core Laboratories Center) and comparison of sequences to an internal reference database using BLASTN, as previously described (34-36). *Listeria sigB* allelic types (AT) were also assigned to each isolate. Isolates were preserved at -80°C in 15% glycerol and information on each isolate can be found at www.FoodMicrobeTracker.com.

Data on Geographical and Meteorological Factors. Data were previously retrieved for each sample location and sample collection date as described by Strawn *et al.* (26). Data on geographical factors were obtained from the US Geological Survey (USGS) EarthExplorer (http://earthexplorer.usgs.gov/), US Department of Agriculture and Soil Survey Geographic database (http://soils.usda.gov/survey/geography/ssurgo/), and Cornell University Geospatial Information Repository (http://cugir.mannlib.cornell.edu/) using the Geographical Resources Analysis Support System (http://grass.osgeo.org). Data on meteorological factors were obtained from the National Oceanic and Atmospheric Administration's National Climate Data Center Local Climatology Database (http://gis.ncdc.noaa.gov/map/lcd/). Further details on the geographical and meteorological factors obtained for each environment are provided in Table 2.5.

Categorical Analysis. Univariate associations between *Listeria* spp. positive samples and field/site, season and sample type were performed by a chi-square test. Confidence intervals (95%) were calculated for each variable assuming a binomial distribution. In addition, the

association between a sample testing positive for *Listeria* spp. (excluding *L. monocytogenes*) and *L. monocytogenes* in each environment was performed by a chi-square test. *P* values less than 0.05 were considered significant. The diversity of allelic types within environment was quantified using Simpson's Index of Diversity (D) (37). All categorical analyses were performed in SAS 9.3 (SAS Institute Inc., Cary, NC).

randomForest (RF) Analysis. RF was performed to identify geographical and meteorological factors for each species of *Listeria* detected in the produce production and natural environment. RF models are a non-parametric statistical tool used to identify important factors in classification of samples as a given species of *Listeria*, but RF models cannot specify the degree of a factor (e.g., level of soil moisture, or distance to a pasture). The outcome for each RF was presence/absence of the target *Listeria* spp. (e.g., *L. innocua, L. seeligeri*) in a sample. Predictor variables for each RF were the 14 geographical and meteorological factors obtained for each sample. RF models were performed using the randomForest package in R 2.13.1 (38) and using the following criteria: 10,000 bootstrap iterations (with replacement) and four randomly selected predictor variables for each split. The misclassification rate was calculated for each RF model using the "out-of-bag" (OOB) samples. OOB samples (approximately 1/3 of dataset) are withheld from each RF model and used to test the RF predictions (i.e., cross validation). Variable importance (VI) scores were calculated for each factor in each RF model. Briefly, factor values are randomly permuted for each tree and the VI score represents the amount of prediction lost (38). Forty-nine of the 734 samples collected in natural environments were excluded from RF analysis due to missing data.

RESULTS

Prevalence of *Listeria* spp. in the Produce Production and Natural Environments. In the produce production environment, the prevalence of *Listeria* spp. was 34% (201/588; Table 3.1). Nearly 15% of samples were positive for *L. monocytogenes* as previously reported (26), while approximately 28% of samples were positive for at least one species of *Listeria* (excluding *L. monocytogenes*; Table 3.1). Approximately 9% of samples were positive for both *L. monocytogenes* and at least one other species of *Listeria* (51/588). Farm, season and sample type were found to be significantly ($P \leq 0.05$) associated with the prevalence of *Listeria* spp. (excluding *L. monocytogenes*) as determined by chi square tests (Table 3.2). Farms 1, 3, and 5 each had a significantly higher prevalence (36%, 32%, and 42%, respectively) of *Listeria* spp. (excluding *L. monocytogenes*), compared to farms 2 and 4 (each 12%; Table 3.2). The prevalence of *Listeria* spp. (excluding *L. monocytogenes*) was significantly higher in winter (41%), compared to fall and summer (24% and 22%, respectively; Table 3.2). Both winter sampling visits (2010 and 2011) yielded the highest prevalence of *Listeria* spp. (excluding *L. monocytogenes*), compared to all other sampling visits (2009-2011). Water samples had a significantly higher prevalence of *Listeria* spp. (excluding *L. monocytogenes*; 51%), compared to soil, drag swab and fecal samples (17%, 21%, and 16%, respectively; Table 3.2). All *Listeria* spp. (excluding *L. monocytogenes*) positive water samples (88/174) were from surface water (e.g., ponds, creeks, or ditches). None of the 28 samples collected from engineered water (e.g., wells, municipal) were positive for any species of *Listeria*.

TABLE 3.1. Frequency of positive *Listeria* spp. (by genus and each species of *Listeria*) samples obtained from produce production (n=588) and natural (n=734) environments.

	Environment	
	Produce Production	Natural
Listeria spp. (samples positive for any species in the genus *Listeria*)	201	245
L. monocytogenes (samples positive for LM with or without co-isolation of at least one sp.)	88	59
L. innocua (samples positive for *L. innocua* with or without co-isolation of at least one sp.)	81	13
L. seeligeri (samples positive for *L. seeligeri* with or without co-isolation of at least one sp.)	45	129
L. welshimeri (samples positive for *L. welshimeri* with or without co-isolation of at least one sp.)	60	117
L. marthii (samples pos. for *L. marthii* with or without co-isolation of at least one sp.)	0	5

TABLE 3.2. Effect of Factors (Farm, Season, and Sample type) on the frequency of positive *Listeria* spp. (excluding *L. monocytogenes*) samples detected in produce production and natural environments.

Factor (Category)		Produce Production Environment		Natural Environment	
		No. Samples	Frequency (Percent)[a]	No. Samples	Frequency (Percent)
Farm/Park					
	1	166	60 (36)^{A}	152	71 (47)^{A}
	2	103	12 (12)^{B}	145	22 (15)^{C}
	3	113	36 (32)^{A}	146	44 (30)^{B}
	4	100	12 (12)^{B}	148	39 (26)^{BC}
	5	106	44 (42)^{A}	143	28 (20)^{BC}
Season					
	Fall	136	33 (24)^{B}	245	57 (23)^{B}
	Winter	125	51 (41)^{A}	NC[b]	-
	Spring	134	37 (28)^{AB}	241	55 (23)^{B}
	Summer	193	43 (22)^{B}	248	92 (37)^{A}

Table 3.2 (Continued)

Sample Type					
	Soil	178	30 (17)[B]	90	28 (31)
	Drag Swab	175	36 (21)[B]	90	32 (36)
	Fecal	61	10 (16)[B]	449	119 (27)
	Water	174	88 (51)[A]	90	20 (22)
	Sediment	NC	-	15	5 (33)
Total		588	164 (28)	734	204 (28)

[a] Different letters represent values that are significantly different ($P < 0.05$). No letters represent values that are not significantly different.

[b] NC represents samples not collected.

In the natural environment, the prevalence of *Listeria* spp. was 33% (245/734; Table 3.1). *L. monocytogenes* was isolated from nearly 8% of samples as previously reported (39), whereas at least one species of *Listeria* (excluding *L. monocytogenes*) was isolated from approximately 28% of samples (Table 3.1). Approximately 3% of samples were positive for both *L. monocytogenes* and at least one other species of *Listeria* (18/734). Study site (e.g., national forests, wildlife refuges) and season were found to be significantly ($P \leq 0.05$) associated with the prevalence of *Listeria* spp. (excluding *L. monocytogenes*; Table 3.2). Study site 1 had a significantly higher *Listeria* spp. (excluding *L. monocytogenes*) prevalence (47%) than study sites 2, 3, 4, and 5 (prevalence of 15%, 30%, 26%, and 20%, respectively; Table 3.2). The prevalence of *Listeria* spp. (excluding *L. monocytogenes*) was significantly higher in summer (37%), compared to fall and spring (each 23%; Table 3.2). No sampling visits were conducted in winter for natural environments, as the study sites are not accessible during the winter months in NYS (due to snow and ice conditions). No significant difference was observed in the prevalence of *Listeria* spp. (excluding *L. monocytogenes*) among sample type (Table 3.2).

Diversity of *Listeria* spp. in the Produce Production and Natural Environments. The 164 *Listeria* spp. positive samples from the produce production environment yielded 426 *Listeria* isolates (excluding *L. monocytogenes*). All isolates were assigned a *sigB* allelic type (AT). If isolates from the same sample were classified as the same AT then only one "representative isolate" was selected for inclusion in the subsequent analyses. The 426 isolates resulted in 186 representative *Listeria* spp. isolates (excluding *L. monocytogenes*). Of the 186 representative isolates, 81, 45, and 60 isolates were identified as *L. innocua*, *L. seeligeri*, and *L. welshimeri*, respectively (Table 3.1). The frequency of *L. innocua* isolates was significantly higher than the frequency of *L. seeligeri* isolates in the produce production environment. There was a high

diversity of ATs within each *Listeria* spp. (D=0.91, 0.87, and 0.88 for *L. innocua*, *L. seeligeri*, and *L. welshimeri*, respectively). There were 22, 10, and 18 different ATs identified for *L. innocua* (n=81), *L. seeligeri* (n=45), and *L. welshimeri* (n=60), respectively. *L. innocua* AT 26, *L. seeligeri* AT 12, and *L. welshimeri* AT 27 were the most common ATs.

The 204 *Listeria* spp. positive samples from the natural environment yielded 504 *Listeria* isolates (excluding *L. monocytogenes*). The 504 *Listeria* spp. isolates (excluding *L. monocytogenes*) resulted in 264 representative isolates for inclusion in the subsequent analyses. Of the 264 representative isolates, 13, 5, 129, and 117 were identified as *L. innocua*, *L. marthii, L. seeligeri*, and *L. welshimeri*, respectively (Table 3.1). *L. marthii* was not included in analyses because it was isolated from only one natural site in a low frequency (5/734; 0.7%). The frequencies of *L. seeligeri* and *L. welshimeri* isolates were significantly higher than the frequency of *L. innocua* isolates in the natural environment. There was a high diversity of ATs within each *Listeria* species (D=0.87, 0.88, and 0.84 for *L. innocua*, *L. seeligeri*, and *L. welshimeri*, respectively). There were 7, 14, and 19 different ATs identified for *L. innocua* (n=13), *L. seeligeri* (n=129), and *L. welshimeri* (n=117), respectively. *L. innocua* AT 23, *L. seeligeri* AT 3, and *L. welshimeri* AT 27 were the most common ATs.

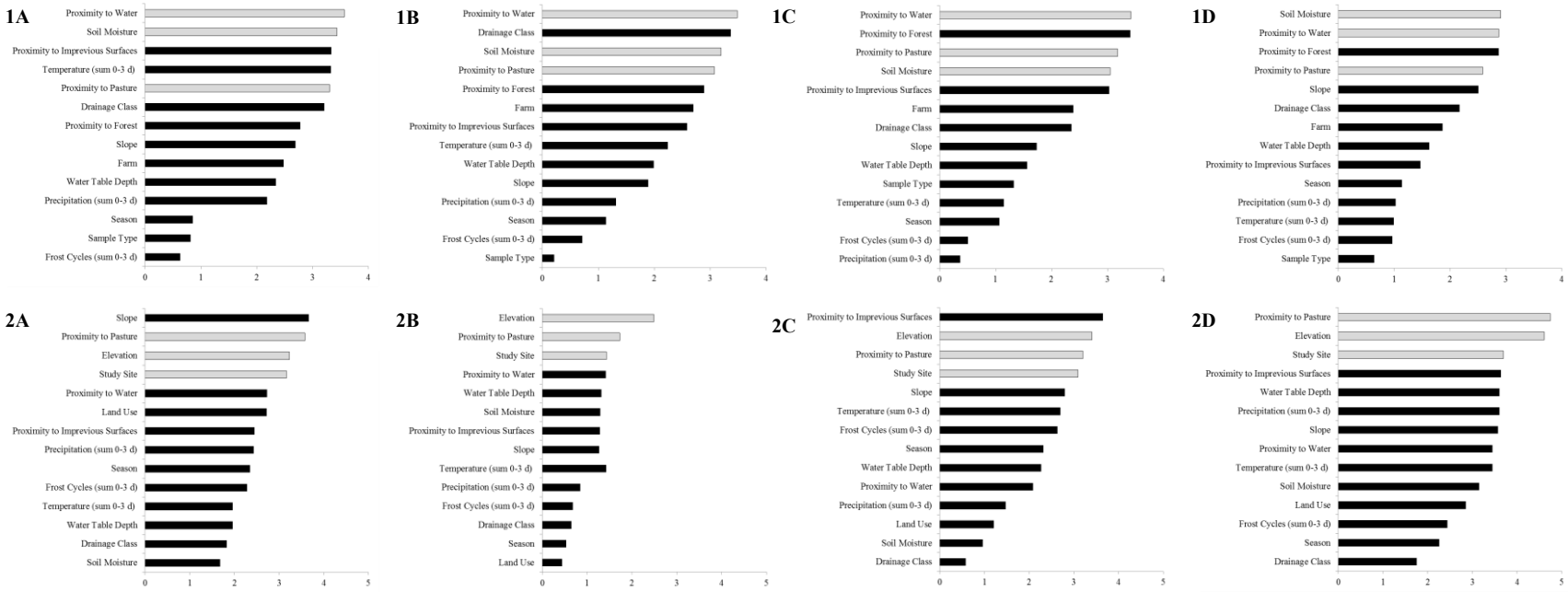
Geographical Predictors of *Listeria* spp. in the Produce Production and Natural Environments. In the produce production environment, three factors (soil moisture, proximity to water, and proximity to pastures) were important for classification of samples as positive for each given species of *Listeria* detected in this study (*L. monocytogenes*, *L. innocua*, *L. seeligeri*, and *L. welshimeri*). These three factors were each ranked in the top five variable importance (VI) scores for each given *Listeria* spp. among all 14 geographical and meteorological factors included in RF models (Fig. 3.1, 1A-1D; grey bars). No other factors were ranked in the top five

VI scores across all *Listeria* spp.; however, some factors were common to two or three species of *Listeria*. Specifically, proximity to forests was identified as an important factor for classification of a sample as positive for *L. innocua*, *L. seeligeri*, or *L. welshimeri*, as determined by VI scores (rank 5, 2, and 3, respectively; Fig. 3.1, 1B-1D). Proximity to impervious surfaces was identified as an important factor for classification of samples as positive for *L. monocytogenes* or *L. seeligeri* (rank 3 and 5, respectively; Fig. 3.1, 1A and 1C). Three factors (temperature, drainage class, and slope) were each important for classification of samples as positive for *L. monocytogenes*, *L. innocua*, or *L. welshimeri*, respectively (Fig. 3.1, 1A, 1B, and 1D).

In the natural environment, three factors (proximity to pastures, elevation, and study site) were important for classification of samples as positive for each given species of *Listeria* detected in this study (*L. monocytogenes, L. innocua*, *L. seeligeri* and *L. welshimeri*). These factors were ranked in the top five VI scores for each given *Listeria* spp. among the 14 geographical and meteorological factors included in RF models (Fig. 3.1, 2A-2D; grey bars). No other factors were ranked in the top five VI scores for each given species of *Listeria*; however, four factors (slope, water table depth, and proximity to water and impervious surfaces) were common to two species of *Listeria*. Slope was identified as an important factor for classification of samples as positive for *L. monocytogenes* or *L. seeligeri*, as determined by VI scores (rank 1 and 5, respectively; Fig. 3.1, 2A and 2C). Water table depth was identified as an important factor for classification of samples as positive for *L. innocua* or *L. welshimeri* (Fig. 3.1, 2B and 2D). Proximity to water was identified as an important factor for classification of samples as positive for *L. monocytogenes* or *L. innocua* (rank 5 and 4, respectively; Fig. 3.1, 2A and 2B). Proximity to impervious surfaces was identified as an important factor for classification of samples as positive for *L. seeligeri* or *L. welshimeri* (rank 1 and 4, respectively; Fig. 3.1, 2C and 2D).

Figure 3.1 Caption. Bar graphs depict variable importance (VI) scores for each geographical and meteorological factor based on randomForest analysis of samples obtained from produce production and natural environments. For each bar graph the x axis represents the variable importance (VI) scores and the y axis represents the factors. The top row of bar graphs labeled 1 (A-D) represents the produce production environment, while the bottom row of bar graphs labeled 2 (A-D) represents the natural environment. Letters A through D represent the four species of *Listeria* detected: A= *L. monocytogenes*, B = *L. innocua*, C = *L. seeligeri* and D = *L. welshimeri*. The VI scores show the loss of predictive power that occurred (over the 10,000 bootstrap irritations) when a factors data values were randomized during for model validation. Geographical and/or meteorological factors with the largest VI score (positioned at the top of each bar graph) are the most important factors for prediction of a species of *Listeria* positive sample. Grey bars represent factors of considerable importance (i.e., the factor must be ranked in the top five highest VI scores for each species of *Listeria*) from the produce production or natural environment.

Figure 3.1, top ↑



DISCUSSION

The goal of this study was to investigate the prevalence of *Listeria* spp. in two distinct environments and to evaluate associations between the detection of different species of *Listeria* and geographical and or meteorological factors. This study also provides data on the distribution and diversity of *Listeria* subtypes in the produce production environment (where field collected data are rare). The prevalence of *Listeria* spp. was similar in produce production and natural environments; however, the prevalence of *L. monocytogenes* was considerably higher in produce production environments compared to natural environments. Of 14 factors, only proximity to pastures was identified as an important factor for classification of samples as *L. monocytogenes-, L. innocua-, L. seeligeri-* and *L. welshimeri-* positive in both produce production and natural environments, suggesting an important role of pastures as a source of *Listeria* spp. The pasture landscape factor represented active pasturages and hay grass fields. Our data also show that certain species of *Listeria* are more prevalent in each environment and *Listeria* spp. isolation is influenced by different environmental factors that exist in these two environments. Sampling locations were in NYS so findings may only be applicable to the sampled regions in NYS; therefore, further studies are needed to determine if these findings presented here can be applied to other regions in the US and abroad.

***Listeria* spp. are found at a high prevalence in produce production and natural environments, but distribution of species is dependent on ecological niche.** Approximately one out of every three samples tested for each environment in this study was *Listeria* positive, with most species identified as non-pathogenic *Listeria*. Previous studies (13-16, 18) have shown that the prevalence of *Listeria* spp. can be high (often >20%) in the environment. For example, a 22% prevalence of *Listeria* spp. was observed in samples (e.g., sponge swabs of vending

machines, sidewalks, railings) obtained from urban environments (16). In another study, an 81% prevalence of *Listeria* spp. was reported in water samples from an estuarine environment (14). In the two previously mentioned studies, non-pathogenic species of *Listeria* were detected and isolated in greater frequencies than *L. monocytogenes*. In our study, we found the highest prevalence of *Listeria* spp. (excluding *L. monocytogenes*; 51%) in water samples from the produce production environment. All of these positive samples were from surface water (e.g., sample from a pond, creek, or ditch) collected near sampled produce fields. Our study findings in the natural environment showed the highest prevalence of *Listeria* spp. (excluding *L. monocytogenes*) in top soil (drag swab samples), while the lowest prevalence was in water samples. Similar findings were reported in one previous study of NYS forest and wildlife refuges, in this prior study a higher prevalence of *Listeria* spp. were observed in soil and vegetation samples, compared to water samples (21). Our data reported here suggest that surface water in agricultural environments is more likely to be positive for *Listeria* spp. *(*excluding *L. monocytogenes*), compared to non-agricultural environments. Further research is needed to identify potential sources and vehicles of contamination of surface waters in produce production environments.

Additionally, we observed that the species of *Listeria* detected was highly dependent on the specific environment. In our study, *L. monocytogenes* and *L. innocua* were the most prevalent species in produce production environments, whereas *L. seeligeri* and *L. welshimeri* were the most prevalent species in natural environments. Other studies have also shown specific species of *Listeria* to be more common in certain ecological niches (7, 16, 20, 21). For example, *L. marthii* has only been isolated from a distinct area in NYS (20). Interestingly, in our study, we isolated *L. marthii* from the same geographic area in NYS as mentioned in the previous study

(20), and failed to isolate *L. marthii* in other more distant sampling areas. Consistent with our data in natural environments, *L. seeligeri* and *L. welshimeri* were found to be overrepresented among *Listeria* isolates obtained from NYS forest and wildlife refuges in a previous study (16). This same study also found *L. monocytogenes* and *L. innocua* most prevalent in *Listeria*-positive samples from urban environments (16). *L. monocytogenes* and *L. innocua* were also isolated more frequently from samples collected in produce production environments in our study. This finding suggests the distribution of *Listeria* isolated from urban and produce production environments may be more closely related than from natural environments. Our findings are consistent with others (e.g., Gray *et al.* (40) and Sauders *et al.* (16)), who have shown that distinct populations of *Listeria* are present in different environments, foods, and/or hosts.

Geographical and meteorological factors predicting *Listeria* spp. prevalence were different between produce production and natural environments. Most factors identified as important predictors of *Listeria* spp. detection were different between the produce production and natural environments. Only one factor (proximity to pastures) was identified as an important factor for the classification of samples as positive for a given species of *Listeria* across both environments. These data suggest that different ecological factors or sources are affecting the isolation of certain species of *Listeria* in produce production and natural environments.

In the produce production environment, soil moisture was identified as a key geographical predictor for each species of *Listeria* detected. Soil properties (soil moisture and loam percentage) have been previously shown to influence the detection of *Listeria* spp. in soil and vegetation samples from non-agricultural environments (21, 24). Interestingly, soil moisture was not identified as an important factor for classification of samples as positive for any species of *Listeria* detected in natural environments in our study, as determined by RF models. In

addition to soil moisture, proximity to water was also identified as an important factor for classification of samples as positive for each species of *Listeria* detected in the produce production environment. This finding is consistent with other studies (25, 41-43) that have demonstrated water to be an important reservoir for *L. monocytogenes* in both agriculture and non-agriculture environments. Our study data showed water was also an important reservoir for other species of *Listeria*, and subsequently may contribute to *Listeria* spp. presence in produce production environments.

In the natural environment, elevation and study site were identified as important geographical predictors for each species of *Listeria* detected. Our findings that elevation and study site were important predictors of *Listeria* spp. prevalence in the natural environment were consistent with findings from others (16, 21). Ivanek *et al.* (21) identified the geographical position of a sampling location (e.g., distance from the equator) as an important factor for the occurrence of a *Listeria* spp. positive sample from natural environments, as determined by classification tree analysis. Additionally, Sauders *et al.* (16) showed certain species of *Listeria* were highly associated with specific sample sites, such as *L. seeligeri* in the Connecticut Hill Wildlife Management area in NYS. Combined, these data suggest geographical factors specific to location of sample collection are influential predictors of *Listeria* spp. detection in NYS forests and wildlife refuges.

In both produce production and natural environments, proximity to pastures was identified as one of the most important factors for classification of samples as positive for each given species of *Listeria* detected from a location. Several studies (9, 30, 44) have found proximity to pastures may be associated with an increased *Listeria* spp. prevalence in non-agricultural environments (e.g., watersheds). Lyautey *et al*. (42) observed a strong association

between proximity of dairy farms and the isolation of *L. monocytogenes* from water samples obtained in a Canadian watershed. The prevalence of *L. monocytogenes* in environmental samples collected from livestock animal operations (e.g., soil, water troughs, bedding, and feedstuff) in two studies (8, 45), both conducted in NYS, was estimated at 24% and 46%. Our data provide evidence that livestock and/or livestock pasture areas may be reservoirs for other species of *Listeria* as well, and potential sources of *Listeria* spp. in both agriculture and non-agriculture environments.

Detection of *Listeria* spp. in produce production environments may not be an effective strategy to predict *L. monocytogenes* contamination in produce fields. Our data also allowed us to evaluate the application of *Listeria* spp. as index organisms for *L. monocytogenes* in produce production environments. The use of *Listeria* spp. as index organisms was evaluated in the context of three previously established criteria (46, 47) including (i) that the index organism should have a higher prevalence than the target pathogen, (ii) the detection of the index organism should have a reasonably strong correlation with the detection of the target pathogen, and (iii) testing for detection of the index organism should be more rapid and/or more cost effective than for the target pathogen.

In our study, *Listeria* spp. were detected in nearly 30% of samples obtained from the produce production environment, where half of those samples were confirmed to be positive for *L. monocytogenes* (only 6% of samples were positive solely for *L. monocytogenes*). *Listeria* spp. thus fulfils the first criterion of an acceptable index organism, i.e., that the index organism should have a higher prevalence than the target pathogen. Additionally, previous studies (e.g., (16, 26, 48) have consistently observed a higher prevalence of non-pathogenic species of *Listeria* as compared to *L. monocytogenes*. The prevalence of *L. monocytogenes* in our study (15%) was

considerably higher than *Salmonella* or STEC. The observation of a high *L. monocytogenes* prevalence in NYS may reduce the need for an index organism. Studies are needed to address the prevalence of *L. monocytogenes* and non-pathogenic species of *Listeria* in other produce growing regions to establish the need for index organisms there. For example, in regions where *L. monocytogenes* prevalence is low, *Listeria* spp. may function as a useful index organism.

In our study, only three of 14 geographical factors (soil moisture, proximity to pastures, and proximity to water) were identified as important factors for classification of samples as positive for each given species of *Listeria* in the produce production environment. This finding suggests that certain environmental conditions increase the likelihood for detection of both selected non-pathogenic *Listeria* and *L. monocytogenes*. This is also supported by our data that showed that *Listeria* spp. positive samples were more likely to be *L. monocytogenes* positive than *Listeria* spp. negative samples in produce production environments, while this did not hold true for natural environments. While some other studies (46, 47) have suggested that *Listeria* spp. may be reliable as an index organism for *L. monocytogenes* in processing environments, a recent study (49) in smoked fish plants suggests limited value of *Listeria* spp. as an index organism for *L. monocytogenes*. Importantly, *Listeria* spp. can differ in their metabolic capabilities (e.g. carbohydrate utilization); for example *L. innocua* shows similar carbohydrate utilization patterns as *L. monocytogenes* and may be a better index organism for *L. monocytogenes* than *Listeria* spp. that differ in their carbohydrate utilization patterns from *L. monocytogenes* (50). Further research is needed to fully address the strength of association between *Listeria* spp. and *L. monocytogenes* in various environments to evaluate whether this second index organism criterion (strong association between index organism and target pathogen) is achieved in different environments. Association between *Listeria* spp. and *L.*

monocytogenes may also differ depending on which *Listeria* spp. (e.g., *L. innocua* versus the more divergent *L. seeligeri*) are present in a given environment (1, 51).

Both traditional and molecular methods can be used to test for *Listeria* in the environment. Traditional methods of *Listeria* detection and isolation require a series of biochemical tests for species differentiation, thus there is a difference in time and cost to identify *Listeria* spp. versus *L. monocytogenes*. The identification of *Listeria* spp. using molecular methods is more rapid and economical as it requires fewer biochemical tests to confirm a sample as positive for *Listeria* (genus) versus positive for a specific species of *Listeria* (27, 52). Most molecular detection methods are targeted to specific organisms and there is no difference in time or cost to identify *Listeria* spp. versus *L. monocytogenes*. Although the third criterion of an acceptable index organism is thus fulfilled when using traditional methods, use of molecular methods may be more suitable for use in the produce industry due to the short shelf life of many produce commodities.

In the context of the three previously established criteria, *Listeria* spp. are generally appropriate index organisms for *L. monocytogenes* (criteria met); however, application of *Listeria* spp. as index organisms for *L. monocytogenes* in the produce production environment is dependent on location (e.g., NYS, elsewhere) and detection method (e.g., traditional, molecular). Our findings reported here suggest limited value to the application of *Listeria* spp. as index organisms for *L. monocytogenes* in NYS produce production environments. Instead, testing directly for *L. monocytogenes* may be more effective due to the relatively high prevalence of the pathogen, compared to other pathogens (e.g., *Salmonella*) of concern in produce. Further research is needed to evaluate the application of *Listeria* spp. as index organisms of *L. monocytogenes* in produce production environments of other regions in the US and elsewhere, as

Listeria spp. (both non-pathogenic species of *Listeria* and *L. monocytogenes*) prevalence differs considerably by region (e.g., (7, 12, 16, 26).

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CHAPTER 4

DEVELOPMENT AND EVALUATION OF FOOD SAFETY MODULES FOR K-12 SCIENCE EDUCATION*

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ABSTRACT

Career and educational opportunities in food science and food safety are under-recognized by K-12 students and educators. Additionally, misperceptions regarding nature of science understanding continue to persist in K-12 students despite being emphasized as an important component of science education for over 100 years. In an effort to increase awareness concerning career and educational opportunities in food science and food safety and to improve nature of science understanding among K-12 students, a series of problem-based learning modules was developed and pilot tested with a total of 61 K-12 students. Results of pre- and post-evaluations and assessments indicated that (i) interest in science, food science, and food safety increased and (ii) content knowledge related to the nature of science, food science, and food safety was improved. We further suggest that these modules provide opportunities for educators in traditional as well as extracurricular settings to demonstrate important concepts contained in the newly released Next Generation Science Standards.

INTRODUCTION

Foodborne illness represents a significant public health burden in the United States. Each year there are an estimated 9.4 million illnesses, 55,961 hospitalizations and 1,351 deaths due to domestically acquired foodborne pathogens (1). Additionally, global food production needs to increase approximately 50% in order to feed the expected global population of 9 billion people by 2050 (2). Despite these staggering statistics, the discipline of food science as a career is not well recognized among high school students (3). As a result, few students are being recruited and trained to tackle these and other issues in the future as food science professionals and the number of qualified food science graduates is insufficient to supply the demand in the food industry, government, and academia (3-5).

Recently there have been several efforts to combat low enrollments in university food science programs around the country. For example, the Institute of Food Technologists provided all 18,000 US high schools with promotional material (6). Additionally, a Food Science Summer Scholars Program was developed by the Cornell Institute of Food Science to recruit undergraduate students from many science disciplines to graduate programs in Food Science (3). Fortunately, the enrollments in university food science programs are now increasing (7) and numerous opportunities exist for graduates (8).

In order to continue recruiting increasing numbers of students into food science/safety training programs, efforts need to build interest at an early age. Previous research suggests that factors such as childhood experiences may be linked to future educational and career choices (9, 10). Our group has participated in several outreach programs for K-12 students through cooperation with Cornell Cooperative Extension, including 4-H Career Explorations and 4-H Camp Bristol Hills.

Regardless of whether students remain in the STEM (Science, Technology, Engineering, Math) pipeline, scientific literacy and nature of science understanding are crucial to success in other educational and career fields as well as citizenship in general. Nature of Science (NOS) understanding is fundamental to scientific literacy and has been recognized as an essential component of science education for over 100 years (11, 12). More recent science education reform efforts (13, 14) continue to emphasize NOS understanding, in recognition of research which has shown that a variety of misconceptions concerning NOS continue to persist among K-12 students (and also among K-12 instructors) (12). Such misconceptions not only lead to difficulties with coursework but may contribute to the rejection of science altogether.

Therefore, the objectives of these efforts were three-fold: (i) to stimulate interest and expose students to career opportunities in food science, food safety and science in general, (ii) to simultaneously increase the scientific literacy and nature of science understanding of the participants in agreement with current science education reform efforts, and (iii) to create a food science/safety curriculum that K-12 educators can use in a variety of traditional or extracurricular settings. The purpose of this paper is to describe the design and report the results of two K-12 food safety workshops as models for accomplishing these objectives.

METHODS

Program Overview. Food safety and food science-based workshops were developed for K-12 students. Workshops were held in conjunction with existing Cornell Cooperative Extension efforts, which handled participant recruitment. Graduate student instructors developed curriculum materials to expose students to principles of food safety and food science and achieve learning outcomes in accordance with science education reform efforts (13, 14). Two workshops were developed for different scenarios and lengths of time (Table 4.1). Pre- and post-program assessments were administered to monitor learning outcomes. Open access to instructional materials (including detailed lesson plans and worksheets) is provided through the Cornell Food Safety Wiki (http://confluence.cornell.edu/display/FOODSAFETY/Middle+and+High+School+Teacher+Food+Safety+Resources).

4-H Lab-based Modules. 4-H Career Explorations is a 2.5-day summer workshop series, with open enrollment, for high school students sponsored by Cornell Cooperative Extension (CCE). There is a wide range of daytime workshops for participants to choose, representing various departments/programs and lab groups across the university. Participants stay in university dorms, eat together in the dining halls and have additional activities in the evenings. Participants break up into small groups to attend workshops during the day. Our group has hosted 12-16 students per year and these students were further divided into four groups for most hands-on activities, each with the support of a graduate student volunteer. The itinerary is shown in Figure 4.1.

Table 4.1. Description and characteristics of the Food Safety Workshops at 4-H Career Explorations and 4-H Camp Bristol Hills.

Characteristics	**Career Explorations**	**Bristol Hills**
Audience	9^{th} – 12^{th} grade	4^{th} – 10^{th} grade
No. of participants	16 per year	7-12 per year
Special needs students?	0	1 per year
Chaperones	2-3 adults	1-2 camp counselors
Participant recruitment	Teachers, 4-H extension agents	Camp scholarships
Venue	University teaching lab	Summer camp lodge and pavilion
Duration	2.5 days	5 days
Instructors	4 graduate students	1 graduate student
Subject matter	Lab-based	Field-based
Timing of activities	Fast-paced (advanced)	Slower-paced (younger students)

Day 1 (2.5 hours) **Module (Duration)**	**Day 2 (7.5 hours[b])** **Module (Duration)**	**Day 3 (3 hours)** **Module (Duration)**
Lab safety overview with Environmental Health & Safety (30 min)	Review (5 min)	Review (15 min)
Introductions, safety, overview and ice breaker (10 min)	Case Study: Outbreak Investigation Part II (45 min)	Analyze data (agar plates, gels) and discuss results (45 min)
Complete pre-assessments (10 min)	Gather microbiological data (15 min)	Conclude investigation (discussion) (15 min)
Food Safety 101 lecturette and discussion (30 min)	Strawberry DNA extraction activity (30 min)	Nature of science demonstration (30 min)
Case Study: Outbreak Investigation Part I (30 min)	Pipet practice (30 min)	Overview of research programs and lab tours (30 min)
Sample collection and processing (35 min)	Polymerase chain reaction (PCR) lab activity (55 min)	Opportunity for questions and complete evaluations (35 min)
Review (5 min)	Lunch (60 min)	Complete post-assessments (10 min)
	DNA fingerprinting lecturette (30 min)	
	Restriction digest lab activity (90 min)	
	DNA replication and PCR modeling activity (30 min)	
	Agarose gel electrophoresis lab activity (45 min)	
	Review (15 min)	

Figure 4.1. Itinerary for 4-H Career Explorations (2013).

Participants completed a pre-assessment after a brief introduction and lab safety training. Students were presented with an outbreak scenario and collected information and evidence to determine the most likely source(s) of the outbreak based on patient symptoms, a list of foods consumed, and characteristics of several foodborne pathogens including information about previous outbreaks, incubation period and pathogenesis. The source of the outbreak was then identified through a mock microbiological sampling, enrichment, and isolation exercise. Students were taught to use molecular subtyping methods to confirm the relatedness of isolates.

4-H Camp-based Modules. 4-H Camp Bristol Hills is a traditional summer camp where campers sign up to participate in one-week (five day) sessions. The 4-H CCE office of Ontario County (NY) hosts the camp but enrollment is open to K-12 students regardless of 4-H membership. Like 4-H Career Explorations, there are a wide variety of activities for campers from which to choose. However, unlike 4-H Career Explorations, few of the other activities (e.g., woodworking, horseback riding, fishing) are STEM-based. Most campers stay in cabins on-site, but some campers (e.g., K-2 and campers with special needs) only participate in day-time activities. Our group hosted 7-12 students per year who chose to participate in our STEM-based camp for three hours each morning. They signed up for other traditional camp activities (e.g., horseback riding, archery, etc.) during the afternoon. The itinerary is shown in Figure 4.2.

Day 1 (3 hours) Module (Duration)	**Day 2 (3 hours) Module (Duration)**	**Day 3 (3 hours) Module (Duration)**	**Day 4 (3 hours) Module (Duration)**	**Day 5 (3 hours) Module (Duration)**
Camp Orientation (35 min)	Review (10 min)	Review (15 min)	Review and discussion (15 min)	Review and discussion (15 min)
Introductions and ice breaker (20 min)	Drag swab activity for microbiological sample collection (15 min)	Ice cream discussion (15 min)	Develop questionnaire/interview questions (15 min)	Strawberry DNA extraction activity (40 min)
Pre-assessments (10 min)	Sample collection activity (30 min)	Alginate gummies demonstration (20 min)	Gather additional information and conduct interviews (35 min)	DNA structure/replication modelling (40 min)
Nature of science demonstration (45 min)	Restroom and water break (10 min)	Restroom and water break (10 min)	Group brainstorming (20 min)	Restroom and water break (10 min)
Sensory evaluation discussion and demonstrations (40 min)	Sample enrichment activity (20 min)	Product development: scenario-based ice cream recipe formulation activity (30 min)	Restroom and water break (10 min)	DNA fingerprinting activity and discussion (20 min)
Food safety and microbiology discussion (20 min)	Pipet practice activity (20 min)	Ice cream and whipped cream/butter manufacturing activity and discussion (45 min)	GPS training, demonstration, and discussion (10 min)	GIS data analysis (10 min)
Review and discussion (10 min)	Microbial detection activity (streak samples on agar plates) (20 min)	Outbreak case study part I, present scenario (15 min)	Field sample collection with GPS (40 min)	Group brainstorming: conclude investigation (10 min)
	What is food processing? Milk demonstration (20 min)	Brainstorming activity: what's going on and what do we do? (20 min)	Group brainstorming: examine agar plates and gather more data (25 min)	Review and discussion (15 min)
	Milk structure modeling activity (20 min)	Review and discussion (10 min)	Review and discussion (10 min)	Complete post assessments (20 min)
	Review and discussion (15 min)			

Figure 4.2. Itinerary for 4-H Camp Bristol Hills (2013). The timing of activities is approximate, as the nature of presenting camp activities with diverse learners requires considerable flexibility.

Like Career Explorations, participants completed a pre-assessment (see Figure 4.2) after brief introductions. Due to the extended duration of 4-H Camp Bristol Hills (compared to Career Explorations), the introductory modules at 4-H Camp Bristol Hills were food science-based. Campers were taught about food chemistry, food microbiology, sensory evaluation, and product development. Campers used knowledge gained during these modules to complete a product development exercise involving the formulation of ice cream, with different restrictions on ingredient availability (e.g., one group had a camper with lactose intolerance). Campers were then presented with a mock, camp-related outbreak scenario. Campers conducted formal interviews with camp staff, "ill patients," and the camp nurse to gather information. The source of the outbreak (the camp-made ice cream) was then established through a mock microbiological sampling, enrichment, and isolation exercise. Students were taught about molecular subtyping methods to confirm the relatedness of isolates but due to the camp setting, subtyping exercises were based on hands-on model simulations rather than laboratory experiments.

RESULTS AND DISCUSSION

The Food Science Career Explorations and Food Science at Camp Bristol Hills programs presented here have provided opportunities for 61 K-12 students to participate in extracurricular food science and food safety activities during the summer break from regular school instruction. Of 61 participants, 42 attended Career Explorations (in 2011, 2012, or 2013) and 19 attended Camp Bristol Hills (in 2012 or 2013). Participants in Career Explorations ranged from grade levels 10-12 and participants in Camp Bristol Hills ranged in grade level from 4-10 (which also included two participants with special needs, i.e., students within autism spectrum disorder). Educational modules for our programs were developed or adapted for use within our food

science and food safety-based curriculum. Assessments were also developed to evaluate learning gains, perceived learning gains, and participant satisfaction. Through these efforts, we have demonstrated that participants (i) have shown increased interest in science, food science and food safety, and (ii) have demonstrated increased knowledge of science, food science, and food safety-based concepts. We further suggest that the educational modules developed for these programs provide opportunities for demonstrating important concepts contained in the NGSS.

Participants showed increased interest in science and food safety. One of the goals of 4-H is to provide career exploration opportunities for students to discover their interests and disinterests, strengths and weaknesses (15). Although each module presented here has specific learning goals, we anticipate that the complete experience provides students with an effective opportunity for self-reflection on future career and educational paths. In exit surveys (Table 4.2), participants indicated that their experience in these workshops increased their enthusiasm for science (54%; 33/61)) and food safety (61%; 37/61). Of the total participants, 52% (32/61) also indicated that they were interested in learning more about the subjects covered in these modules. Interestingly, the participants at Camp Bristol Hills were much more interested in learning more about these subjects (89%; 17/19). A majority of participants (77%; 46/60; one participant did not respond) also believed that the inquiry-based instructional approach used in these modules improved their learning.

Table 4.2. Results of exit surveys indicated that participants showed increased interest in science and food safety.

Participants indicated their agreement with the following statements:	Percent who agree or strongly agree[a]
1. This experience increased my enthusiasm for science in general.	54 (33/61)
2. This experience increased my enthusiasm for the science of food safety	61 (37/61)
3. I am interested in learning more about this subject.	52 (32/61)
4. This instructional approach improved my learning.	77 (46/60)[b]
5. I would have learned **more** in a classroom than in this camp.[c]	11 (2/19)

[a](5-point scale; strongly disagree, disagree, neither agree nor disagree, agree, strongly agree)
[b]One participant did not respond
[c]This question was only asked to Camp Bristol Hills participants

Experiences that increase interest and enthusiasm for science are critical to the process of learning, and may also influence future educational and career choices (9, 10). The 4-H Career Explorations workshop is held on the Cornell campus in a university laboratory and classroom space. This authentic place-based learning has been advocated as a way for students to learn science in an engaging setting and allows the concepts of the nature of science to be experienced first-hand (16). The authentic and stimulating setting at Camp Bristol Hills offers similar opportunities. Camp Bristol Hills participants were also asked to compare their camp experience with traditional classroom instruction and 63% (12/19) of participants believed that they learned more from these modules than they would have learned in their traditional classroom setting. Previous work has shown that the experience of working with practicing scientists stimulates interest in science and learning and promotes students to consider science as a viable career option (16).

Participants demonstrated increased knowledge of food science and food safety-based concepts. Evaluations of pre- and post-program assessments indicated that participants showed an increased understanding of concepts related to foodborne illnesses, foodborne outbreak investigations, DNA, PCR, the nature of science, and food processing (Table 4.3). Due to differences in material covered in the Career Explorations and Camp Bristol Hills programs, knowledge of molecular techniques were only assessed for Career Explorations participants and food processing questions were only covered in the Camp Bristol Hills assessments. Topics that were covered in both programs were assessed with identical questions, with the exception of one open ended question concerning foodborne illness. In total we obtained 61 pairs of pre-and post-program assessments from participants in either Career Explorations (N = 42) or Camp Bristol Hills (N = 19).

Of the questions posed to participants in both Career Explorations and Camp Bristol Hills, the largest gains were seen in knowledge of DNA replication (19% improvement from pre- to post-assessments; Table 4.2). Similar gains were observed in the identification of foodborne illness symptoms and the Nature of Science (17% improvement each). Students demonstrated an overall higher level of knowledge related to the Nature of Science as compared to other subject areas assessed but important knowledge gaps in certain areas were evident. For example, pre-assessments indicated that only 43% of students viewed science as a complex social activity. Most concerning was that only 57% of students believed that science is based on evidence. Post-assessment scores indicated a 23 and 13% improvement in these questions, respectively. Responses to questions concerning general characteristics of DNA showed an overall 13% improvement from pre- to post-assessments. The largest increases related to the general characteristics of DNA were seen in questions concerning the stability and composition of DNA (20 and 18% improvement, respectively). A majority of students experienced difficulty understanding the process of outbreak investigations, with only 23% correct answers on post-assessments, which was a 13% improvement from pre-assessments.

Table 4.3. Results of pre- and post-assessments indicated that content knowledge related to the nature of science, food science, and food safety was improved.

Learning outcome	Percent of correct responses (total)		Percent improvement
	Pre	Post	
1. described personal habits to reduce foodborne illness[a,]	81	81	0
2. defined the term "foodborne illness"[b]	42	58	16
3. identified symptoms of foodborne illness[a, b]	59	76	17
4. identified incubation period of select pathogens[a]	27	58	31
5. identified general characteristics of DNA[a, b]	59	72	13
6. identified steps of DNA replication[a, b]	48	67	19
7. identified steps of PCR[a]	50	62	12
8. identified steps of outbreak investigation[a, b]	10	23	13
9. provided primary components of milk[b]	0	60	60
10. described the conversion of milk to cheese[b]	11	53	42
11. distinguished between the terms "aroma" and "flavor"[b]	0	5	5
12. identified characteristics of the nature of science[a, b]	64	81	17

[a] indicates question posed to participants of Career Explorations (n=42)
[b] indicates question posed to participants of Bristol Hills (n=19)

Overall, comparisons of pre- and post-assessment scores indicated vast improvement in all areas. Liceaga and others (17) concluded that using problem-based learning (PBL) in a food science course reinforced course material and taught students to think critically. Additionally, Duffrin (18) found that using PBL in an introductory food science course increased communication and problem-solving skills. In these modules, PBL was used in the form of guided inquiry in which the instructor served as a facilitator to assist students in asking the right research questions and generating adequate conclusions in order to develop solutions. In PBL, students take ownership of their learning and demonstrate independence while instructors simply act as another colleague on the problem-solving team (17, 19). PBL provides opportunities for deeper and more meaningful learning because students have constructed the knowledge themselves within a context and in response to a need (19, 20). Previous work also has shown that PBL is effective at motivating students to learn and enjoy the process of learning (18, 20).

The educational modules used in these programs provide opportunities to demonstrate important concepts contained in the Next Generation Science Standards (NGSS). The development of the modules presented here was guided by current science teaching reform efforts that seek to emphasize understanding of the process of scientific inquiry (13, 14). Although the proposed Next Generation Science Standards (NGSS) were released after the development of these educational modules, post-hoc examination suggests that these modules seem well suited for demonstrating the concepts emphasized in the NGSS (Table 4.4). Broadly, the NGSS emphasize the integration of three dimensions, which include (i) core ideas, (ii) practices, and (iii) cross-cutting concepts (21). Additionally, the incorporation of real world applications of science (e.g., agriculture, forensics) is expressly encouraged to build student interest and demonstrate the use and practicality of scientific principles (21). Specifically, the

food safety and food science modules presented here provide a meaningful and engaging setting to introduce many of the core ideas, practices, and cross-cutting concepts outlined in the NGSS (Table 4.1). Additional work (with students and educators) is needed to assess the efficacy of these modules for achieving learning outcomes in accordance with the newly released NGSS.

Through these efforts, we have demonstrated that inquiry-based food science and food-safety-based education modules for K-12 increase interest in the pursuit of future study in these subjects, increase knowledge and understanding of important science-based and consumer-related concepts, and provide opportunities for educators (in both traditional and extracurricular settings) to convey important concepts related to the NGSS and the Nature of Science. The NGSS provides considerable flexibility for the development and use of novel curriculum such as the one presented here, which is needed to stimulate and build interest in science before talented students seep out of the STEM pipeline. Through the implementation of NGSS-based food science curricula that couples practice with content and emphasizes the process of scientific inquiry, students will gain valuable critical thinking and problem solving skills, as well as content-based knowledge, thus providing a larger talent pool adequately prepared for challenging educational and career opportunities in food science and related fields.

Table 4.4. Food science and food safety modules provide opportunities to demonstrate important concepts contained in the Next Generation Science Standards (NGSS).

NGSS standard	NGSS performance expectation[1]	Application in 4-H modules
PS1-1 Matter and its interactions	Develop models to describe the atomic composition of simple molecules and extended structures.	Participants constructed a hands-on model of DNA molecules and demonstrated the process of DNA replication.
PS1-3 Matter and its interactions	Gather and make sense of information to describe that synthetic materials come from natural resources and impact society.	Participants made and used agarose gels to analyze PCR products. A variety of chemically modified agaroses are available with different melting points.
LS1-1 From molecules to organisms: structures and processes	Conduct an investigation to provide evidence that living things are made of cells; either one cell or many different numbers and types of cells.	i) Participants grew and isolated bacteria on agar plates (each colony is made up of millions of single-celled bacteria). ii) Participants extracted DNA from strawberries (all living things are made up of cells and each cell contains DNA)
LS1-5 From molecules to organisms: structures and processes	Construct a scientific explanation based on evidence for how environmental and genetic factors influence the growth of organisms.	Participants conducted an outbreak investigation and learned that genetic and environmental factors influence the growth of organisms (e.g., cold growth of *L. monocytogenes*) and also used genetic subtyping methods to identify target organisms.
LS2-1 Ecosystems: Interactions, Energy, and Dynamics	Analyze and interpret data to provide evidence for the effects of resource availability on organisms and populations of organisms in an ecosystem.	Participants conducted field sampling and GIS analysis to determine environmental factors that may have influenced the presence of target organisms.
LS2-2 Ecosystems: Interactions, Energy, and Dynamics	Construct an explanation that predicts patterns of interactions among organisms across multiple ecosystems.	Participants used GIS analysis to predict other "high-risk" landscapes.
LS2-4 Ecosystems: Interactions, Energy, and Dynamics	Construct an argument supported by empirical evidence that changes to physical or biological components of an ecosystem affect populations.	The GIS case study scenario involved flooding of cropland. Target pathogens were present in high numbers after flooding and gradually declined to a baseline level.

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