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Research Paper

Small Produce Farm Environments Can Harbor Diverse *Listeria monocytogenes* and *Listeria* spp. Populations

ALEXANDRA BELIAS,[1] LAURA K. STRAWN,[2] MARTIN WIEDMANN,[1] AND DANIEL WELLER[1,3,4]*

[1]*Department of Food Science, Cornell University, 354 Stocking Hall, Ithaca, New York 14853 (ORCID: https://orcid.org/0000-0002-6308-8025 [D.W.]);* [2]*Department of Food Science and Technology, Eastern Shore Agricultural Research and Extension Center, Virginia Tech, 33446 Research Drive, Painter, Virginia 23420;* [3]*Department of Statistics and Computational Biology, University of Rochester, Rochester, New York 14627; and* [4]*Department of Environmental and Forestry Biology, SUNY College of Environmental Science and Forestry, Syracuse, New York, 13210, USA*

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ABSTRACT

A comprehensive understanding of foodborne pathogen diversity in preharvest environments is necessary to effectively track pathogens on farms and identify sources of produce contamination. As such, this study aimed to characterize *Listeria* diversity in wildlife feces and agricultural water collected from a New York state produce farm over a growing season. Water samples were collected from a pond ($n = 80$) and a stream ($n = 52$). Fecal samples ($n = 77$) were opportunistically collected from areas <5 m from the water sources; all samples were collected from a <0.5-km^2 area. Overall, 86 (41%) and 50 (24%) of 209 samples were positive for *Listeria monocytogenes* and *Listeria* spp. (excluding *L. monocytogenes*), respectively. For each positive sample, one *L. monocytogenes* or *Listeria* spp. isolate was speciated by sequencing the *sigB* gene, thereby allowing for additional characterization based on the *sigB* allelic type. The 86 *L. monocytogenes* and 50 *Listeria* spp. isolates represented 8 and 23 different allelic types, respectively. A subset of *L. monocytogenes* isolates ($n = 44$) from pond water and pond-adjacent feces (representing an ~5,000-m^2 area) were further characterized by pulsed-field gel electrophoresis (PFGE); these 44 isolates represented 22 PFGE types, which is indicative of considerable diversity at a small spatial scale. Ten PFGE types were isolated more than once, suggesting persistence or reintroduction of PFGE types in this area. Given the small spatial scale, the prevalence of *L. monocytogenes* and *Listeria* spp., as well as the considerable diversity among isolates, suggests traceback investigations may be challenging. For example, traceback of finished product or processing facility contamination with specific subtypes to preharvest sources may require collection of large sample sets and characterization of a considerable number of isolates. Our data also support the adage "absence of evidence does not equal evidence of absence" as applies to *L. monocytogenes* traceback efforts at the preharvest level.

HIGHLIGHTS

- There is considerable *Listeria* diversity in the farm environment investigated.
- *Listeria* subtypes were reintroduced or persisted over the growing season.
- Four *L. monocytogenes* PFGE types were shared between feces and pond samples.

Key words: Agricultural water; Feces; *Listeria*; Pulsed-field gel electrophoresis; *sigB*

It is estimated that *Listeria monocytogenes* is responsible for ~1,600 illnesses, 1,455 hospitalizations, and 255 deaths annually in the United States *(31),* and several high-profile outbreaks and recalls have been traced to *L. monocytogenes* contamination of fresh produce *(8, 24, 44)*. For example, a 2011 outbreak was traced back to *L. monocytogenes* contamination of cantaloupes that resulted in 147 illnesses and 33 deaths *(2)*. In addition, there have been >30 recalls since 2016 due to *L. monocytogenes* contamination of produce and ready-to-eat produce products *(36)*. Although the majority of produce outbreaks and recalls due to *L. monocytogenes* contamination are traced back to the processing environment, *L. monocytogenes* can be introduced, or reintroduced, in the processing environment from the preharvest environment (e.g., on produce, in soil, on harvesting equipment, or on workers shoes *(32)*) or could be introduced onto product in the field and remain on product until consumption.

After detection of a *Listeria*-positive sample during routine monitoring or an outbreak investigation, processors or investigators often conduct extensive sampling to identify potential contamination sources (i.e., traceback analysis). Identifying how a *Listeria* isolate was introduced onto product or into the processing environment is a critical step in performing a root cause analysis and establishing effective corrective actions (to control potential contamina-

* Author for correspondence. Present address: Department of Biostatistics and Computational Biology, University of Rochester, Rochester, NY 14620, USA. E-mail: wellerd2@gmail.com.

tion pathways). To determine whether an isolate originated in the preharvest environment, investigators and operators must collect environmental samples from farms that supply the product. However, the ubiquity of *Listeria* in the environment *(1, 7, 11, 12)* may make it challenging to prove a causal relationship between *Listeria* isolated from the preharvest environment and *Listeria* isolated from postharvest environments and/or product. Previous studies of preharvest environments isolated *Listeria* from soil *(3, 25, 34, 35, 39, 40),* wildlife feces *(5, 19, 37, 40),* agricultural water *(1, 6, 17, 20, 33),* and biological soil amendments *(25)*. As such, a more comprehensive understanding of the prevalence, diversity, and distribution of *Listeria* on produce farms is needed to better understand on-farm sources of *Listeria,* understand transfer of *Listeria* between sample types on produce farms, and facilitate traceback analysis. Although some studies have investigated the diversity of *Listeria* in preharvest produce environments *(3, 19, 20, 33–35),* these studies have examined diversity over large geographic areas. For example, Chapin et al. *(3)* and Strawn et al. *(34)* investigated *Listeria* diversity on five New York state farms located up to 205 km from each other, whereas Stea et al. *(33)* examined *Listeria* diversity within an agricultural watershed in Nova Scotia, Canada, with a drainage area of 135 km^2. Because there are limited data on *Listeria* diversity at small spatial scales (e.g., within individual farms), we aimed, in this study, to address this knowledge gap by examining the diversity of *Listeria* on a single produce farm.

Agricultural water and wildlife intrusion in produce fields have been identified as key sources and pathways for dispersal of *Listeria* contamination in preharvest environments *(14, 26, 37, 39)*. Multiple studies have isolated *L. monocytogenes* from agricultural water and wildlife feces collected in produce-growing environments *(6, 7, 19, 25, 34, 39)*. For example, Weller et al. *(39)* isolated *L. monocytogenes* from 45% ($n = 11$) of wildlife feces collected on 10 produce farms in New York state. Falardeau et al. *(7)* isolated *L. monocytogenes* from 10% ($n = 223$) of agricultural water samples collected from two watersheds in British Colombia, Canada. Furthermore, Cooley et al. *(6)* isolated *L. monocytogenes* from 43% ($n = 1,405$) of water samples collected from California's Central Coast agricultural region. As such, the primary objective of the study reported here was to characterize and compare the *L. monocytogenes* and *Listeria* spp., excluding *L. monocytogenes,* diversity in agricultural water and wildlife feces collected from a single produce farm in New York state.

MATERIALS AND METHODS

Study design and sample collection. The study was conducted between May and July 2014 on a produce farm in the Finger Lakes region of New York state. Two separate irrigation water sources are located on the farm: (i) a pond and (ii) a stream. The water sources are within a rectangular 0.5-km^2 (124-acre) area; the sampling sites in the pond and stream are ~775 m (2,546 ft) apart.

Agricultural water and fecal samples were collected on 26 days. Fifty-two and 80 water samples were collected from the stream and pond, respectively; the *Listeria* prevalence and number of *sigB* allelic types (ATs) for the 52 stream samples and the 13 fecal samples collected near the stream were previously reported by Weller et al. *(40)*. However, to better understand the *Listeria* diversity on the studied farm, these data were also included here. All water samples were collected as described in Weller et al. *(40)*. Briefly, 250 mL of water was collected into a sampling cup (Nalgene, Rochester, NY) by using a sampling pole and was stored on ice until processing. Fecal samples ($n = 77$) located within 5 m of the pond ($n = 64$) or stream ($n = 13$) were collected opportunistically, as described previously *(40)*; data for the fecal samples collected near the pond were not previously reported. A 5-m buffer around the pond and stream was used for practical reasons. All samples were stored at 4°C and processed within 3 h of collection.

***Listeria* enrichment and isolation.** All samples were processed as described previously *(40)*. Briefly, 10 g of the fecal samples was aliquoted and transferred to separate Whirl-Pak bags (Nasco, Fort Atkinson, WI). The water samples were each passed through separate 0.45-μm-pore-size filters, and each filter was transferred to a separate Whirl-Pak bag. Each sample was enriched in 90 mL of buffered *Listeria* enrichment broth (BD, Franklin Lakes, NJ) at 30°C. After 4 h, *Listeria* selective enrichment supplement (Oxoid, Cambridge, UK) was added to each sample. Each sample was incubated for a total of 48 h at 30°C. After 24 and 48 h of incubation, the enrichments were substreaked onto modified Oxford agar (MOX; BD) and *L. monocytogenes* plating medium (LMPM; Biosynth International, Itasca, IL). MOX plates were incubated at 30°C and LMPM plates were incubated at 35°C for 48 h. After incubation, characteristic *Listeria* colonies on 24- and 48-h MOX plates (i.e., gray, donut-shaped colonies) were streaked onto LMPM for isolation and incubated at 35°C for 48 h. One colony per presumptive *Listeria*-positive LMPM plate (up to four colonies total: one from the 24-h LMPM plate, one from the 48-h LMPM plate, one from the LMPM plate substreaked from the 24-h MOX plate, and one from the LMPM plate substreaked from the 48-h MOX plate) was selected, substreaked onto brain heart infusion agar (BD), and incubated at 37°C for 24 h. If blue colonies were present (characteristic of *L. monocytogenes, L. ivanovii,* hemolytic *L. innocua* due to phospholipase C action; *(28)*) on a given plate, they were preferentially selected to be substreaked on brain heart infusion agar. If a blue colony was not present, a round white colony was selected to be substreaked on brain heart infusion agar; this colony phenotype is characteristic of *Listeria* spp. other than *L. monocytogenes, L. ivanovii,* and hemolytic *L. innocua*. If no round white or blue colonies were present on any of the LMPM plates, the sample was considered negative for *Listeria*. If a round white or blue colony was present, *sigB* PCR was performed as a confirmation step and speciation of *Listeria,* as described below.

***sigB* sequencing and allelic typing.** All isolates per presumptive-positive *Listeria* sample (i.e., up to four isolates per sample) were confirmed as *Listeria* via PCR amplification of *sigB,* as described by Nightingale et al. *(22)*; all samples with presumptive-positive *Listeria* isolates in this study were confirmed as positive via *sigB* PCR. Sequencing of the *sigB* gene was then performed on one isolate per positive sample for further characterization: if the colony characterized was identified as *L. monocytogenes,* the sample was considered *L. monocytogenes* positive; if the colony characterized was identified as *Listeria* spp. other than *L. monocytogenes,* the sample was considered *Listeria* spp. positive. Although only subtyping one isolate per sample may lead to an underestimation of the true diversity present in the

TABLE 1. *Frequency and prevalence of* Listeria *spp. and* L. monocytogenes *in fecal and agricultural water samples*

Sample type[a]	Location	No. of samples	No. of samples positive (prevalence in %) for:				
			Listeria spp.[b]	*L. innocua*	*L. seeligeri*	*L. welshimeri*	*L. monocytogenes*
Fecal	—[c]	77	11 (14)	6 (8)	4 (5)	1 (1)	22 (29)
	Near pond	64	9 (14)	4 (6)	4 (6)	1 (2)	13 (20)
	Near stream	13	2 (15)	2 (15)	0 (0)	0 (0)	9 (69)
Water	—	132	39 (30)	14 (11)	20 (15)	5 (4)	64 (48)
	Pond	80	25 (31)	8 (10)	15 (19)	2 (3)	31 (39)
	Stream	52	14 (27)	6 (12)	5 (10)	3 (6)	33 (63)

[a] In addition to the water samples collected from the pond water, one sediment sample was collected and it tested positive for *L. innocua*.
[b] *Listeria* spp. includes *L. innocua, L. seeligeri,* and *L. welshimeri*.
[c] —, total across a given sample type.

sample types investigated here, this approach allowed us to get a preliminary estimate of the diversity present. There were 22 samples with both blue and white colonies present on LMPM plates (of 209 total samples); for 21 of these samples, the blue colony was selected for characterization (2 of 3 fecal samples, 8 of 8 stream samples, and 11 of 11 pond samples). Because only *L. monocytogenes* was selected for pulsed-field gel electrophoresis (PFGE), preferentially selecting these blue colonies provided us with the larger sample size for further subtyping. A BLAST search was performed against an internal database (http://www.foodmicrobetracker.com/login/intro.aspx) to determine its *sigB* AT of each sequence. All isolates were archived as 15% glycerol stocks and stored at −80°C. A *sigB* AT phylogeny was constructed based on the 660-bp nucleotide sequence of the *sigB* gene by using the maximum likelihood method in MEGA7 version 7.0.26; the nucleotide substitution Tamura three-parameter model was used and 1,000 bootstraps were performed.

PFGE analysis. All confirmed *L. monocytogenes* isolates from the pond and the feces collected near the pond ($n = 44$) were characterized by PFGE. Only isolates from pond water and feces were characterized by PFGE as a subset analysis to investigate diversity over a small spatial scale (i.e., the ~5,000 m^2 around the pond). PFGE was performed using *Asc*I and *Apa*I restriction enzymes, according to the Centers for Disease Control and Prevention PulseNet protocol *(10)*.

Statistical analysis. All statistical analyses were performed using R version 3.5.1 *(27)*. Chi-square tests were used to determine whether *L. monocytogenes* and *Listeria* spp. (excluding *L. monocytogenes*) prevalence differed between sample types. The Bonferroni correction was used to account for the three comparisons made for *Listeria* spp. (excluding *L. monocytogenes*) and for the three comparisons made for *L. monocytogenes* (pond versus fecal, pond versus stream, fecal versus stream; $\alpha = 0.05/3 = 0.0167$). Although separate data for the feces collected near the pond and feces collected near the stream are provided in this study, these sample types were combined into a single fecal sample category for analysis due to the small number of fecal samples collected near the stream ($n = 13$ of 77 total fecal samples). To determine the number of unique ATs and PFGE types, AT richness and PFGE type richness were estimated using the breakaway package version 4.6.7 *(43),* which calculates the transformed species richness estimation with 95% confidence intervals (CIs) as outlined in Rocchetti et al. *(29)* and Willis and Bunge *(42)*. To estimate AT and PFGE type diversity for each sample type, Simpson's index of diversity was calculated using the vegan package version 2.5-3 *(23)*; 95% CIs were calculated for the Simpson's index of diversity, according to the methods described by Hunter and Gaston *(13)*. The effective number of subtypes was also calculated to communicate Simpson's index of diversity in a more intuitive form (i.e., the estimated number of subtypes present in a sample type given a certain value of Simpson's index of diversity *(15)*).

RESULTS AND DISCUSSION

Seek and you shall find. If a reasonably large number of samples are collected from the preharvest environment in New York state and tested for *Listeria* ("seek"), it is likely *Listeria* will be "found." The overall prevalence of *Listeria* in the study reported here was 136 (65%) of 209; the prevalence of *Listeria* spp. (excluding *L. monocytogenes*) was 50 (24%) of 209, and the prevalence of *L. monocytogenes* was 86 (41%) of 209 (Table 1). Although the *Listeria* spp. (excluding *L. monocytogenes*) prevalence in the pond (25 of 80, 31%) and stream (14 of 52, 27%) samples was not significantly different ($P = 0.735$), the *L. monocytogenes* prevalence in the pond (31 of 80, 39%) and stream (33 of 52, 63%) samples was significantly different, even after the Bonferroni correction ($P = 0.009$; Table 1). The frequency of *Listeria* isolation from agricultural water has been reported in several previous studies conducted in New York state *(3, 34, 35, 38, 39)* and other produce-growing regions *(1, 6, 7, 18, 33)*. In previous New York state studies, the *L. monocytogenes* prevalence ranged between 9% *(39)* and 59% *(35)* in pond samples, and between 10% *(38)* and 50% *(39)* in stream samples. As such, the frequency of *L. monocytogenes* isolation from pond samples in the current study falls within the range reported in the literature, whereas the frequency of *L. monocytogenes* isolation from the stream samples in the current study is higher than in previous studies. The higher *L. monocytogenes* prevalence in the stream sampled here, compared with those sampled in other studies, may be due to presence of two dairy operations immediately upstream of the sampling sites along the stream. In addition, run-off from the dairy operations could explain the significantly higher *L. monocytogenes* prevalence in the stream compared with the pond. Previous studies have indicated upstream livestock operations are associated with an increased prevalence of *L. monocytogenes* in stream water *(7, 19, 41)*. Furthermore, another study suggested cattle may

TABLE 2. *Diversity of* Listeria *spp.* sigB *allelic types (ATs) in agricultural water and fecal samples*

Sample type	No. of *Listeria* spp.[a]	No. of ATs	Estimated subtype richness (95% CI)[b,c]	Simpson's index (95% CI)[c]	Effective no. of ATs[d]
Fecal					
—[e]	11	8	35 (8–2,871)	0.84 (0.75–0.94)	6
Water					
—	39	14	23 (14–259)	0.84 (0.76–0.92)	6
Pond	25	11	32 (11–1,445)	0.79 (0.67–0.92)	5
Stream	14	8	11 (8–39)	0.84 (0.76–0.91)	6

[a] Excluding *L. monocytogenes*.
[b] Estimated using weighted linear regression.
[c] 95% CI, 95% confidence interval.
[d] Effective number of ATs is calculated based on Simpson's index of diversity.
[e] —, total across a given sample type.

increase the prevalence and may distribute *L. monocytogenes* in the farm environment *(22)*. Of note, the current study only tested 250 mL of water per sample, whereas other studies collected upward of 1 L per sample. As such, the prevalence reported for the pond and stream investigated in the current study may be an underestimation of the true prevalence. Regardless, although the current study supports agricultural water as a source of *L. monocytogenes* in the New York state preharvest produce environment, our findings also suggest future studies on agricultural water need to consider the impact of spatiotemporal factors (e.g., upstream land use) on downstream microbial water quality.

The prevalence of *Listeria* spp. (excluding *L. monocytogenes*) and *L. monocytogenes* in the fecal samples collected here were 11 (14%) and 22 (29%) of 77, respectively (Table 1). Based on fecal shape and consistency, the majority of the fecal samples appeared to be produced by Canada geese (*Branta canadenesis*; $n = 71$). This is logical given the large flock of Canada geese that nested in the wetlands adjacent to the pond throughout the study. The remainder of the feces were produced by canids ($n = 5$) and an unknown animal ($n = 1$). The *Listeria* spp. (excluding *L. monocytogenes*) prevalence was 9 (13%) of 71 in the goose and 2 (40%) of 5 in the canid fecal samples. The *L. monocytogenes* prevalence was 20 (28%) of 71 in the goose and 2 (40%) of 5 in the canid fecal samples. Neither *Listeria* spp. (excluding *L. monocytogenes*) nor *L. monocytogenes* was isolated from the one unidentified fecal sample. Although multiple studies have isolated *Listeria* from wildlife feces *(5, 19, 25, 34, 37, 39, 40),* relatively few studies have examined the prevalence of *L. monocytogenes* in Canada goose feces specifically *(5, 19)*. The *L. monocytogenes* prevalence in the goose fecal samples collected as part of the current study was greater than that of previous studies *(5, 19)*. For example, a study conducted in Ontario, Canada, did not detect *L. monocytogenes* in any of the 18 goose fecal samples collected *(19),* whereas Converse et al. *(5)* isolated *Listeria* spp. from 47 (10%) of 495 of goose fecal samples. Although, the prevalence of *L. monocytogenes* in wildlife fecal samples varied between studies, our findings support previous studies' findings *(33, 36, 37)* that wildlife can act as source of *L. monocytogenes* in preharvest environments.

Absence of evidence does not equal evidence of absence. The composition of the *Listeria* population in the agricultural water and fecal samples was characterized using *sigB* allelic typing. Allelic typing was used to speciate the isolates and provide a preliminary estimate of diversity. In total, 136 isolates were selected for *sigB* allelic typing (i.e., one isolate per *Listeria*-positive sample); *L. monocytogenes* isolates were preferentially selected for subtyping based on colony morphology (blue colonies on LMPM). Of the 136 isolates, 20, 24, 6, and 86 isolates were identified as *L. innocua, L. seeligeri, L. welshimeri,* and *L. monocytogenes,* respectively. We identified 15 *sigB* ATs among the 50 *Listeria* spp. (excluding *L. monocytogenes*) isolates (~1 unique AT per 3 isolates) and 8 ATs among the 86 *L. monocytogenes* isolates (~1 unique AT per 11 isolates; Tables 2 and 3; Supplemental Table S1). In addition, we estimated two diversity parameters, richness and the Simpson's index, for *Listeria* spp. (excluding *L. monocytogenes*; Table 2) and *L. monocytogenes* (Table 3) in the pond, stream, and fecal samples. For example, the estimated *L. monocytogenes* AT richness for the pond water, stream water, and fecal samples was 8 (95% CI = 7 to 15), 9 (95% CI = 6 to 73), and 5 (95% CI = 5 to 8) ATs, respectively, indicating the presence of a considerable diversity on the farm investigated. However, it should be noted that this may be an underestimation of the true diversity present in these sample types on this produce farm, because only one isolate per sample type was characterized by allelic typing. Even with this limitation, our data indicate considerable *Listeria* diversity in the small area of the produce farm investigated. The proportion of ATs identified relative to the total number of isolates subtyped in the current study was similar to the proportion reported by previous studies conducted on New York state produce farms *(3, 34)*. For example, applying the same analysis as in the current study, Chapin et al. *(3)* identified 50 *sigB* ATs among 186 *Listeria* spp. (excluding *L. monocytogenes*) isolates (~1 AT per 4 isolates) and Strawn et al. *(34)* identified 12 *sigB* ATs among 107 *L. monocytogenes* isolates (~1 AT per 8 isolates); both studies isolated *Listeria* from soil, drag swabs, water, and fecal samples collected from the five produce farms across New York state. Because all samples collected in the study reported here were from a <0.5-km^2 area, compared with

TABLE 3. *Diversity of* Listeria monocytogenes sigB *allelic types and pulsotypes in agricultural water and fecal samples*

Sample type	Location	No. of *L. monocytogenes*	Subtyping method[a]	No. of subtypes	Estimated subtype richness (95% CI)[b,c]	Simpson's index (95% CI)[c]	Effective no. of subtypes[d]
Fecal	—[e]	22	AT	5	5 (5–8)	0.63 (0.46–0.80)	3
	Near pond	13	PT	7	9 (7–33)	0.78 (0.63–0.93)	5
Water	—	64	AT	7	7 (7–8)	0.54 (0.41–0.68)	2
	Stream	33	AT	6	9 (6–73)	0.41 (0.20–0.62)	2
	Pond	31	AT	7	8 (7–15)	0.66 (0.49–0.82)	3
	Pond	31	PT	19	29 (19–207)	0.93 (0.91–0.95)	15

[a] AT, allelic type; PT, PFGE type. PFGE was only performed on *L. monocytogenes* isolates and not on nonpathogenic *Listeria* spp. isolates.
[b] Estimated using weighted linear regression.
[c] 95% CI, 95% confidence interval.
[d] Effective number of subtype is calculated based on Simpson's index of diversity.
[e] —, total across a given sample type.

past studies that sampled larger areas (between 33 and 205 km apart), the similarity in the ratio of unique ATs relative to the total number of isolates subtyped in this and these previous studies *(3, 34)* suggests *Listeria* richness is similar across spatial scales. However, additional research is required to confirm this finding, especially in other geographical areas.

Furthermore, multiple *Listeria* (including *L. monocytogenes*) *sigB* ATs were isolated from a single sample type on a given day on multiple occasions. In the pond, stream, and fecal samples, there was an average of 1.9, 1.5, and 1.7 *Listeria* (including *L. monocytogenes*) *sigB* ATs isolated per sampling day, respectively. There was also a maximum of three, two, and four *Listeria* (including *L. monocytogenes*) *sigB* ATs isolated per sampling day from the pond, stream, and fecal samples, respectively, indicating considerable *Listeria* diversity in the area sampled even on a given day. Six of the eight *L. monocytogenes sigB* ATs were isolated from at least one sample type on multiple dates (see Fig. 1, showing the isolation of *L. monocytogenes sigB* ATs over time; equivalent data for *Listeria* spp. [excluding *L. monocytogenes*] are shown in Supplemental Fig. S1). Notably, AT 57 was isolated from all three sample types (i.e., pond water, stream water, and feces) on multiple occasions and was isolated from at least one sample type on 23 of the 26 sampling dates. AT 57 represents 13 PFGE types (Fig. 2), consistent with previous reports that PFGE is considerably more discriminatory than *sigB* allelic typing *(4)*. The high prevalence of a single AT (i.e., AT 57) makes it likely that less prevalent ATs are not isolated; for example, AT 57 may outcompete other ATs. In addition, the considerable AT diversity observed also makes it likely that samplings that do not include large sample numbers will not detect and isolate some *Listeria sigB* ATs. Hence, traceback investigations could mistakenly conclude that a given *Listeria* subtype is not present in a given preharvest environment, particularly in environments similar to the one investigated here.

In addition, a *sigB* AT phylogeny was constructed to investigate whether there was any relationship between *sigB* AT isolation and sample type (Fig. 3). The phylogeny suggests that neither specific *sigB* ATs nor specific *sigB* clades are associated with a given sample type. A Fisher's exact test also found no significant relationship between

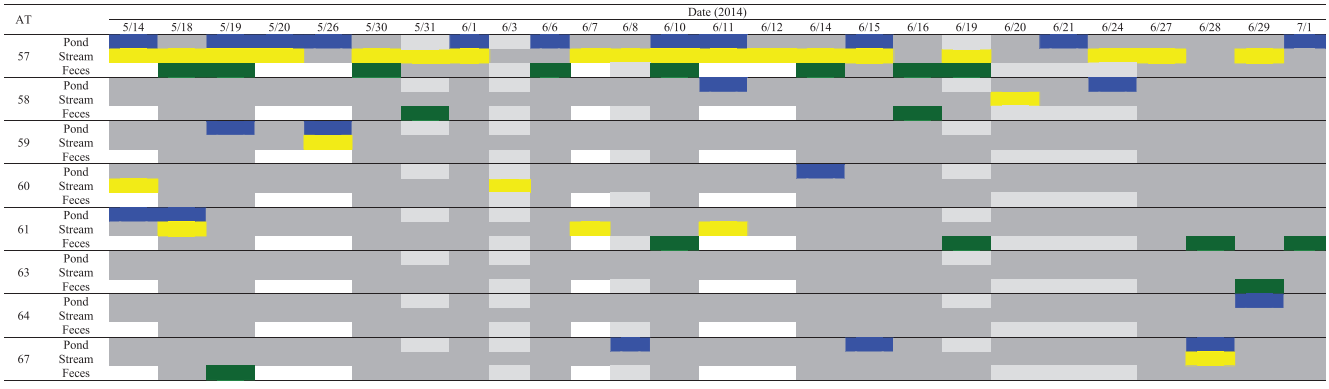

FIGURE 1. *Date of* L. monocytogenes sigB *AT isolation from water and fecal samples. In total, 31, 33, and 22 isolates from pond, stream, and fecal samples were allelic typed, respectively. The colored squares indicate at least one isolate of the given AT was isolated on the given date from pond (blue), stream (yellow), or fecal (green) samples; dates when a sample was collected, but the specified AT was not isolated are represented by dark gray square, and dates when a sample was collected but was negative for* Listeria *are represented by light gray square. At each sampling event, three pond and three stream samples were collected. Fecal samples were collected opportunistically (i.e., when feces were present). If no fecal samples were collected, the square is white. The dates of* Listeria *spp. (excluding* L. monocytogenes*)* sigB *AT isolation is shown in Figure S1.*

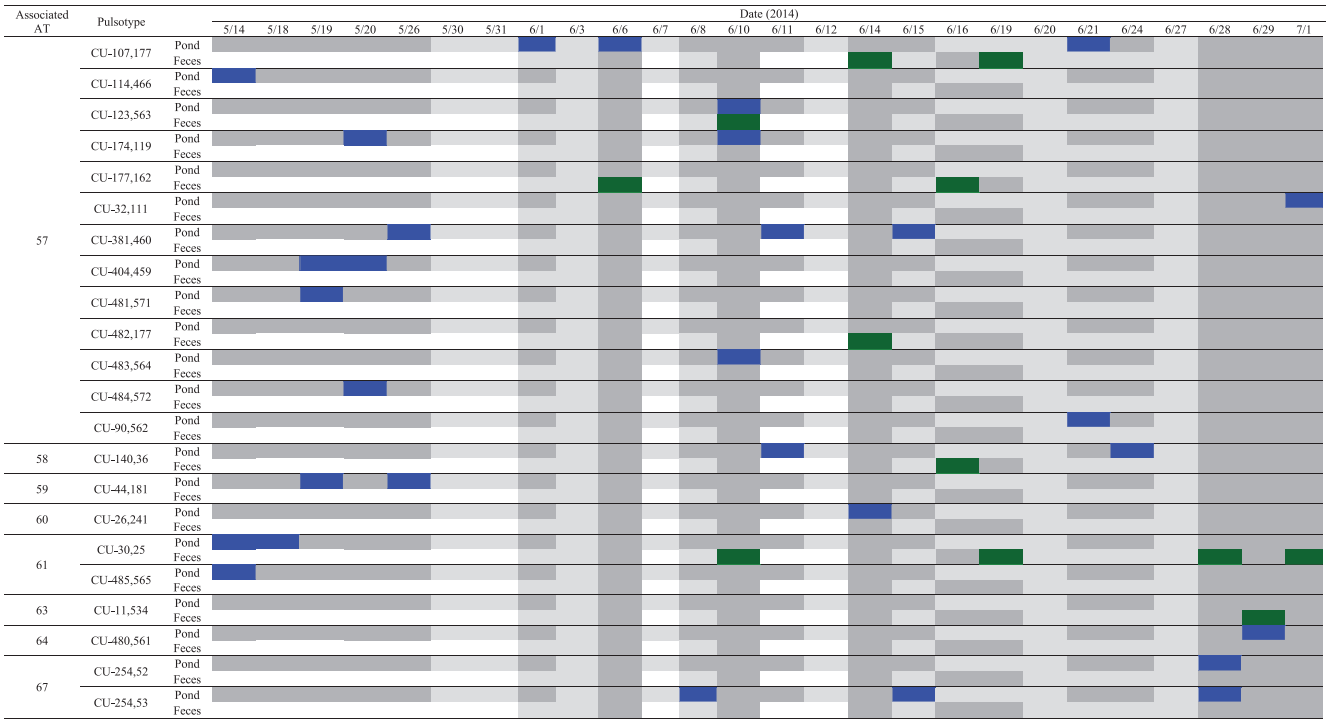

FIGURE 2. *Date of* L. monocytogenes *PFGE type isolation from pond and fecal samples collected near the pond. In total, PFGE was performed on 31 and 13 isolates from pond and fecal samples, respectively. The colored squares indicate at least one isolate of the given PFGE type was isolated on the given date from pond (blue) or fecal (green) samples; dates when a sample was collected, but the specified PFGE type was not isolated are represented by dark gray squares, and dates when a sample was collected but was negative for* L. monocytogenes *are represented by light gray squares. At each sampling event, three pond samples were collected. Fecal samples were collected opportunistically (i.e., when feces were present). If no fecal samples were collected, the square is white.*

sigB AT and sample type ($P = 0.30$). Although our data could also be interpreted as suggesting that none of the ATs isolated in this study have an enhanced fitness in the different habitats sampled here, studies with larger data sets are needed to further probe niche adaptation of different *Listeria* species and clades.

In addition to characterization by *sigB* AT, we performed PFGE on the *L. monocytogenes* isolates from the pond ($n = 31$) and feces collected near the pond ($n = 13$) to better understand the diversity of the isolates at a very small spatial scale (~5,000 m^2). We identified 19 PFGE types among the 31 *L. monocytogenes* isolates obtained from pond water samples (~1 PFGE type per 2 isolates) and 7 PFGE types among the 13 *L. monocytogenes* isolates obtained from fecal samples (~1 PFGE type per 2 isolates). The estimated PFGE type richness for *L. monocytogenes* isolated from the pond water and fecal samples was 29 (95% CI = 19 to 207) and 9 (95% CI = 7 to 33) PFGE types, respectively (Table 3). Two concurrent studies *(19, 20)* conducted in a 200-km^2 agricultural watershed in Ontario, Canada, also performed PFGE on *L. monocytogenes* isolates from agricultural water and wildlife feces; these studies used the same PFGE protocol as the current study *(10)*. In the two Canadian studies, Lyautey et al. *(19, 20)* identified 21 PFGE types from 75 *L. monocytogenes* isolates from water samples (~1 PFGE type per 3 isolates) and 18 PFGE types from 84 *L. monocytogenes* isolates from fecal samples (~1 PFGE type per 5 isolates). As such, the ratio of PFGE types identified relative to the number of *L. monocytogenes* isolates subtyped in the current study, which was conducted in a 40,000-fold smaller area, was larger than the ratio found in the Canadian studies *(19, 20)* for both the fecal samples and water samples. Although this may suggest the *L. monocytogenes* population in the Finger Lakes region of New York is more diverse than Ontario, Canada, the greater diversity on the farm sampled in the study reported here may also be due to factors unique to the sampled farm. For example, because two dairy farms are located uphill of the sampled farm, continuous introduction of *L. monocytogenes* from the dairies may have resulted in a more diverse population of *L. monocytogenes,* compared with the other areas. To identify potential drivers of the greater PFGE type diversity observed in the study reported here, compared with Lyautey et al. *(19, 20),* a systematic survey of *Listeria* populations across and within regions is needed. Regardless, the diversity of *Listeria* identified in this study, and other studies, suggests it often may be difficult to determine whether an isolate found in a processing environment originated in a particular preharvest environment, as multiple subtypes may be present in a single environment. In fact, to either rule in or out any particular source in an environment with a considerable diversity, such as the source investigated in the current study, a large number of samples would have to be collected and a large number of isolates would have to be subtyped, including multiple isolates per sample, as suggested previously *(9, 30)*. Consequently, the "absence of evidence" for presence of a given *Listeria* subtype (i.e., the *Listeria* subtype was not

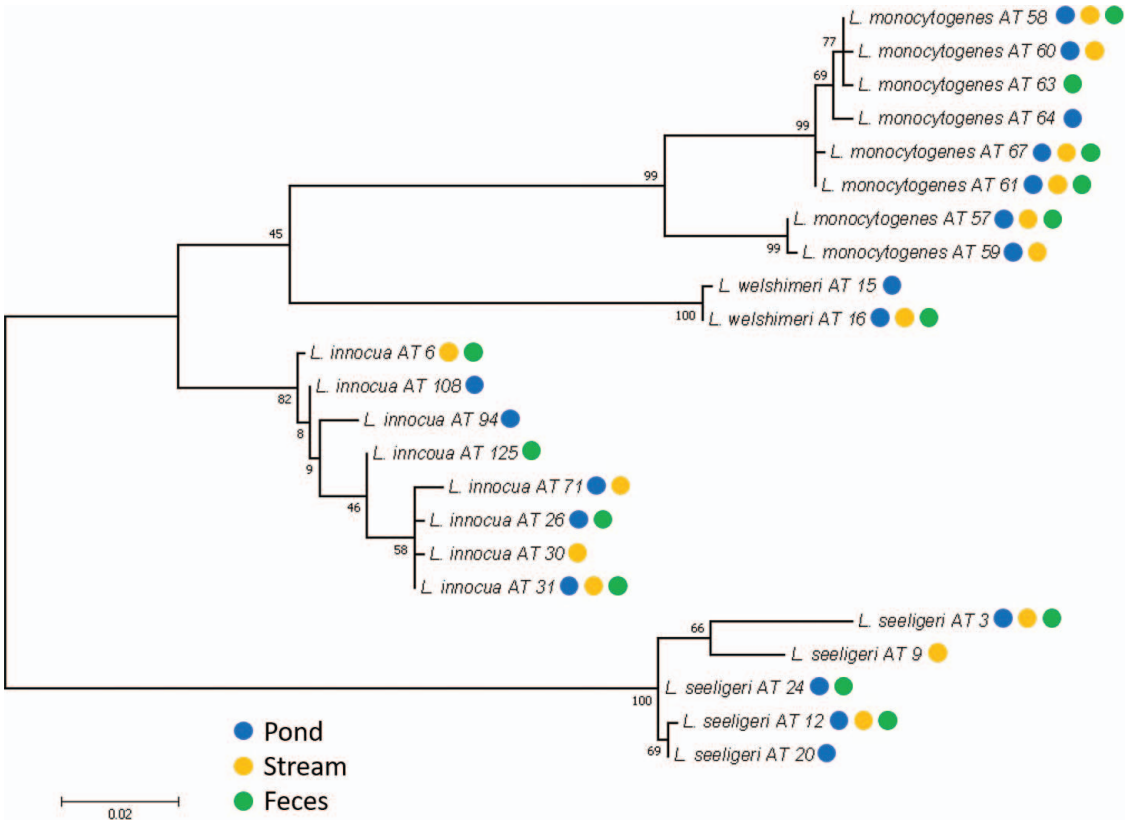

FIGURE 3. *Maximum likelihood phylogeny of* Listeria *isolated from pond, stream, and fecal samples from a single produce farm in New York state based on a 660-bp fragment of the* sigB *gene. Values on the branches are bootstrap values from 1,000 replicate trees. The scale displayed below the tree represents the genetic distance shown in the branch length.*

detected in any of the samples collected) in the preharvest environment in New York state and other preharvest environments with prevalent *Listeria* does not necessarily provide evidence that the given subtype is truly absent in this environment, particularly if sample size is small.

Should I stay or should I go. The *L. monocytogenes* PFGE data collected here indicate the survival ("stay") or continuous reintroduction ("go") of *L. monocytogenes* into the farm environment. Specifically, 8 of the 19 PFGE types isolated from the pond and 3 of the 7 PFGE types isolated from the feces collected near the pond were isolated on more than one date (Fig. 2). The repeated isolation of multiple PFGE types suggests these PFGE types were surviving or were continuously reintroduced in these sample types. For example, PFGE type CU-30,25 was isolated a total of eight times in this study, making it the most commonly isolated PFGE type (Fig. 2). This could indicate that the PFGE type CU-30,25 has an increased fitness in this environment or that there is a source of this PFGE type continuously contaminating the environment. In addition, 4 of the 22 PFGE types identified here were isolated from both the pond and feces collected near the pond (Fig. 2). Lyautey et al. *(19, 20)* found similar results in two of their studies: 5 of the 18 PFGE types from wildlife feces in the South Nation watershed in Ontario, Canada, were also isolated in water samples collected from the same watershed. Because the same PFGE types can be found in agricultural water and feces collected in proximity of the sampled water in both studies, this supports transfer of *L. monocytogenes* between feces and agricultural water and/or a common source leading to the contamination of both sample types in the investigated environment.

Work smarter not harder. Allelic typing of the *sigB* gene has been used to speciate *Listeria* isolates and can provide initial characterization of *Listeria* strains present *(16),* but it is not of high enough discriminatory power to elucidate the actual diversity of a *Listeria* population in an environment. For example, in the current study AT 57 represents 13 different PFGE types, AT 61 represents 2 different PFGE types, and AT 67 represents 2 different PFGE types (Fig. 2). Strawn et al. *(34)* also performed PFGE on *L. monocytogenes* isolates with matching *sigB* ATs from a single sample type in a survey of soil, feces, drag swabs, and water samples from five farms in New York state (i.e., repeatedly isolated *sigB* ATs). For three of the four repeatedly isolated *sigB* ATs, they found multiple PFGE types corresponding to each individual AT. PFGE hence clearly provides improved discriminatory power (over *sigB* allelic typing), facilitating investigations of persistence, transfer between sample types, and continuous reintroduction *(4)*. Interestingly, in our study, all isolates of the same PFGE type also had the same AT (Fig. 2). For example, all isolates of PFGE type CU-371,460 were AT 57, all isolates of PFGE type CU-107,177 were AT 57, all

isolates of PFGE type CU-254,53 were AT 67, etc. This finding has not been directly reported in other studies; however, Liao et al. *(16)* have determined the *sigB* gene is genetically stable, because it is not a frequent site of homologous recombination or positive selection. As such, we expect this relationship between *sigB* ATs and PFGE types to hold true across different environments. Although *sigB* allelic typing is not as discriminatory as PFGE, it can still be useful in performing traceback analysis, as it can be used as an initial screen to determine which isolates should be selected for PFGE, or other fine resolution typing methods (e.g., whole genome sequencing) during a traceback investigation. As a result, to determine which isolates from an environment match an isolate from another environment (e.g., processing environment), allelic typing should first be performed ("work smarter"). Next, isolates from the preharvest environment that have the same AT as the isolate from the processing environment should be characterized by PFGE or whole genome sequencing ("work harder") to determine which are truly the same. This will save time and money, as PFGE and whole genome sequencing are considerably more expensive and time intensive to perform.

Overall, the data presented here and in previous studies *(39)* indicate *Listeria* is prevalent in the New York state preharvest produce environment; thus, it is likely for *Listeria* from the preharvest environment to contaminate produce and/or be introduced to the processing environment (from the farm). The considerable *Listeria* diversity observed in the small area within the produce farm studied, and the evidence of transfer between sample types or continuous reintroduction of *Listeria,* indicate that it often may be difficult to successfully trace isolates from finished products or the processing environment (e.g., environmental swab) back to farm sources (e.g., feces, water), unless a large number of samples are collected and a large number of isolates are subtyped. However, it should be noted that these conclusions may be limited to similar preharvest produce environments that are continuously contaminated with livestock runoff. As such, additional research is needed to determine whether the findings reported as part of this pilot study hold true for other produce farms and produce growing regions.

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SUPPLEMENTAL MATERIAL

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