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Research Paper

Detection and Prevalence of *Listeria* in U.S. Produce Packinghouses and Fresh-Cut Facilities

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ABSTRACT

Listeria monocytogenes (LM) contamination of produce can often be traced back to the environment of packinghouses and fresh-cut facilities. Because there is limited information on the detection, prevalence, and distribution of this pathogen in produce operations, environmental "routine sampling" plans for LM and other *Listeria* spp. were developed and implemented in three packinghouses and five fresh-cut facilities in the United States. For routine sampling, a total of 2,014 sponge samples were collected over six to eight separate samplings per operation, performed over 1 year; vector and preproduction samples ($n = 156$) were also collected as needed to follow up on positive findings. In addition, a single "validation sampling" visit by an outside expert was used to evaluate the routine sampling. Among the 2,014 routine sponge samples collected, 35 and 30 were positive for LM and *Listeria* species other than LM (LS), respectively. LM prevalence varied from 0.8 to 5.8% for packinghouses and <0.4 to 1.6% for fresh-cut facilities. Among the 394 validation sponge samples, 23 and 13 were positive for LM and LS, respectively. Validation sampling found statistically significantly higher LM prevalence compared with routine sampling for three of eight operations. For all samples collected, up to eight isolates per sample were characterized by sequencing of *sigB,* which allowed for classification into *sigB* allelic types. Among the 97 samples with more than one *Listeria* isolate characterized, 28 had more than one *sigB* allelic type present, including 18 sponges that were positive for LM and another *Listeria* species and 13 sponges that were positive for more than one LM subtype. This indicates that collection of multiple isolates is necessary to capture *Listeria* diversity present in produce operations. Additionally, 17 of 77 sponges that were positive for LM were positive at only one enrichment time (i.e., 24 or 48 h), indicating that LM testing after two different enrichment times provides enhanced sensitivity.

HIGHLIGHTS

- Specific sampling areas show repeat *Listeria* positives across produce operations.
- Validation sampling can be used to investigate the efficacy of routine sampling.
- Produce operation environmental samples can contain multiple LM subtypes.

Key words: Environmental monitoring; FDA *Bacteriological Analytical Manual*; Food safety; *Listeria*; Produce

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Listeria monocytogenes (LM), a pathogenic bacterium that is found in the natural environment *(18),* has been linked to foodborne disease outbreaks that often have high case-fatality rates *(19)*. Over the past 10 years, LM has been implicated in outbreaks tied to produce, including outbreaks linked to melons, packaged salads, and caramel apples *(3, 8)*. Although produce can be contaminated preharvest by LM, outbreaks are often due to postharvest contamination from the packing and processing operations because LM can survive in facilities over time (e.g., for months and years), typically persisting in harborage points that are not adequately addressed during the cleaning and sanitation process *(6)*. Food Safety Modernization Act rules, as detailed in the Code of Federal Regulations (21 CFR part 117.165 (a)(3)), stipulate that a food facility at risk for an environmental pathogen (such as LM) must perform activities to verify that preventive controls are effectively implemented *(24),* such as pathogen environmental monitoring (PEM). *Listeria*-specific PEM can help food companies that are considered "at risk" (i.e., those that make ready-to-eat foods that support the growth of LM) to seek out these harborage points; this can enable companies to apply corrective actions to mitigate the hazard and prevent contamination of the food. Although the U.S. Food and Drug Administration (FDA) released draft guidance that outlines basic PEM requirements *(25),* that document is not specific to produce. Other resources and recommendations on PEM program development have initially targeted foods that were historically common culprits for LM outbreaks, such as deli meats *(2, 20)*. More recently, university-based extension programs and trade organiza-

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tions such as United Fresh have released produce-specific guidance on how to develop a *Listeria* PEM program *(4, 21–23)*. However, there is still a lack of research to support these guidance documents, as compared with other food sectors. A 2018 scoping review of research and guidance materials related to *Listeria* PEM programs revealed that, of 74 references relating to environmental monitoring, only four were related to the fresh produce sector, compared with 20 and 19 in the meat and dairy sectors, respectively *(28)*. Since then, only a few additional research studies have been completed on *Listeria* in produce packinghouses *(5, 11, 15)*. Given the diversity of the produce sector and the variety of produce operations, there is a need for additional data on *Listeria* in the built environment of produce operations, including information on prevalence, detection, and distribution of LM in produce operations, to improve *Listeria* PEM programs and their implementation. To address this need, *Listeria* environmental monitoring programs were designed, implemented, and evaluated in eight produce operations located across the United States over the course of 1 year. Samples were collected from sites located in zones 2 and 3. Zone 2 sites are those in close proximity to food or food contact surfaces without directly contacting food; zone 3 sites are not directly adjacent to food contact surfaces, but are still located in the processing or packing area *(25)*. Zone 1 sites (food contact surfaces) were not sampled. This article provides the baseline prevalence and distribution of *Listeria* within these operations and details potential challenges associated with culture-based detection of *Listeria* in produce operations.

MATERIALS AND METHODS

Operation recruitment and characteristics. Eight produce operations were selected for this study: three packinghouses and five fresh-cut facilities. None of these operations had previously been used in published studies by any of the authors. For the purposes of this article, a fresh-cut facility is defined as an operation where produce is processed in some way that physically alters the commodity (e.g., sliced, peeled). In contrast, a packinghouse is defined as an operation where produce is washed and packed, without being physically modified. Note, however, that the packinghouses selected for this study pack product for multiple growers that are under multiple managements; therefore, they are defined and registered as "facilities," as required by the FDA under section 415 of the Federal Food, Drug and Cosmetic (FD&C) Act.

The eight operations included in this study were located in one of four different states on either the East or West Coasts of the United States. Operations were recruited through different methods, such as during workshops or via e-mail. Given the sensitive nature of the research topic, operations could not be chosen randomly and were instead chosen based on (i) their being characterized as a specialty crop operation that specifically handles fruits or vegetables and (ii) their willingness to participate.

Although ranging in size (from 25 to 600 employees), all operations sell >$500,000 worth of produce annually. Four of the operations are seasonal (all three packinghouses and fresh-cut facility G). All operations did environmental sampling before the start of the project period, including, at a minimum, ATP testing and *Listeria* testing of environmental samples; testing was performed either by a third party or by using an in-house rapid method, or both.

Sample collection. A member of the project team visited each operation once at the start of the project period to create a master site list specific to the operation in collaboration with one or more representatives from the operation. The master site list for each operation ranged from 52 to 109 sites representing either zone 2 or zone 3. This list contained all possible sites that could be selected for sampling during a given sampling date; for each sampling date a specific "sampling site list" was created, which included the subset of sites that were used for sample collection at that specific date. Master site lists were tailored to each operation due to the variability from operation to operation. In addition to likelihood of contamination, factors such as size of operation, layout, practices, and historical data were considered during site selection. Specific sites were selected if they appeared to be difficult to clean (e.g., drains, sandwich junctures, squeegees), difficult to access (e.g., between, behind, or underneath equipment), or wet (e.g., from flume overflow or condensation); if they had a buildup of organic debris (e.g., cull collection points and catch pans); or if they were a high risk to product (e.g., zone 2 equipment frames). Practices such as presence of moveable equipment (e.g., maintenance carts, forklifts) and traffic patterns of people and product were also considered in site selection. During the first visit, an initial sampling was conducted on a subset of sites included in the master site list; this opportunity was used to demonstrate proper aseptic sampling techniques for operation representatives who were less familiar with sampling using sponges. The amount of surface area swabbed on a given site varied depending on the site itself, and therefore, training did not specify a predefined site area size. Sample collection was performed with individually packaged sponges hydrated with 10 mL of Dey-Engley neutralizing buffer, with two decontaminated gloves per sponge (3M, St. Paul, MN). Regardless of the operation size, at least 40 sponge samples were collected during the initial visit. Approximately every 2 months, each operation was sent a cooler containing the materials for sampling, including the sponges, ice packs, and a return label. Operation representatives would prefreeze the ice packs before packing them back in the cooler along with the collected samples and sending them to the project team's research laboratory via overnight shipping. Representatives were also provided with a sampling site list, which contained approximately 40 sampling sites from the master site list, preselected by the project team for sampling. The sampling site lists rotated through the sites on the master site list. However, sites that returned positive results were included on the next round's sampling list.

On the provided sampling site list, sample collectors were able to record (i) the date and time of sampling (instructions provided by the project team called for collection at least 3 to 4 h into production), (ii) if a site was missing and why, and (iii) any additional sites sampled; sample collectors were instructed to collect additional samples if high-risk sites for *Listeria* harborage or presence were identified during sampling (e.g., pooled water, equipment newly identified as showing potential unhygienic design features). Samples were processed within 24 to 48 h, with the exception of one set, whose arrival was delayed due to weather, which could not be processed for 72 h. In each operation, samples were collected at least six times. These sample collection events will be referred to as routine sampling "rounds."

If a site was found to be positive during a routine sampling round and the operation asked for assistance with follow-up, additional sponges were supplied for "vector" swabbing, which

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targeted sites located in the immediate area of the positive site or sites that may have led to the contamination. Vector sites were chosen at the discretion of the operation representatives, based on consultation with the project team. If, during an operation's given sampling round, >10% of sponge samples were positive for *Listeria,* or if a site was positive during two consecutive sampling rounds, then additional samples were collected after cleaning but before the start of production (i.e., preproduction).

Throughout the duration of the project, communication with each operation occurred between one member of the project team and one representative of each operation (typically the person responsible for food safety). Presumptive positives were communicated via phone following processing of samples from each sampling round. As requested, the project team also provided help with result interpretation as well as individualized support on corrective actions and *Listeria* control strategies; commonly discussed control strategies included intensive and modified cleaning and sanitation protocols, identification and removal of equipment, maintenance procedures, repairs (e.g., of floor cracks), color coding of equipment, and traffic patterns. Assessment of corrective actions and their effectiveness was, however, considered to be beyond the scope of the project due to the complexities of having to ensure consistent implementation and to ensure that a given intervention, and not other concomitant factors and changes, affected LM and LS detection. Therefore, individual operations were not asked to report back corrective actions.

Sample processing. Samples were tested using the FDA BAM method, as detailed in chapter 10 of the *Bacteriological Analytic Manual (9)*. Briefly, sponges were added to 90 mL of buffered Listeria enrichment broth (BD, VWR International, Radnor, PA), followed by stomaching for 30 s at 230 rpm using a Stomacher 400 Circulator (Seward, Basingstoke, UK). Enrichments were then incubated at 30°C for 4 h before they were briefly removed for the addition of 360 μL of *Listeria* selective enrichment supplement (Thermo Scientific Oxoid, Fisher Scientific, Hampton, NH) to each sample. The samples were returned to 30°C to resume incubation. Two plating media were used to test enrichments: (i) modified Oxford agar (MOX; Difco Oxford Medium, BD, VWR; Thermo Scientific Oxoid Oxford Listeria selective supplement, Fisher Scientific) and (ii) Listeria monocytogenes plating media (LMPM; Listeria monocytogenes Chromogenic detection system, R&F Products, Downers Grove, IL). On MOX, *Listeria* species colonies, including LM, show a dark color and the medium surrounding the colony also turns black. LMPM turns LM and *Listeria ivanovii* colonies blue, sometimes with a white halo, whereas other *Listeria* species appear white. After both 24 and 48 h of incubation of the original enrichment, 50 μL of each enrichment was streaked onto both MOX and LMPM media, ultimately resulting in four plates per sample (one 24-h MOX, one 24-h LMPM, one 48-h MOX, one 48-h LMPM). The streaking technique used the whole plate, split into three sections: the first section, a lawn, took up 50% of the plate; the second section was streaked out from the first section using a new loop and constituted another 25% of the plate; and for the third section, the other 25% of the plate, the same loop (flipped over) was used. MOX plates were incubated at 30°C for 48 h, whereas LMPM plates were incubated at 35°C for 48 h.

Isolate characterization. For each plate, up to two presumptive-positive colonies (i.e., colonies with color and morphology consistent with *Listeria* on LMPM or MOX) were substreaked onto brain heart infusion agar (VWR) followed by incubation at 37°C for 24 h. Therefore, up to eight isolates could be collected per sponge. For LMPM plates, only blue colonies were selected for isolation (i.e., if only white colonies were present, no colonies were selected from LMPM plates). PCR amplification of a 780-bp fragment of the *sigB* gene was used for confirmation of each presumptive isolate. PCR was conducted using an enzymatic hot start methodology (see https://github.com/CCCeliaLiao/sigBdata), using AmpliTaq Gold DNA Polymerase with GeneAmp 10X Gold Buffer and $MgCl_2$ (Thermo Fisher Scientific, Waltham, MA). PCR products were purified using 5 units of exonuclease I and 1 unit of shrimp alkaline phosphatase (both from Thermo Fisher Scientific). The mixture was heated in a thermocycler at 37°C for 45 min and then at 80°C for 15 min. Purified PCR products were submitted to the Biotechnology Resource Center at Cornell University for Sanger sequencing of the 780-bp *sigB* gene fragment. The sequences were then trimmed for analysis of the 660-bp internal fragment to determine the *sigB* allelic type (AT) for each isolate. The *sigB* gene was used for initial subtyping and characterization due to its genetic stability and accuracy in species and lineage classifications, as previously detailed *(16)*. Sequences were edited and assembled using Sequencher sequence analysis software (version 5.4.6, Gene Codes Corporation, Ann Arbor, MI) and were analyzed using MEGA7 *(13)*. The assignment of ATs to each sequence was performed as previously described, using a *sigB* database available on Food Microbe Tracker (http://www.foodmicrobetracker.com) *(16, 26)*. All *Listeria* isolates were preserved at –80°C in 15% glycerol.

Validation of environmental monitoring programs. At the end of the year 1 sampling period, each operation was revisited for a final sampling to validate the routine monitoring program. Samples were collected by an outside "expert"; experts were defined as individuals that had training and experience with *Listeria* environmental sampling using sponges. These experts were not part of the project team or associated with the operation; they had not seen the operation previously and were selected based on convenient availability. The experts used the same materials and methods for sample collection as described for the routine sampling. For site selection, each expert was given a list of the routine sites (i.e., the sites on the master site list) and historical results (i.e., which sites had previously tested positive for *Listeria*), although they were also allowed to collect samples from other sites. The results from the validation sampling were compared against the routine sampling. The experts were asked to collect at least 40 sponge samples, although they were provided with up to 100 sponges and were encouraged to collect more than 40 sponges based on their evaluation of the operation. Overall, 40 to 66 samples were collected for each operation's validation sampling.

Statistics. A nested generalized linear model using R Studio (version 1.2.1335) was used to determine whether there was a statistically significant difference between the number of *Listeria*-positive samples from zone 2 versus zone 3, with Result∼Operation/Zone and following a binomial distribution. Similarly, to determine whether there was a statistically significant difference in LM positives between the routine and validation sampling, a nested generalized linear model was fit to the data with Result∼Operation/Treatment following a binomial distribution via logistic link function. Treatment was used in the analysis to represent either the "routine" or "validation" sampling categories. The family binomial was used because the result was either 1 or 0, depending on whether or not LM was detected in the sample. A Fisher's exact test was used to determine if fresh-cut facilities

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TABLE 1. *Number of sponge samples collected from produce operations during routine and validation sampling, by zone*

	No. of samples (% of total swabs per operation and sampling purpose)			
	Routine[a]		Validation[b]	
Operation	Zone 2	Zone 3	Zone 2	Zone 3
A	52 (30.4)	119 (69.6)	14 (25.5)	41 (74.5)
B	158 (62.7)	94 (37.3)	14 (31.8)	30 (68.2)
C	100 (46.9)	113 (53.1)	22 (33.3)	44 (66.7)
D	99 (40.2)	147 (59.8)	23 (46.0)	27 (54.0)
E	116 (35.7)	209 (64.3)	15 (37.5)	25 (62.5)
F	132 (41.8)	184 (58.2)	28 (52.8)	25 (47.2)
G	82 (32.9)	167 (67.1)	12 (27.3)	32 (72.7)
H	80 (33.1)	162 (66.9)	12 (28.6)	30 (71.4)
Total	819 (40.7)	1,195 (59.3)	140 (35.5)	254 (64.5)

[a] Does not include sponge samples that were collected during validation sampling, vector sampling, or preproduction.
[b] Represents sponge samples collected during production at the end of the project year, from sites chosen by an outside expert. The expert was provided with the master site list and historical sampling results.

were significantly more likely to have sponge samples with more than one AT compared with packinghouses. McNemar's test was used to determine if there was a statistically significant difference between the number of positive samples detected at the 24- or 48-h enrichment times.

RESULTS

Prevalence of *L. monocytogenes* and other *Listeria* species varied across different produce packinghouses and fresh-cut operations. Over the course of 1 year, between 171 and 325 routine sponge samples were collected from each of the three packinghouses and the five fresh-cut facilities, for a total of 2,014 routine sponge samples collected. Among these 2,014 samples, 819 (41%) were from zone 2, whereas the remaining 1,195 (59%) were from zone 3 (Table 1). LM prevalence for the routine sponge samples collected in the packinghouses ranged from 2 (0.8%) of 252 to 10 (5.8%) of 171, whereas LS prevalence (*Listeria* species excluding LM) ranged from 5 (2.0%) of 252 to 8 (4.7%) of 171. LM prevalence for the fresh-cut facilities ranged from 0 ($<$0.4%) of 249 to 4 (1.6%) of 246, whereas LS prevalence ranged from 0 ($<$0.4%) of 249 to 5 (1.5%) of 325 (Table 2). For these calculations, instances in which both LM and LS were detected in the same sponge were counted in both the LM and LS prevalence calculations.

Among the 819 sponges collected from zone 2, 1.1 and 1.0% were positive for LM and LS, respectively. By comparison, 2.2 and 1.9% of zone 3 sponges were positive for LM and LS, respectively. Neither LM nor LS prevalence differed significantly between zones 2 and 3 ($P > 0.05$, using a generalized linear model).

All three packinghouses and two of the fresh-cut facilities had instances in which LM or LS was found at a given site during more than one sampling round (Table 3). A total of 21 sites across five operations showed repeat positives; only 3 of these 21 sites represented zone 2, all other sites with repeat positives represented zone 3. For 15 of 21 sites, positive samples represented a mixture of LM- and LS-positive sponges, including sponges that were positive for both LM and LS as well as sponges from different collection dates that were positive for LM or LS (Table 3). Sample sites with repeat positives in more than one operation included drains, forklifts, and bin dumper areas (Table 3). For example, two operations had repeat positives for sponges collected from forklifts, with two additional operations showing repeat positives on sites designated as "forklift stop by bin dumper" (bin dumper refers to the equipment that is used to transfer product from a large container onto a washing or processing line). Overall, sampling sites close to "bin dumping" or "bin-in feed" areas showed repeat positives in five operations (Table 3). In addition, sampling sites representing drains showed repeat positives in three operations, with three drain-related sites showing repeat positives in packinghouse A. Whereas all isolates were characterized by *sigB* allelic typing, the data are consistent with findings from previous studies *(1, 5, 16)* that *sigB* sequencing alone has limited discriminatory power and is typically insufficient for establishment of persistent contamination, as supported by the fact that LM with *sigB* AT57 was found in all six operations that had LM positives. However, for two sites ("square drain 1" in packinghouse A and "rot bin under rollers" in packinghouse C; see Table 3), isolates with identical *sigB* ATs were obtained on three separate sampling dates; these sites had high priority for follow-up, including follow-up higher resolution subtyping.

For the three packinghouses, the number of sites with repeat positives ranged from three to eight, whereas the five fresh-cut facilities showed zero to two sites with repeat positives (three of five fresh-cut facilities had no repeat positives sites). Hence, for all three packinghouses, additional preproduction sampling was performed to further assess the effectiveness of cleaning and sanitation procedures. In addition, a substantial number of vector samples were collected in all three packinghouses. Overall, both vector and preproduction sampling showed higher LM and LS prevalence than routine sampling; for example, routine

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TABLE 2. *Percentage of sponges that were positive for* Listeria *for the different produce operations*

	Routine[a]			Validation[b]			Vector (not including preproduction vectors)[c]			Preproduction follow-up swabs (including preproduction vector)[d]			All (including validation)[e]		
Operation	LM (%)[f]	LS (%)[f]	No. of samples collected	LM (%)	LS (%)	No. of samples collected	LM (%)	LS (%)	No. of samples collected	LM (%)	LS (%)	No. of samples collected	LM (%)	LS (%)	No. of samples collected
Packinghouse															
A	5.8	4.7	171	1.8	1.8	55	10.3	7.7	39	25.6	12.8	39	8.2	5.6	304
B	0.8	2.0	252	18.2	9.1	44	3.4	3.4	29	<16.7	16.7	6	3.3	3.3	331
C	5.6	4.2	213	15.2	9.1	66	14.3	9.5	21	7.1	7.1	14	8.3	5.7	314
Fresh-cut facility															
D	1.6	0.8	246	6	2	50	—	—	0	—	—	0	2.4	1.0	296
E	1.2	1.5	325	<2.5	<2.5	40	—	—	0	—	—	0	1.1	1.4	365
F	0.9	0.3	316	1.9	1.9	53	<12.5	<12.5	8	—	—	0	1.1	0.5	377
G	<0.4	<0.4	249	<2.3	<2.3	44	—	—	0	—	—	0	<0.3	<0.3	293
H	<0.4	<0.4	242	<2.4	<2.4	42	—	—	0	—	—	0	<0.4	<0.4	284
All operations	1.7	1.5	2,014	5.8	3.3	394	8.2	6.2	97	18.6	11.9	59	3.0	2.2	2,564

[a] Represents sponge samples that were from the master site list and collected during production (i.e., not collected during the validation sampling, vector sampling, or preproduction).
[b] Represents sponge samples collected by an outside expert during production at the end of the project year; the expert was provided with the original master site list and historical sampling results and was allowed to select any sites for sample collection (including sites not on the original master site list).
[c] Represents vector sponge samples collected during production from sites that were chosen due to a previous round's positive sampling result, including surrounding areas (i.e., the so-called vectors). Vector swabbing results are not shown for operations that elected to perform vector swabbing internally.
[d] Represents sponge samples collected prior to the start of production, collected only in situations in which multiple repeat positive results had been detected from routine and vector swabbing.
[e] Represents any sponge samples collected in the operation, specifically for the project (i.e., routine, validation, vector, preproduction).
[f] Percentage of sponges that were positive for LM (refers only to the species *L. monocytogenes*) or LS (refers to all species of *Listeria,* excluding *L. monocytogenes*); samples with co-isolation of *L. monocytogenes* and another species of *Listeria* were counted in both the LM and LS columns. In instances in which no sponges were positive, the limit of detection is provided (i.e., $1/n \times 100$). —, sponges were not collected in the given category.

sampling showed a prevalence of 1.7% for LM, whereas vector and preproduction sampling showed an LM prevalence of 8.2 and 18.6%, respectively (see Table 2). While all three packinghouses had sites that tested positive for *Listeria* preproduction, in packinghouse B, only one preproduction sample was positive for LS, and in packinghouse C, one site each was positive for LM and LS. However, for packinghouse A, 10 (25.6%) of 39 preproduction sponges were positive for LM; 8 of these 10 LM-positive sponges were collected after cleaning, but before sanitizing. The positive sites included drains, which did not appear easily cleanable. Interestingly, sampling results for the three packinghouses showed a trend toward fewer positives among the sites with repeat LM or LS findings toward the second half of the sampling year (i.e., the end of the packing season). For example, for packinghouse A, the seven sites with repeated positives had a cumulative 14 of 19 LM or LS positives over the first three routine samplings, but only 4 of 20 LM or LS positives over the last three routine samplings. Note that, during the packing season, a number of changes can occur; possible corrective actions may be taken following repeated positives at a given site, and there may also be changes in product sources (e.g., direct receipt from field harvest in the fall versus receipt from long-term storage facilities later in the season).

Validation sampling *Listeria* prevalence was either higher or lower than routine sampling prevalence. To assess the validity of routine sampling protocols and strategies implemented in the eight operations participating in this study, in-depth validation sampling was performed in each operation. A total of 394 validation sponge samples were collected across these operations (40 to 66 samples per operation; Table 2) with 140 (36%) and 254 (65%) sponges collected from zones 2 and 3, respectively (Table 1). Overall, 23 (5.8%) of 394 and 13 (3.3%) of 394 validation sponges were positive for LM and LS, respectively; 7 (5.0%) of 140 and 16 (6.3%) of 254 zone 2 and 3 samples were LM positive, respectively; and 1 (0.71%) of 140 and 12 (4.7%) of 254 zone 2 and 3 samples were LS positive, respectively. Four operations showed numerically higher LM prevalence during the validation sampling compared with the routine sampling: packinghouses B and C, and fresh-cut facilities D and F (Table 2). Packinghouses B and C, as well as fresh-cut facility D, all had a statistically significant ($P < 0.05$)

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TABLE 3. *Sites with repeat isolation of* Listeria *from sponge samples collected from produce operations over 1 year*[a]

Zone	Site description[b]	Visit								
		1	2	3	4	5	6	7	8	Validation
Packinghouse A										
3	Forklift stop by bin dumper	Neg	LS	LM	Neg	Neg	Neg	NA	NA	Neg
3	Square drain 1	LM/LS	LM/LS	LM/LS	LM	Neg	LS	NA	NA	—
3	Square drain 2	LM/LS	Neg	LM	Neg	Neg	Neg	NA	NA	Neg
2	Catch pan underneath brush bed	LM	LS	Neg	Neg	Neg	Neg	NA	NA	Neg
3	Square drain 3	LM	Neg	LM	Neg	Neg	Neg	NA	NA	—
3	Outlet of PVC pipe from catch pan	LM	LM	LM/LS	Neg	Neg	Neg	NA	NA	Neg
3	Metal edge of dock	Neg	—	—	—	LS	LM/LS	NA	NA	LM
Packinghouse B										
3	Floor under bin in-feed	—	—	LM	LS	Neg	Neg	Neg	NA	Neg
3	Floor under bin out-feed	LS	Neg	LM	Neg	Neg	Neg	Neg	NA	Neg
3	Forklift	LS	Neg	Neg	Neg	Neg	—	Neg	NA	LS
Packinghouse C										
3	Floor crack in hallway	Neg	—	—	LM	Neg	Neg	NA	NA	LM
3	Forklift stop by bin dumper	LM	LM	LM	Neg	Neg	Neg	NA	NA	LM
3	Drain under bin dumper	Neg	—	—	LS	—	Neg	NA	NA	LM/LS
2	Fan over brush bed	LM	LS	Neg	Neg	Neg	Neg	NA	NA	Neg
2	Support by waxer	—	LM	Neg	Neg	LS	Neg	NA	NA	Neg
3	Rot bin under rollers	LM	LM	LS	Neg	LS	LM	NA	NA	LM/LS
3	Trench drain under waxer	—	Neg	LS	LS	Neg	Neg	NA	NA	Neg
3	Tracks for bin filler car	LS	LM	Neg	Neg	Neg	Neg	NA	NA	Neg
Fresh-cut facility E										
3	Floor underneath bin dumper	Neg	—	Neg	Neg	—	LM	LS	NA	Neg
3	Trench drain in front of flumes	Neg	LM	Neg	Neg	Neg	Neg	LM	NA	—
Fresh-cut facility F										
3	Forklift by bin dumper	—	Neg	—	Neg	Neg	Neg	LM	Neg	LS

[a] Neg, site was tested, but was negative for *Listeria* species, including *Listeria monocytogenes*; LS, site was positive for *Listeria* species, not including *L. monocytogenes*; LM, site was positive for *L. monocytogenes*; NA, not applicable (indicates the operation did not have that sampling round, i.e., not all operations were sampled the same number of times); LM/LS, site was positive for *L. monocytogenes* and at least one other *Listeria* species; —, site was not tested.
[b] Sites listed only include sites listed on master site list (i.e., vector sites with repeat positives not included).

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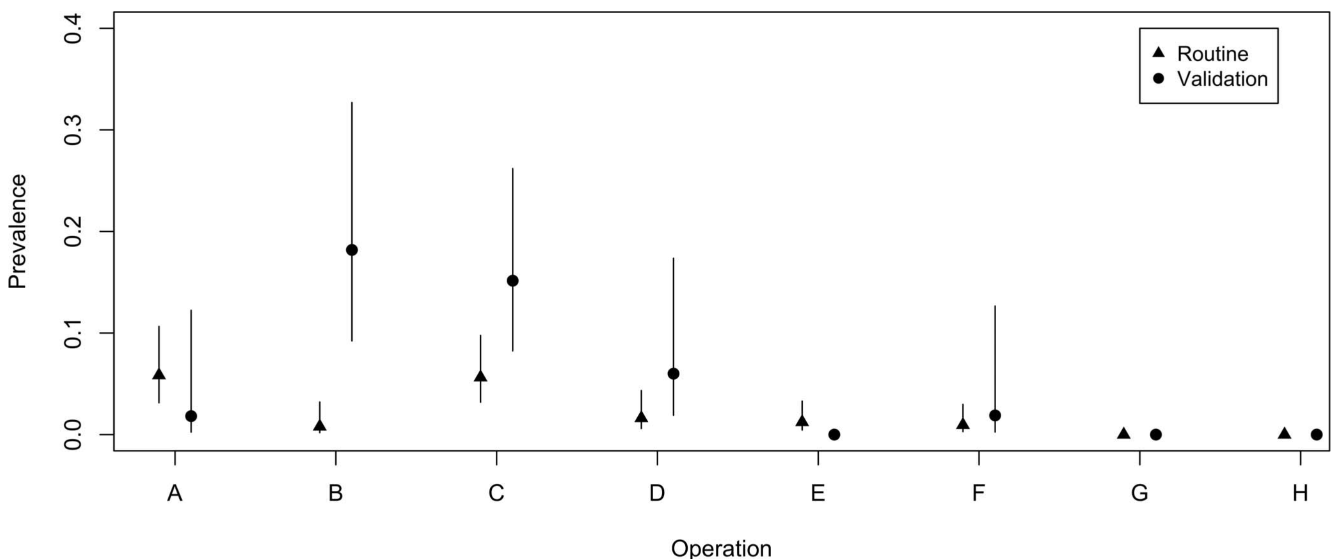

FIGURE 1. *Routine and validation prevalence of* L. monocytogenes, *with 95% confidence intervals derived from the nested generalized linear model for eight produce operations: three packinghouses (A, B, and C) and five fresh-cut facilities (D, E, F, G, and H). Prevalence is the probability of a sample testing positive for* L. monocytogenes.

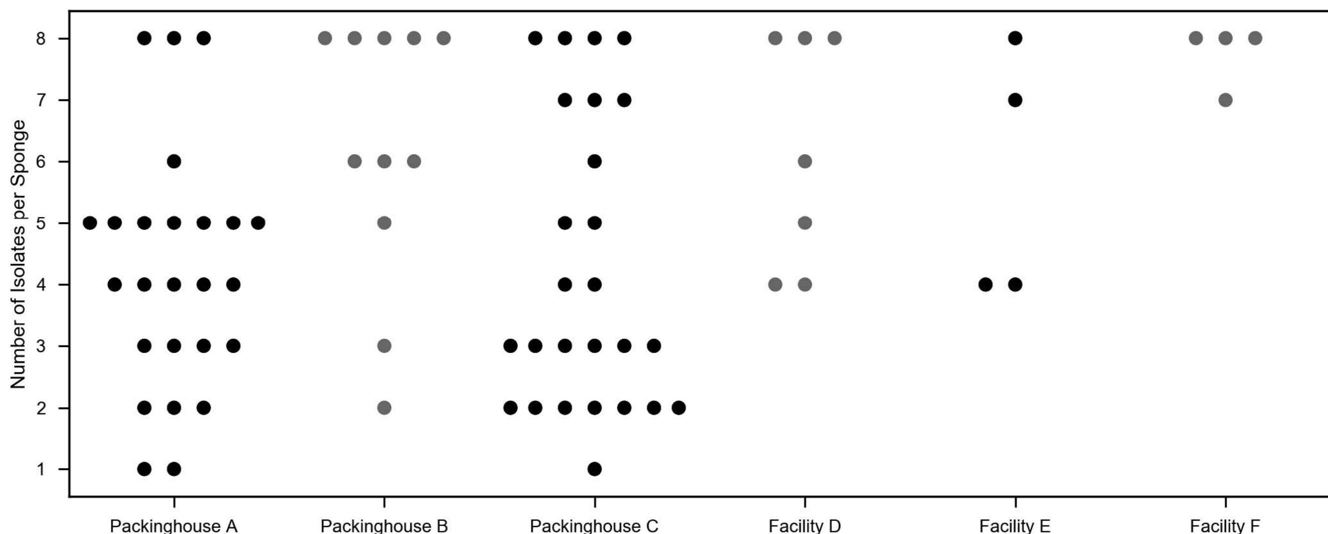

FIGURE 2. *Distribution of the number of* Listeria *colonies that could be recovered for LM-positive sponge samples, from produce packinghouses and fresh-cut facilities. The figure reports the number of colonies that were isolated for a given sponge, including* Listeria *species, given* L. monocytogenes *was present; colonies were not collected from LMPM plates in instances in which only LS was present. Each circle represents one sponge (*n = 77*). Up to eight colonies were isolated collectively from the four plate media (24- and 48-h MOX, and 24- and 48-h LMPM) required for testing each sponge (up to two colonies per plate).*

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difference between the routine and validation prevalence (Fig. 1). Two operations (packinghouse A and fresh-cut facility E) had numerically lower LM prevalence (not statistically significant) in the validation sampling compared with the routine sampling. For fresh-cut facilities G and H, no *Listeria*-positive samples were found throughout the duration of the project, including the validation sampling.

A substantial proportion of positive sponge samples had more than one type of *Listeria*. Over the project duration, a total of 2,564 sponge samples were collected and processed; this number includes all sponges from routine, validation, vector, and preproduction swabbing. Of these 2,564 sponges, 115 (4.5%) were positive for *Listeria* species (including LM), with 59 positive for only LM, 38 positive only for LS (non-LM *Listeria* species), and 18 positive for both LM and LS. From these 115 sponges, 461 *Listeria* isolates were collected and sequenced to determine the *sigB* AT, which also was used to classify isolates into species. Overall, 40 unique ATs were detected, which included 15 *L. seeligeri* ATs, 4 *L. welshimeri* ATs, 10 *L. innocua* ATs, and 11 *L. monocytogenes* ATs. The *sigB* AT data were also used to identify instances in which isolates from a given sponge included multiple distinct subtypes (defined here as isolates with different *sigB* ATs). If multiple isolates from the same sample showed the same AT, only one isolate was designated as representative and used to calculate the species distribution among sponge samples. Based on 152 isolates classified as representative, isolates were classified as LM (92 of 152, 60.5%), *L. seeligeri* (39 of 152, 25.7%), *L. innocua* (14 of 152, 9.2%), and *L. welshimeri* (7 of 152, 4.6%).

For each sponge sample, enrichments were tested using four agar plates (i.e., 24-h MOX, 24-h LMPM, 48-h MOX, 48-h LMPM); up to two presumptive *Listeria* isolates were collected from each MOX plate and up to two presumptive LM isolates from each LMPM plate. Therefore, if a sponge had LM present, up to eight isolates could be collected for a given sponge sample, whereas up to four isolates could be collected for a sponge that tested positive for only LS (non-LM *Listeria* spp.). However, not all eight isolates (or all four, in cases of sponges positive for only LS) could be collected for a given sponge, because not all sponges that were LM or LS positive had two isolates present on all four plates. This occurred in instances in which the sample was only positive at one time point or in which background microbiota interfered with isolation. Only 19 (24.6%) of 77 of the LM-positive sponge samples had the maximum of eight isolates collected (Fig. 2). For the sponges positive for only LS, 6 (15.8%) of 38 had the maximum of four isolates collected. For the 115 sponges that were positive for LM, LS, or both, 18 sponges (15.7%) had only one *Listeria* isolate recovered. Additionally, for three samples, LM was detected on MOX, but not on LMPM; this supports the idea that use of both media can enhance recovery of LM over use of LMPM only.

Of the 97 sponge samples that had more than one isolate collected, 28 (29%) had more than one *sigB* AT present. Of these 28 sponge samples, 20 had two ATs present, seven had three ATs present, and one had four ATs present. For the three fresh-cut facilities, 17 to 25% of samples with more than one isolate had more than one AT. In contrast, for the three packinghouses, 11 to 54% of samples with more than one isolate had more than one AT (Table 4). Importantly, 13 (13%) of 97 sponge samples that had more than one isolate collected had more than one LM *sigB* AT present (Table 4), with nearly all of those samples (12 of 13) being from packinghouses. Overall, there was no significant difference ($P > 0.05$) in the number of sponge samples with more than one *sigB* AT between fresh-cut facilities (4 of 20, 20%) and packinghouses (24 of 77, 31%).

TABLE 4. *Number of samples with more than one allelic type present, given more than one* Listeria *colony was isolated*[a]

	Packinghouse				Fresh-cut facility				
	A ($n = 24$)	B ($n = 19$)	C ($n = 34$)	All packinghouses ($n = 77$)	D ($n = 8$)	E ($n = 6$)	F ($n = 6$)	All fresh-cut facilities ($n = 20$)[b]	All operations ($n = 97$)
No. of sponges with >1 AT/sponge (%)	13 (54)	2 (11)	9 (27)	24 (31)	2 (25)	1 (17)	1 (17)	4 (20)	28 (29)
No. of sponges with multiple LM ATs (%)	7 (29)	1 (5.3)	4 (12)	12 (16)	0 (0.0)	0 (0.0)	1 (17)	1 (5.0)	13 (13)
No. of sponges with multiple LS ATs (%)	1 (4.2)	1 (5.3)	1 (2.9)	3 (3.9)	0 (0.0)	1 (17)	0 (0.0)	1 (5.0)	4 (4.1)
No. of sponges with ≥1 LS AT and ≥1 LM AT (%)	5 (21)	0 (0.0)	4 (12)	9 (12)	2 (25)	0 (0.0)	0 (0.0)	2 (10)	11 (11)

[a] Throughout the table, *n* indicates the number of sponges that had more than one *Listeria* colony isolated; this number is the denominator for the % values shown in the subsequent rows. AT, *sigB* allelic type; LM, *Listeria monocytogenes*; LS, *Listeria* species, not including *L. monocytogenes*.
[b] Fresh-cut facilities G and H had zero *Listeria* positives and, therefore, are not included in this table.

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Enrichment time affects ability to detect *L. monocytogenes*. Among the 115 *Listeria*-positive sponges, 9 were positive for *Listeria* spp. (LM or LS) only after 24 h of enrichment (i.e., not positive at 48 h) and 27 were positive for *Listeria* spp. only after 48 h of enrichment (i.e., not positive at 24 h). Therefore, 36 (31.3%) of 115 sponge samples that were positive for *Listeria* spp. were only positive at one enrichment time (Table 5). There was a significant difference between the two enrichment times ($P < 0.05$, McNemar's test). Of the 77 sponges that were positive for LM, 5 were positive for LM at only 24 h, whereas 12 were positive for LM at only 48 h, resulting in 17 (22.1%) that were only positive for LM at one enrichment time; the number of positives found at each enrichment time was not significantly different.

DISCUSSION

A 1-year longitudinal study that involved development, implementation, and evaluation of environmental monitoring programs in eight produce operations was used to characterize *Listeria* prevalence and contamination patterns in packinghouses and fresh-cut facilities and to assess possible impacts of different culture-based testing approaches on *Listeria* detection and characterization. The data generated indicate that (i) implementation of standardized sampling schemes can provide baseline *Listeria* prevalence data and help identify key sampling sites that should be targeted for improved *Listeria* control in produce operations, (ii) validation sampling can be used to assess routine sampling and confirm or refute existence of robust *Listeria* control strategies, and (iii) appropriate culture-based detection approaches are essential for *Listeria* PEM programs that provide for accurate assessment of *Listeria* presence and diversity.

TABLE 5. *Number of sponges that were positive for* Listeria *at various enrichment times*[a]

Sponges detected positive for:	No. (%) of sponges
LM	
Only after 24 h of enrichment[b]	5 (6.5)
Only after 48 h of enrichment[b]	12 (15.6)
Only at one enrichment time (either 24 or 48 h)	17 (22.1)
At either or both enrichment times	77 (100)
Listeria spp. (including LM)	
Only after 24 h of enrichment	9 (7.8)
Only after 48 h of enrichment	27 (23.5)
Only at one enrichment time (either 24 or 48 h)	36 (31.3)
At either or both enrichment times	115 (100)

[a] LM, *Listeria monocytogenes*; *Listeria* spp., *Listeria* species, including *L. monocytogenes*.
[b] Non-LM *Listeria* species may have been present at the other enrichment time.

Implementation of standardized sampling schemes can provide baseline *Listeria* prevalence data and help identify key sampling sites that should be targeted for improved *Listeria* control in produce operations. Whereas a number of PEM guidance documents *(21, 25)* discuss testing for *Listeria* spp. (which would detect both LM and LS with no indication as to which of these organism groups is present in a given sample), the study reported here tested for both LM and LS. This approach provides for additional information that can be used to elucidate LM and LS detection and contamination patterns across operations; these data also may be valuable for regions or operations where PEM programs may target LM. LM was found in six of eight of the produce operations participating in this study, with an average LM prevalence of 1.7%, ranging from <0.4 to 5.8% among operations. More specifically, there was a lower overall prevalence among the five fresh-cut facilities, compared with the three packinghouses. One cannot necessarily extrapolate from these findings to all packinghouses, because packinghouses and fresh-cut facilities in the study reported here differ in many characteristics, including, possibly, implementation of corrective actions. However, the data suggest that continued efforts to control *Listeria* in packinghouses are important. Overall, the

prevalences of *Listeria* spp. and LM found in the study reported here are lower than or similar to those found in other studies, acknowledging that there are complexities in formal comparison between studies (e.g., variation in implementation of corrective actions, variation in operation age). For example, in 2014, Leong et al. *(14)* reported an LM prevalence of 9.4% in finished product and environmental samples collected in six fresh-cut vegetables facilities in Ireland *(14)*. In a 2017 study, Leong et al. *(15)* reported an average LM prevalence of 9.5% among 474 environmental samples collected in five small vegetable processing facilities. Based on 1,588 environmental samples collected at 11 East Coast U.S. packinghouses, Estrada et al. *(5)* reported an average LM prevalence of 3.8%, with a range of 0.0 to 11.4%. Jorgensen *(11)* reported an average LM prevalence of 4.3%, based on 350 environmental samples collected from seven packinghouses in the northwest United States. The study reported here showed that none of the sponges collected in two fresh-cut facilities tested positive for LM (or any *Listeria* spp.); this is consistent with Leong et al. *(15),* Estrada et al. *(5),* and Jorgensen *(11),* whose studies each report at least two participating operations with no detected LM over the course of 1 year or season. Together, and consistent with a large body of studies in processing operations for other commodities (meat, seafood, dairy), these studies suggest that LM can be detected in the majority of produce operations, but with a range of expected LM prevalences based on facility and sampling plan specifics. Importantly, the combined data suggest that some produce operations have implemented *Listeria* control practices that are effective in assuring consistently low LM and LS prevalence.

In some regions, particularly North America, *Listeria* spp. testing is used as part of PEM programs; therefore, it is important to understand the relationship between LM presence and LS presence in environmental samples. For the operations sampled in the study reported here, 77 (67%) of 115 samples positive for *Listeria* spp. also tested positive for LM. This was consistent with Estrada et al. *(5),* who found that, of their 102 *Listeria*-positive samples from produce packinghouse environments, 60 (59%) were positive for LM. Jorgensen *(11)* found a similar ratio; 15 (63%) of 24 *Listeria*-positive sponges were positive for LM. By comparison, an article that summarized testing results obtained for ~18,000 environmental samples collected from 12 ready-to-eat meat and poultry plants reported that 876 (42%) of the 2,101 *Listeria*-positive samples tested positive for LM *(20)*. Importantly, a number of studies *(10, 12, 27)* suggest the relationship between LM and LS detection in environmental samples differs considerably by facility; for example, Tompkin *(20)* found that the percentage of samples positive for *Listeria* spp. that also tested positive for LM ranged from 5 to 96%, depending on the facility from which samples were collected. Overall, these studies further support that there is considerable likelihood (likely >50% across operations) that *Listeria* spp.–positive samples collected in produce operations are positive for LM. This also further supports the current industry practice *(21)* that requires detection of any *Listeria* spp.–positive sample as part of PEM programs to trigger corrective actions of an intensity that assumes that the sample is positive for LM *(17)*. However, even if an environmental sample is confirmed to be LS positive but LM negative, equally stringent corrective actions still need to be implemented because non-LM *Listeria* often behaves similarly to LM and, therefore, can be a good indicator of where LM may be able to survive and grow (i.e., harborage points) *(17)*.

Whereas the data from this study found a numerically higher prevalence of LM and LS in zone 3 sites compared with zone 2 sites, the difference was not significant ($P > 0.05$). By comparison, Beno et al. *(1)* found that zone 2 sites in cheese processing plants were significantly less likely to be positive for LM than zone 3 sites, consistent with other prior studies, as discussed by Beno et al. *(1)*. The study reported here found that a considerably larger number of zone 3 sites (as compared with zone 2 sites) show repeat isolation of LM or LS. More specifically, zone 3 sites found near the bin dumper area, including drains, forklifts, and forklift stops, were found to be repeatedly positive in multiple operations. This provides important insights into both high-risk areas for LM presence as well as sampling sites that should be included in *Listeria* PEM lists. The sites identified in the study reported here are also consistent with previous studies, including Estrada et al. *(5),* who reported that drain sites show the highest *Listeria* prevalence in produce operations and who also reported repeat isolation of *Listeria* spp. from one "moveable non-food contact surface." Similarly, Jorgensen *(11)* reported that, in all five produce operations studied that had *Listeria*-positive samples, at least one drain was a positive sample site, and that, in one operation in particular, all production area drains were positive for *Listeria* spp. for all four sampling rounds.

Validation sampling can be used to assess routine sampling and confirm or refute existence of robust *Listeria* control strategies. Validation sampling data generated in the study reported here suggest that four operations had robust routine sampling plans and sampling techniques, because the LM prevalence of the validation sampling was less than or equal to that of the routine prevalence. Importantly, two of these operations yielded no LM or LS positives in both routine and validation sampling, further supporting that these facilities have implemented an effective *Listeria* control program. These were two large facilities, one seasonal and one year-round, that processed a limited number of commodities as compared with some of the other fresh-cut facilities in this study. It would be valuable to identify specific practices that facilitated effective *Listeria* control in these two facilities; however, given the diversity of operation-specific characteristics, formal statistical analysis could not be performed to identify the risk factors that may have contributed to the two operations that had no detected *Listeria*. Future studies should include formal investigations of the effectiveness of corrective actions; this may be challenging because it is difficult to implement corrective actions in a way that

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controls for other concomitant changes (e.g., implementation of improved sanitation practices in some drains with others serving as an untreated control group).

The other four operations in this study showed a numerically higher prevalence during the validation sampling compared with the routine sampling. This suggests that the routine sampling plans for these four operations should be reevaluated, perhaps with additional sites added to the routine site list. Three of these operations had a statistically significant ($P < 0.05$) difference in LM prevalence between the routine sponge samples versus the validation sponge samples collected by the outside expert, with the greatest difference occurring with packinghouse B. One theory for the extreme difference found in packinghouse B is that the master site list for this operation was the only site list that was developed preproduction (i.e., when equipment was not on or running), whereas sites identified during the validation sampling were selected during production. This suggests that site lists should be developed during production, particularly if the operation develops wet areas during production, so that sites may be selected based on worst-case-scenario conditions. The data presented in the study reported here are comparable to those from another study that described routine versus validation sampling in a food facility *(1)*. Beno et al. *(1)* found that, of seven small cheese processing facilities, two facilities had significantly higher prevalence detected during validation sampling, whereas two facilities had a significantly lower prevalence. The ultimate goal of validation sampling is to determine how well the routine sampling may reflect the operation's true LM prevalence and the location of LM, if present. Ideally, an outside expert should find less *Listeria* than the routine monitoring program, because the routine program is designed and executed by personnel familiar with the operation. On the other hand, an outside expert should have more knowledge and experience with sampling and be less biased compared with the operation personnel; this could explain the instances in which the expert found more *Listeria* compared with the routine results. Therefore, the validation sampling results demonstrate the importance of sampling by an outside party, which can increase confidence in results and also identify potentially problematic sampling schemes. It can also serve to periodically validate the efficacy of a routine *Listeria* environmental monitoring program *(1, 28)*. However, a single validation sampling may not necessarily be appropriate to assess routine sampling conducted over a preceding year; discrepancies between routine and validation sampling should be followed up by a more comprehensive sampling process review and, if deemed appropriate, a root cause analysis investigation (e.g., of the routine sampling procedures if the validation sampling provides considerably larger proportion of positive samples).

Appropriate culture-based detection approaches are essential for *Listeria* PEM programs that provide for accurate assessment of *Listeria* presence and diversity. Whereas the data presented in the study reported here clearly indicate the importance of appropriate sampling plans and sample collection procedures for effective *Listeria* PEM programs, there also is a need to continue to evaluate the impact of testing approaches on their ability to detect *Listeria* and recover *Listeria* isolates for further characterization and subtyping, which is often used as part of PEM programs. One aspect of culture-based *Listeria* detection methods, including those detailed in FDA BAM and the International Organization for Standardization (ISO), is that these methods call for testing of enrichments at two time points (i.e., 24 and 48 h), which adds considerable expense to *Listeria* testing. The data presented in the study reported here indicate that 31.3% of the *Listeria*-positive samples were positive at only one enrichment time (24 or 48 h), with 7.8 and 23.5% of samples positive at only 24 and only 48 h, respectively. In another study, Fortes et al. *(7)* found that three and five samples, respectively, were positive only after the 22-h and only the 48-h enrichment when testing with a molecular platform was performed at different enrichment times (compared with 68 samples positive for both enrichment times) *(7)*. By comparison, an evaluation of the ISO 11290-1 method reported that 30.7% of samples were negative with the first enrichment but positive with the second enrichment *(14)*. These data indicate that the classical culture-based methods that plate after two different enrichment times provide enhanced sensitivity and that rapid detection methods that save time by only using a single time point (24 or 48 h) could result in false negatives. Therefore, selection of appropriate, validated detection methods remains essential for PEM programs, particularly for root cause analyses. The findings not only support the requirement to test enrichments at two time points, as detailed in BAM and ISO culture methods, but also suggest that under some circumstances testing at two time points may be appropriate for molecular methods, which typically only involve testing of a single enrichment time point.

Another concern with detection methods used to test PEM samples is the ability of a given protocol to recover the diversity of *Listeria* species and subtypes found in a given sample. This is a particular concern if (i) culture-based methods without differential plating media for LM are used or (ii) isolates are used for subsequent subtyping. Therefore, the data generated in the study reported here were used to evaluate the potential species and subtype diversity found within a given sponge sample. Among all 2,564 sponge samples collected, 28 (29%) of 97 samples with more than one *Listeria* isolate characterized had more than one *sigB* AT present. Among the 77 sponge samples that were positive for LM, 18 (23%) had an additional *Listeria* species detected and 13 samples had at least two LM *sigB* ATs detected. This is comparable to results from a previous study that investigated produce packinghouses and showed that 21 (21%) of 102 *Listeria*-positive samples contained two or more different ATs *(5)*. This indicates that collection of multiple isolates is necessary to capture *Listeria* diversity present in environmental samples collected in produce operations. In addition, the frequent coexistence of LM and LS in a given sample supports the importance of either (i) a molecular screen of the

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enrichment media or (ii) chromogenic differential media (e.g., LMPM) when testing environmental samples specifically for LM (which may be desired in root cause analysis or outbreak investigation). Importantly, the FDA BAM method specifies that up to five colonies (up to two colonies if using differential chromogenic agar) should be restreaked and tested (using classical tests, rapid biochemical test kits, or PCR analyses) to confirm isolates to the species level *(9)*. The data presented in the study reported here reinforce the need for collection, speciation, and subtyping of multiple isolates to capture *Listeria* diversity present in produce operations. Overall, the data presented in the study reported here suggest that choice of appropriate detection methods, including protocol details (testing time points, number of isolates characterized, etc.), continues to be important in the design and implementation of PEM programs.

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