Listeria monocytogenes in Ready-to-Eat Food Processing Plants: Persistence Indicators and Control Strategies

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ABSTRACT

Listeria monocytogenes can persist in food processing environments, resulting in the predominant source of *L. monocytogenes* post-processing ready-to-eat (RTE) food contamination. Butt's "Seek and Destroy" strategy is a systematic method for finding sites of persistent growth ("niches"); it may reduce *L. monocytogenes* prevalence in RTE food and expedite detection and response to *L. monocytogenes* outbreaks.

Applying this strategy, we sampled environments at two smoked fish plants to identify persistent *L. monocytogenes* ribotypes, and to identify and eliminate or manage niches. Persistence was measured with binomial statistics: one statistic compared ribotype recurrences to reference distributions; the second measured ribotype occurrences as a risk factor. Persistent ribotypes and persistence sites were identified to guide interventions. Poisson regression showed borderline decreases in *L. monocytogenes* isolation at both plants ($p=0.026$ and $p=0.076$). One niche on a food contact surface was eliminated; others were not. These methods should facilitate identification of microbial persistence.

BIOGRAPHICAL SKETCH

A native of Long Island, New York, Tom Malley attended Boston University as a Merit Scholar where, advised by Dr. Lynette L. Linden, Tom earned a Bachelor of Science in Manufacturing Engineering from College of Engineering in 1984. Tom worked with Drs. Spencer W. Thomas, Thomas C. Henderson, and Thomas G. Stockham, Jr., earning a Master of Science in Computer Science from the Computer Science Department at The University of Utah in 1988. His thesis, focused on the integration of radiosity and ray-tracing techniques, was entitled "A Shading Method for Computer-Generated Images." Tom worked for Evans & Sutherland Computer Corporation from 1987 to 1993 as a scientific applications programmer developing computer graphics software for the Conceptual Design and Rendering System; the system was subsequently sold to Parametric Technologies Corporation. Tom earned a Doctor of Veterinary Medicine from Colorado State University in 1998 and completed an internship in General Medicine and Surgery at the E. & M. Bobst Hospital of The Animal Medical Center in New York in 1999.

Dedicated to Mary, Elizabeth, and the rest of my family and friends.

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The views expressed in this article are those of the authors and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.

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CHAPTER 1

THE SEEK AND DESTROY STRATEGY: LISTERIA MONOCYTOGENES PROCESS CONTROLS IN THE READY-TO-EAT (RTE) MEAT AND POULTRY INDUSTRY

Abstract

Listeria monocytogenes is an important cause of foodborne illness and death in the United States. The USDA and U.S. FDA enforce a zero tolerance policy for *L. monocytogenes* in ready-to-eat (RTE) foods. Post-processing environmental contamination is the primary source of *L. monocytogenes* contamination in RTE meat and poultry. Contamination of RTE meat and poultry is difficult to avoid because *L. monocytogenes* can persist in food processing environments and can be difficult to detect and eradicate; persistent *L. monocytogenes* strains have been identified as the main source of post-processing RTE food contamination. In addition, multiple human listeriosis outbreaks have been linked to persistent contamination in processing plants. As a result, eradication of plant persistent strains is a critical activity for regulatory compliance by RTE meat and poultry processors toward reducing *L. monocytogenes* prevalence in RTE meat and poultry products and reducing the incidence of human listeriosis. The "Seek and Destroy" strategy is a systematic approach to finding sites of persistent growth ("niches") in food processing plants, with the goal of either eradicating or monitoring and mitigating effects of niches. The Seek and Destroy strategy employs environmental *Listeria* testing, and it has been used effectively to address persistent contamination in food processing plants. Thus, it is important to maintain a regulatory environment that encourages aggressive environmental *Listeria* testing, especially for RTE food contact surfaces. As microbial testing technologies are becoming faster, cheaper, and more specific, testing at the species or subtype level

in combination with the Seek and Destroy strategy may improve efficiency and efficacy of *Listeria monocytogenes* niche eradication and reduce *L. monocytogenes* prevalence in RTE meat and poultry products.

Introduction

Listeria monocytogenes causes an estimated 2-4 cases of listeriosis per million persons per year in most developed countries, and the vast majority (99%) of listeriosis infections are foodborne *(32).* The estimated death rate for laboratory confirmed human listeriosis in the United States is 15.9% *(32)*, while in Europe, the fatality rate was 20.5% among reported cases in 2008 *(12)*; USDA and U.S. FDA enforce a zero tolerance policy for *L. monocytogenes* in RTE food *(33)*. In the 2003 U.S. FDA *L. monocytogenes* risk assessment *(41)*, RTE meat and poultry products were the food vehicle in 5 of 12 outbreaks of listeriosis in the United States with known food vehicles between 1970 and 2002, and RTE deli meats were ranked highest for relative risk for predicted cases of listeriosis for the United States on a per serving and per annum basis. Other selected food groups ranked high in relative risk ranking per serving for the United States include frankfurters (when not reheated), pâté and meat spreads, unpasteurized fluid milk, smoked seafood, and cooked RTE crustaceans; the moderate relative risk per serving category included high fat and other dairy products, soft unripened cheese, and pasteurized fluid milk. In the risk assessment, fruits and vegetables were in the low risk category; however outbreaks in 2011 due to (i) *L. monocytogenes* contaminated cantaloupe in the U. S. *(8)* (causing 146 illnesses including 30 deaths and 1 miscarriage) and (ii) *Escherichia coli* O104:H4 contaminated fenugreek sprouts in Europe *(4, 30)* (causing at least 3,602 illnesses including 47 deaths) highlighted produce as a potential cause of foodborne illness.

Environmental *L. monocytogenes* is a major source of post-processing contamination, and persistent *L. monocytogenes* contamination is responsible for the majority of environmental contamination events. *L. monocytogenes* contamination in the processing environment is the principal source of *Listeria* contamination of processed RTE foods *(35)*. Multiple longitudinal or intervention studies in food processing environments have characterized environmental *L. monocytogenes* isolates through molecular subtyping *(2, 13, 14, 15, 17, 20, 26, 29, 34)*. Overall, these studies support the concept that environmental contamination is the major source of post-processing contamination of food products and that plant-persistent molecular subtypes are isolated in the majority of post-processing contamination.

In a multi-year study, Lundén et al. *(21)* collected 596 *L. monocytogenes* isolates to study contamination by persistent and non-persistent *L. monocytogenes* strains in three meat and one poultry processing plants. Based on pulsed-field gel electrophoresis (PFGE) type assignments and recurrence of PFGE types, 19 of 47 PFGE types were identified as persistent (isolated five or more times over a period of three or more months). A higher proportion of persistent *L. monocytogenes* PFGE types were isolated from heat-treated products than from raw products, emphasizing the importance of persistent strains as sources of contamination of final heat-treated products.

Rørvik et al. *(31)* examined multilocus enzyme electrophoretograms in a salmon slaughterhouse and smoked salmon processing plant. Among samples from salmon taken after smoking and before further processing, *L. monocytogenes* was not isolated. All (7 of 7) of the *L. monocytogenes* isolates from the finished vacuum-packed smoked salmon were of the same electrophoretic type as the predominant (representing 37 of 42 environmental samples) electrophoretic type in the smokehouse, strongly suggesting post-processing contamination. The predominant

electrophoretic type was also persistent, having been isolated in the facility during the whole investigation period.

Ojeniyi et al. *(27)* examined *L. monocytogenes* prevalence in turkey processing from pre-harvest through slaughter, processing and finally production of both raw and RTE products in Denmark. No *L. monocytogenes* was isolated from turkey housing prior to slaughter, yet *L. monocytogenes* was repeatedly isolated in both the cleaned and disinfected abattoir and in intra-operational processing areas. The majority of environmental and food isolates belonged to just three PFGE types: 47 of 48 *L. monocytogenes* isolates from 22 repeated environmental sample sites and 11 of 12 *L. monocytogenes* isolates from raw and smoked or cooked turkey products. The predominant PFGE types were isolated in the facility over the entire 18 week duration of the study and in food samples over a 9 week period. These data suggest that (i) the turkey pre-harvest environment was not contaminated with *L. monocytogenes*, (ii) three specific PFGE types represented the predominant persistent *L. monocytogenes* contaminants in the plant, and (iii) the predominant persistent PFGE types from the plant environment were the predominant PFGE types of isolates from food products. The authors also noted that some PFGE types from turkey products and the processing plant matched PFGE types from human listeriosis cases.

In a study of smoked fish plants, Lappi et al. *(20)* applied ribotyping to environmental and food samples. *L. monocytogenes* isolates were identified as persistent when their associated ribotypes were significantly associated with a given plant or if the ribotype was isolated multiple consecutive times in a given plant. Among 172 *L. monocytogenes* isolates, 22 unique ribotypes were identified. Persistent subtypes were identified in the majority of *L. monocytogenes* environmental isolates from three of three plants where *L. monocytogenes* was isolated from environmental samples. In addition, six of eight ribotypes from finished food products in the three

plants were plant-persistent ribotypes. The fourth plant in the study had no *L. monocytogenes* positive environmental isolates and two *L. monocytogenes* positive results from finished food product samples.

Collectively, these studies illustrate that *L. monocytogenes* strains persist in some processing plants, and plant environmental *L. monocytogenes* strains are associated with the majority of post-processing contamination. Additionally, persistent environmental *L. monocytogenes* molecular subtypes represent the majority of isolates from environmental sampling and finished food products.

Food processing plant environmental isolates have been linked RTE foodborne listeriosis outbreaks. In 2010 in the state of Louisiana, eight listeriosis patients were identified as being infected by *L. monocytogenes* serotype 1/2a with indistinguishable isolate PFGE patterns *(10)*. Ultimately, 7 patients were hospitalized and 2 patients died. In the course of the outbreak investigation, hog head cheese samples were collected from two grocery stores and environmental samples were collected from the processing establishment. *L. monocytogenes* PFGE patterns from isolates from a refrigeration unit at the processing establishment matched *L. monocytogenes* isolates from a grocery store sample as well as the PFGE patterns from patient isolates. Multiple-locus variable-number tandem repeat analysis and multilocus genotyping further supported the links between the isolates with identical PFGE patterns.

In 1999, 25 cases of listeriosis in Finland were part of a *L. monocytogenes* outbreak due to butter *(22)*; the patients were mainly immunosuppressed and 6 patients died. The outbreak isolates were all serotype 3a and the PFGE patterns *from the L. monocytogenes* isolates from all the patients were indistinguishable from one another. As part of the investigation, *L. monocytogenes* was isolated from 7 g packages of butter containing 11,000 CFU/g. The outbreak strain was isolated from the butter processing plant in two butter packing machines, the screw conveyor of the

butter wagon, and from two floor drains beneath the butter wagon at the small packaging line. This outbreak strain had been previously isolated in butter samples from the same dairy in 1997; the processing line was cleaned and microbiological sampling was intensified, but *L. monocytogenes* was not isolated again before February 1999.

"Seek and destroy" strategy. Butts *(6)*outlined the "Seek and Destroy" strategy: an aggressive, systematic process to identify persistence "niches" - locations where microbes (in this case *L. monocytogenes*) survive despite cleaning and sanitation measures. The Seek and Destroy strategy is employed when food processors want to (i) respond to a positive test for *L. monocytogenes* or its indicator organisms (*Listeria* spp. or *Listeria*-like organisms), (ii) qualify equipment or a process (e.g. new equipment 90-day assessment), or (iii) perform process validation (documenting performance and assuring that a process will meet requirements). The first step in the Seek and Destroy strategy is to clearly identify the physical area of interest. A review should be performed to determine if any events with a high risk of causing contamination have occurred. High risk events include drain backups; movement or modification of a packaging line; equipment breakdown; transfer of personnel between raw and cooked products; construction in or adjacent to critical processing areas; increases in environmental temperature or moisture; transfer of equipment out of storage, from another plant, or from another processing line; use of high pressure water or air on a floor or in a drain; wet in-process cleanup; rinsing or cleaning equipment on the floor; equipment transfer between raw and cooked products; and cooked product transported through a raw product area *(16)*.

The core of the Seek and Destroy strategy is the following process, performed by cleaning staff monitored by and interacting with supervisors, managers, and quality assurance staff as appropriate. Each piece of equipment in the physical area under

review is cleaned and sanitized according to standard sanitary operating procedures (SSOPs). These steps include cleaning, disassembly, flood sanitation (if applicable), re-assembly, and sanitizing. Next, the equipment is set up (monitored for good manufacturing practices [GMPs]) and run without food. Microbiological sample swabs are collected, particularly in suspect areas or where organic material may have accumulated. The equipment is disassembled again (as it would be for routine cleaning), while continuing collection of sample swabs in suspect areas. This is followed by full disassembly of the equipment, while continuing to swab suspect areas. All disassembled components are cleaned, then flood or heat sanitized. The equipment is re-assembled and, if appropriate, placed into operation. The results are assessed on the basis of (i) adequacy of routine or full disassembly, (ii) visible organic material, (iii) cleaning methods or improvements, and (iv) swab results (e.g. aerobic plate counts [APCs] or *Listeria* species presence or absence).

The Seek and Destroy strategy applies to the whole processing plant environment. The fundamental concepts of the Seek and Destroy strategy can be extended beyond a single machine or small sampling area to an entire processing plant. Key principles of the Seek and Destroy strategy are still relevant: (i) a strong pathogen environmental monitoring program provides baseline data; (ii) sampling at different times relative to processing shifts indicates cleaning and sanitation efficacy or potential persistent contamination; and (iii) additional sampling locations, particularly during equipment cleaning or disassembly, clarify potential niches.

When sampling larger domains, molecular subtyping becomes a critical support to assessing *L. monocytogenes* isolates. Molecular subtyping methods, such as ribotyping *(5)*or PFGE *(23)*, offer strong evidence for relatedness between *L. monocytogenes* isolates; thus, at the plant level, molecular subtyping results can identify relatedness between *L. monocytogenes* positive samples. Molecular subtyping

results are extremely useful when (i) increased prevalence makes relationships between samples from different areas unclear, (ii) potential contamination of raw materials obfuscates whether contamination is due to in-plant persistence or raw materials, (iii) repeated *L. monocytogenes* positive samples over time may be due to repeated sporadic contamination or in-plant persistence, and (iv) sources of *L. monocytogenes* contamination in finished product must be rapidly identified and eliminated or managed.

Aspects of the Seek and Destroy strategy have been applied in a number of food production categories: cold smoked pork *(3)*, fish or smoked seafood *(1, 9, 14, 19, 20, 25, 26, 27, 34)*, chicken *(2)*, meat and poultry *(21)*, and turkey *(27)*. These studies illustrate some of the challenges of large scale application of the Seek and Destroy strategy, including (i) identification and collection of appropriate food and/or environmental samples, (ii) application of appropriate microbiological tests and/or subtyping methods, (iii) identification of root causes of contamination, and (iv) elimination or control of identified sources of contamination.

Successful application of the Seek and Destroy strategy in food processing plants. Unfortunately, there is rarely incentive for food processing plants to publicize the details of specific internal investigations of environmental *L. monocytogenes* contamination. However, whether or not the techniques were identified as using the Seek and Destroy strategy, some published studies show the process has been successfully applied to food processing environments. In one study, regular samples were taken in a smoked seafood processing plant *(20)*. Molecular subtyping of isolates tied persistence of a *L. monocytogenes* subtype to floor mats in the finished product area. Removal of the floor mats led to resolution of the persistent *L. monocytogenes* subtype. This ribotype was identified finished product samples, and was persistent within the processing plant. In a long term study of environmental samples in a chilled

food processing plant *(18)*, *L. monocytogenes* was repeated isolated from a cooler on a processing line. The line was shortened to remove conveyors, and the cooler was eliminated; these actions led to elimination of two persistent molecular subtypes and reduced *L. monocytogenes* prevalence. These data show that application of the Seek and Destroy strategy at the plant level can reduce *L. monocytogenes* environmental prevalence and potentially eliminate *L. monocytogenes* from specific sampling sites.

USDA policies for environmental sampling and positive results. This discussion applies to food processing establishments under USDA jurisdiction that produce RTE meat or poultry products exposed to the processing environment after the basic lethality procedure (e.g., cooking); these establishments must comply with the USDA Food Safety and Inspection Service's (FSIS) *Listeria* interim final rule ("*Listeria* rule") *(36)*. These establishments are required to identify processes in one of three categories – Alternatives 1, 2, and 3, depending on the application of a post-lethality treatment and/or application of antimicrobial agents or processes. *Post-lethality treatments* (e.g. steam pasteurization, hot water pasteurization, radiant heating, or high pressure processing) are processes applied to RTE products that have been cooked and where subsequent processing (e.g., slicing, dicing, or repackaging) poses a risk of cross-contamination. *Antimicrobial agents or processes* suppress or limit *L. monocytogenes* growth throughout the refrigerated shelf life (e.g., addition of lactates or diacetates). Processes identified under Alternative 1 employ both a post-lethality treatment and an antimicrobial agent or process; the facility must initially validate and document that the establishment's post-lethality treatment is effective in eliminating or reducing *L. monocytogenes*. Processing under Alternative 2 employs either a post-lethality treatment ("Choice 1") or an antimicrobial agent or process ("Choice 2"), while processing under Alternative 3 employs neither a post-lethality treatment nor an antimicrobial agent or process. Alternative 3 sanitation program requirements also

depend on whether the product is a "Deli or Hotdog Product" or not. The alternative under which a process is identified dictates the minimum requirements for food contact surface and other environmental microbiological testing; the lowest sampling requirements apply to Alternative 1 and the greatest sampling requirements apply to Alternative 3.

Under USDA regulations, when microbial tests for *Listeria* species from food contact surfaces are positive, the required response depends on whether the process is identified under Alternative 1, 2, or 3 *(36)*. For processes identified under Alternative 1, establishments must investigate, take corrective actions to eliminate the source, and complete verification of corrective actions. Also, the establishment should review its sanitation program and post-lethality treatment. Facilities operating processes under Alternative 1 may periodically test food contact surfaces for *Listeria* species or *L. monocytogenes* to verify SSOP effectiveness.

Follow-up to a positive *L. monocytogenes* or *Listeria* indicator microbiological test on a food contact surface for processes identified under Alternative 2 are similar to those under Alternative 1. However, under Alternative 2, the sanitation program must include a microbiological test plan, and periodic testing of food contact surfaces is required. Appropriate tests indicate the presence or absence of *L. monocytogenes* or its indicator organisms (*Listeria* species or *Listeria*-like organisms); however aerobic plate counts (APCs), total plate counts (TPCs), or coliform counts are not considered appropriate indicators for *L. monocytogenes*. The sanitation program must also identify conditions which trigger hold-and-test procedures following a positive result for *L. monocytogenes* or indicator organisms on post-lethality food contact surfaces.

Processes identified under Alternative 3 have to have a documented testing program, and must specify the conditions for hold and test procedures following a positive result on a food contact surface. In response to an initial positive food contact

surface test for *L. monocytogenes* or its indicator organisms, establishments producing deli products or hot dog products must verify that corrective actions are effective. If follow-up testing subsequent to a positive food contact surface test for *L. monocytogenes* or its indicator organisms is positive for *L. monocytogenes* or an indicator organism, then the establishment must hold lots of product that may have become contaminated until correction of the problem. If tests indicate the presence of *L. monocytogenes* (as opposed to other *Listeria* species), the affected product lot is considered adulterated, and must be recalled if necessary, destroyed, or reworked to destroy *L. monocytogenes*.

USDA effectively offers strong incentives for producers to operate under Alternative 1 (when feasible) rather than Alternative 2, and Alternative 2 rather than Alternative 3. Alternative 1 allows less environmental sampling and with proper documentation, product holds and recalls are unlikely.

The USDA rule effectively penalizes establishments when food contact surface tests for *L. monocytogenes* or *Listeria*-like organisms are positive, and every food contact surface microbiological test has some (perhaps small) chance of resulting in a positive for *Listeria* spp. or *L. monocytogenes*; thus testing food contact surfaces for *L. monocytogenes* or its indicator organisms poses a potential liability for processors. In the best case for processors, positive results require follow-up and documentation, while in the worst case, for processes identified under Alternative 3, recalls and/or product rework or destruction may be necessary. Two successive positive results for *L. monocytogenes* or *Listeria* spp. on food contact surfaces for processes operating under Alternative 3 can cost the processor significant expenses if product holds, recalls, testing, rework, or product destruction are indicated. As an overall result of the *Listeria* rule, processors have incentives to (i) where possible, identify processes with Alternative 1 in preference to Alternative 2 in preference to alternative 3, (ii) maintain

high levels of sanitation, especially on food contact surfaces and particularly when operating under Alternative 3, and (iii) avoid *L. monocytogenes* or *Listeria*-like indicator tests on food contact surfaces except when required, due to potential liabilities associated with a positive test result.

U.S. FDA policies for environmental sampling and positive results. U.S. FDA recommends that "any processor of a refrigerated or frozen RTE (RF-RTE) food, including processors of fresh-cut fruits and vegetables, and processors of frozen fruits and vegetables" follow their *Guidance for Industry: Control of* Listeria monocytogenes *in Refrigerated or Frozen Ready-To-Eat Foods; Draft Guidance (38).* In the context of environmental microbiological sampling, this guidance instructs processors evaluate each plant, product, and process to determine monitoring points. U.S. FDA also recommends that RTE food facilities establish and implement a written plan for collection of environmental sample from critical surfaces and areas in order to detect locations that harbor *Listeria* species or specifically *L. monocytogenes (38).* For critical food contact surfaces, representative sets of surfaces should be tested for *L. monocytogenes* or *Listeria* species weekly, and all critical food contact surfaces are to be tested at least monthly, while for critical non-food contact surfaces, representative samples are to be tested at least every two weeks, and all critical non-food contact surfaces should be tested at least once per quarter. If *Listeria* species are detected on critical food contact surfaces or in food products, U.S. FDA recommends taking corrective actions, including (i) additional sampling, (ii) cleaning and sanitizing, (iii) follow-up sampling after cleaning and sanitizing until the results demonstrate elimination of *Listeria* species and *L. monocytogenes*, (iv) review of procedures, (v) review of scenarios to identify causes, (vi) checking maintenance records, (vii) interviews and observations with employees on procedure compliance, (viii) correction of problems that could lead to contamination, and (ix) record

corrective actions taken. Under circumstances of the detection or assumption of L. *monocytogenes* on a critical food contact surface or food (whether the food supports the growth of *L. monocytogenes* or not), U.S. FDA recommendations include (i) segregation and holding of relevant food; (ii) corrective actions for the relevant food, which may include listericidal treatments, reprocessing the food, diverting or destroying the food, and recalling finished refrigerated or frozen food which has been distributed, and (iii) keep records of corrective actions *(38)*. Additional guidance may apply for specific RTE products under U.S. FDA regulations, such as seafood products *(39)* or fresh-cut fruits and vegetables *(40)*.

U.S. FDA implements a zero tolerance policy; the effects of positive environmental *Listeria* test results are comprehensive and are designed to ensure safe products. It would be ideal if responses to potentially contaminated foods incorporated whether or not the foods support the growth of *L. monocytogenes*, because post-purchase growth of the organism can increase *L. monocytogenes* doses to consumers, so the risks are not the same. More importantly, under U.S. FDA recommendations, facilities in certain circumstances may have to initiate recalls based on a single critical food contact surface *L. monocytogenes* result. While these policies are clearly intended to improve public health, they may also deter food processing establishments from microbiological testing of important environmental and food contact surfaces or food products, unless such testing is required by regulations or guidance. A regulatory environment that effectively encourages proactive environmental testing and the application of the Seek and Destroy strategy is needed to reduce human listeriosis cases.

Environmental microbial testing can be beneficial in reducing *L. monocytogenes* prevalence. An ideal regulatory environment will effectively promote environmental microbial testing, including both food contact surfaces and the rest of the environment.

Multiple longitudinal studies have demonstrated that molecular subtyping of environmental isolates at multiple sampling sites over time can clarify sporadic versus persistent contamination and identify common subtypes between different sampling sites within a food processing facility *(13, 18, 20)*. These data guide measures such as Seek and Destroy strategy to reduce the prevalence of *L. monocytogenes* in food processing facilities. Disincentives to collect microbiological environmental samples may ultimately have a negative effect on efforts to reduce the prevalence of *L. monocytogenes*.

Aggressive application of the Seek and Destroy strategy in the past might have prevented outbreaks. Analysis of isolates from outbreaks in 1988 and 2000 linked to a single processing plant offered strong evidence for plant environmental persistence of the outbreak strain *(28)*. Given the 12 year duration of plant persistence, this suggests that practices in the plant did not successfully find and eradicate one or more *L. monocytogenes* persistence sites. The 1998 U.S. outbreak of *L. monocytogenes* due to hot dogs was postulated to have resulted from environmental contamination *(24)*. The outbreak strain may have previously colonized a ceiling refrigerator unit in the frankfurter hopper room, and removal of the unit increased contamination within the room. In a 1994 outbreak of *L. monocytogenes* due to gravad trout, persistent environmental contamination in the processing plant packing machine was suspected, and outbreak isolates were closely related under restriction enzyme analysis to strains isolated six months prior to the outbreak by the local health authority *(11)*. An outbreak due to plant persistent *L. monocytogenes* took place in Finland; *L. monocytogenes* was isolated from the processing plant environment in 1997; unresolved plant contamination resulted in an outbreak due to *L. monocytogenes* with the same PFGE patterns in 1999 *(22)*. These outbreaks indicate that adaptive environmental microbiological testing for *L. monocytogenes* such as the Seek and

Destroy strategy should be promoted aggressively with the intention of identifying and either eradicating or at least monitoring *L. monocytogenes* niches.

Conclusions. The U.S. RTE meat and poultry industry has made significant progress in reducing *L. monocytogenes* prevalence in RTE products from 1990 (4.61%) to 2010 (0.32%) *(37)*. While these improvements came with significant effort and expense, the efforts appear to have had a positive impact on U.S. public health; listeriosis incidence was decreased 38% in a comparison of 2003 to 1996 through 1998 *(7)*. It is critical that regulations promote collection of data regarding the location of foodborne pathogens within food processing establishments.

The acquisition of subtype data on bacterial isolates is becoming less expensive, and approaches to understanding the relationships between environmental isolates are evolving. It is desirable to know not only if a location is contaminated with *L. monocytogenes*, but whether the contamination is persistent. It is also important to know if *L. monocytogenes* at one location is highly genetically similar to *L. monocytogenes* at another location within the same plant, which might suggest transfer surfaces or other routes of contamination. A regulatory climate which promotes identification of persistent *L. monocytogenes* environmental contamination and promotes active responses including the Seek and Destroy strategy may reduce *L. monocytogenes* prevalence and reduce the burden of listeriosis on public health. In addition, in the long term, development of "real-time" *L. monocytogenes* detection methods will promote environmental testing. For example, a real-time environmental test might identify *L. monocytogenes* contamination on equipment before processing start-up, and the equipment could be re-sanitized and re-tested to avoid processing food in a contaminated environment. Until such tests are available, improvements to the regulatory environment to promote environmental microbiological testing can help reduce *L. monocytogenes* contamination in food and reduce the incidence of listeriosis.

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REFERENCES

1. Autio, T., S. Hielm, M. Miettinen, A. M. Sjöberg, K. Aarnisalo, J. Björkroth, T. Mattila-Sandholm, and H. Korkeala. 1999. Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. *Appl. Environ. Microbiol.* 65:150-155.
2. Berrang, M. E., R. J. Meinersmann, J. F. Frank, and S. R. Ladely. 2010. Colonization of a newly constructed commercial chicken further processing plant with *Listeria monocytogenes*. *J. Food Prot.* 73:286-286.
3. Bērziņš, A., S. Hellström, I. Siliņš, and H. Korkeala. 2010. Contamination Patterns of *Listeria monocytogenes* in Cold-Smoked Pork Processing. *J. Food Prot.* 73:2103-2109.
4. Bielaszewska, M., A. Mellmann, W. Zhang, R. Köck, A. Fruth, A. Bauwens, G. Peters, and H. Karch. 2011. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *The Lancet infectious diseases* 11(9):671-676.
5. Bruce, J. 1996. Automated system rapidly identifies and characterizes microorganisms in food. *Food Technol.* 50:77-81.
6. Butts, J. 2003. Seek & destroy: identifying and controlling *Listeria monocytogenes* growth niches. Available at: http://www.foodsafetymagazine.com/articlePF.asp?id=3284&sub=sub1. Accessed 26 May 2012.
7. CDC. 2011. Statistics - Listeriosis. Available at: http://www.cdc.gov/listeria/statistics.html. Accessed 31 May 2012.
8. Cosgrove, S., A. Cronquist, G. Wright, T. Ghosh, R. Vogt, and P. Teitell. 2011. Multistate Outbreak of Listeriosis Associated with Jensen Farms Cantaloupe—

United States, August–September 2011. *Morbidity and Mortality Weekly Report* 11:2768-2769.

9. Dauphin, G., C. Ragimbeau, and P. Malle. 2001. Use of PFGE typing for tracing contamination with *Listeria monocytogenes* in three cold-smoked salmon processing plants. *Int. J. Food Microbiol.* 64:51-61.
10. Delaune, E., T. Sokol, R. Ratard, B. Kissler, L. Allen, S. Seys, T. Ward, B. Silk, K. Jackson, and L. Graves. 2011. Outbreak of invasive listeriosis associated with the consumption of hog head cheese, Louisiana, 2010. *Morbidity and Mortality Weekly Report* 60:401-405.
11. Ericsson, H., A. Eklöw, M. Danielsson-Tham, S. Loncarevic, L. Mentzing, I. Persson, H. Unnerstad, and W. Tham. 1997. An outbreak of listeriosis suspected to have been caused by rainbow trout. *J. Clin. Microbiol.* 35:2904-2907.
12. European Food Safety Authority. 2010. The community summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in the European Union in 2008. *EFSA Journal* 8:1496.
13. Ho, A. J., V. R. Lappi, and M. Wiedmann. 2007. Longitudinal monitoring of *Listeria monocytogenes* contamination patterns in a farmstead dairy processing facility. *J. Dairy Sci.* 90:2517-2524.
14. Hoffman A.D, Gall K.L, Norton D.M, and Wiedmann M. 2003. *Listeria monocytogenes* contamination patterns for the smoked fish processing environment and for raw fish. *J. Food Prot.* 66:52-52.
15. Holah, J. T., J. Bird, and K. E. Hall. 2004. The microbial ecology of high-risk, chilled food factories; evidence for persistent *Listeria* spp. and *Escherichia coli* strains. *J. Appl. Microbiol.* 97:68-77.

16. Huffman, R., J. Butts. 2008. Lessons Learned in the Meat Industry: Control of *Listeria* in RTE Meat and Poultry Products. Available at: http://www.cmc-cvc.com/english/documents/PresentationAMI_000.pdf. Accessed 8 May 2012.
17. Kabuki, D. Y., A. Y. Kuaye, M. Wiedmann, and K. J. Boor. 2004. Molecular subtyping and tracking of *Listeria monocytogenes* in Latin-style fresh-cheese processing plants. *J. Dairy Sci.* 87:2803-2812.
18. Keto-Timonen, R., R. Tolvanen, J. Lunden, and H. Korkeala. 2007. An 8-year surveillance of the diversity and persistence of *Listeria monocytogenes* in a chilled food processing plant analyzed by amplified fragment length polymorphism. *J. Food Prot.* 70:1866-1866.
19. Kışla, D., Y. Üzgün, and M. A. Demirhisar. 2007. Incidence and sources of *Listeria monocytogenes* in a traditional hot‐smoked rainbow trout processing plant in Turkey. *Int. J. Food Sci. Tech.* 42:1376-1381.
20. Lappi, V. R., J. Thimothe, K. K. Nightingale, K. Gall, V. N. Scott, and M. Wiedmann. 2004. Longitudinal studies on *Listeria* in smoked fish plants: impact of intervention strategies on contamination patterns. *J. Food Prot.* 67:2500-2500.
21. Lunden, J. M., T. J. Autio, A. M. Sjoberg, and H. J. Korkeala. 2003. Persistent and nonpersistent *Listeria monocytogenes* contamination in meat and poultry processing plants. *Journal of food protection;* 66:2062-2069.
22. Lyytikäinen, O., T. Autio, R. Maijala, P. Ruutu, T. Honkanen-Buzalski, M. Miettinen, M. Hatakka, J. Mikkola, V. Anttila, T. Johansson, L. Rantala, T. Aalto, H. Korkeala, and A. Siitonen. 2000. An Outbreak of *Listeria Monocytogenes* Serotype 3a Infections from Butter in Finland. *J. Infect. Dis.* 181:1838-1841.
23. Maule, J. 1998. Pulsed-field gel electrophoresis. *Mol. Biotechnol.* 9:107-126.
24. Mead, P. S., E. F. Dunne, L. Graves, M. Wiedmann, M. Patrick, S. Hunter, E. Salehi, F. Mostashari, A. Craig, P. Mshar, T. Bannerman, B. D. Sauders, P.

Hayes, W. Dewitt, P. Sparling, P. Griffin, D. Morse, L. Slutsker, and B. Swaminathan. 2006. Nationwide Outbreak of Listeriosis Due to Contaminated Meat. *Epidemiol. Infect.* 134:744-751.

25. Miettinen, M., L. Palmu, K. Bjorkroth, and H. Korkeala. 2001. Prevalence of *Listeria monocytogenes* in broilers at the abattoir, processing plant, and retail level. *J. Food Prot.* 64:994-999.
26. Norton, D. M., M. A. McCamey, K. L. Gall, J. M. Scarlett, K. J. Boor, and M. Wiedmann. 2001. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Appl. Environ. Microbiol.* 67:198-205.
27. Ojeniyi, B., J. Christensen, and M. Bisgaard. 2000. Comparative investigations of *Listeria monocytogenes* isolated from a turkey processing plant, turkey products, and from human cases of listeriosis in Denmark. *Epidemiol. Infect.* 125:303-308.
28. Orsi, R. H., M. L. Borowsky, P. Lauer, S. K. Young, C. Nusbaum, J. E. Galagan, B. W. Birren, R. A. Ivy, Q. Sun, and L. M. Graves. 2008. Short-term genome evolution of *Listeria monocytogenes* in a non-controlled environment. *BMC Genomics* 9:539.
29. Peccio, A., T. Autio, H. Korkeala, R. Rosmini, and M. Trevisani. 2003. *Listeria monocytogenes* occurrence and characterization in meat-producing plants. *Lett. Appl. Microbiol.* 37:234-238.
30. Robert Koch Institut. 28 March 2012. EHEC/HUS O104:H4 Outbreak: Germany, May/June 2011. Available at: http://www.rki.de/EN/Home/EHEC_Report.pdf?__blob=publicationFile. Accessed 29 May 2012.
31. Rørvik, L. M., D. A. Caugant, and M. Yndestad. 1995. Contamination pattern of *Listeria monocytogenes* and other *Listeria* species. in a salmon slaughterhouse and smoked salmon processing plant. *Int. J. Food Microbiol.* 25:19-27.

32. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States--major pathogens. *Emerging infectious diseases* 17:7-15.
33. Shank, F. R., E. L. Elliot, I. K. Wachsmuth, and M. E. Losikoff. 1996. US position on *Listeria monocytogenes* in foods. *Food Control* 7:229-234.
34. Thimothe J, Nightingale K.K, Gall K, Scott V.N, and Wiedmann M. 2004. Tracking of *Listeria monocytogenes* in smoked fish processing plants. *J. Food Prot.* 67:328-328.
35. Tompkin, R. 2002. Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* 65:709-725.
36. U. S. Department of Agriculture, Food Safety and Inspection Service. 2006. Compliance guidelines to control *Listeria monocytogenes* in post-lethality exposed ready-to-eat meat and poultry products. Available at: http://www.fsis.usda.gov/oppde/rdad/frpubs/97-013f/lm_rule_compliance_guidelines_may_2006.pdf. Accessed 31 May 2012.
37. U. S. Department of Agriculture, Food Safety and Inspection Service. 2011. Microbiological Testing Program, Ready-to-Eat Meat and Poultry Products. Available at: http://www.fsis.usda.gov/Science/Micro_Testing_RTE/index.asp. Accessed 8 May 2012.
38. U. S. Food and Drug Administration. 2003. Guidance for Industry: Control of *Listeria monocytogenes* in Refrigerated or Frozen Ready-To-Eat Foods; Draft Guidance. Available at: http://www.fda.gov/food/guidancecomplianceregulatoryinformation/guidancedocuments/foodprocessinghaccp/ucm073110.htm. Accessed 8 May 2012.
39. U. S. Food and Drug Administration. 2011. Fish and Fishery Products Hazards and Controls Guidance Available at:

http://www.fda.gov/food/guidancecomplianceregulatoryinformation/guidancedocuments/seafood/fishandfisheriesproductshazardsandcontrolsguide/default.htm. Accessed 8 May 2012.

40. U. S. Food and Drug Administration. 2008. Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables. Available at: http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ProduceandPlanProducts/ucm064458.htm. Accessed 10 May 2012.
41. U. S. Food and Drug Administration, Center for Food Safety and Applied Nutrition. 2003. Quantitative assessment of relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. Available at: http://www.fda.gov/downloads/food/scienceresearch/researchareas/riskassessmentsafetyassessment/ucm197330.pdf. Accessed 10 May 2012.

CHAPTER 2

IMPLEMENTATION OF STATISTICAL TOOLS TO SUPPORT IDENTIFICATION AND MANAGEMENT OF PERSISTENT *LISTERIA MONOCYTOGENES* CONTAMINATION IN SMOKED FISH PROCESSING PLANTS

Abstract

Listeria monocytogenes persistence in food processing plants is a key source of post-processing contamination of ready-to-eat foods. Thus, identification and elimination of sites where *L. monocytogenes* persists (typically referred to as "niches") is critical. Two smoked fish processing plants were used as models to develop and implement environmental sampling plans to (i) identify persistent *L. monocytogenes* subtypes (*Eco*RI ribotypes) using two novel statistical methods, and to (ii) identify and eliminate likely *L. monocytogenes* niches. The first statistic, a binomial test based on ribotype frequencies, evaluates *L. monocytogenes* ribotype recurrences relative to reference distributions; the second statistic, a binomial test based on previous positives, measures ribotype occurrences as a risk factor for subsequent isolation of the same ribotype. When applied to data from the initial four months of sampling, both statistics identified persistent ribotypes in both plants. The binomial test based on ribotype frequencies also showed significant evidence for persistence of a given ribotype at specific sampling sites. Two adaptive sampling strategies guided plant interventions: sampling multiple times before and during processing; and vector swabbing (i.e., sampling of additional sites in different directions ["vectors"] relative to a given site). Among sites sampled for 12 months, a Poisson model regression showed borderline significant monthly decreases in *L. monocytogenes* isolates at both plants (p=0.026 and p=0.076). Our data also indicate elimination of an *L. monocytogenes* niche on a food contact surface; additional niches on non-food contact

surfaces were not eliminated. While our data illustrate the challenge of identifying and eliminating *L. monocytogenes* niches, particularly at non-food contact sites in small and medium size plants, the methods for identification of persistence we describe should broadly facilitate science-based identification of microbial persistence.

Introduction

According to the 2003 FDA *L. monocytogenes* Risk Assessment, smoked seafood was designated high risk because smoked seafood has relatively high rates of *L. monocytogenes* contamination and smoked seafood supports the growth of *L. monocytogenes* during extended refrigerated storage *(68)*. As with ready-to-eat (RTE) foods from other categories, such as deli meats, dairy, and produce, a major source of smoked seafood contamination occurs in post-processing, particularly from persistent microbial contamination in food processing plants *(31)*. Avoidance of post-processing contamination requires effective employee training programs, prerequisite programs and sanitation standard operating procedures (SSOPs), as well as appropriate environmental monitoring programs. As *L. monocytogenes* strains have been found to persist in food production plants for periods of multiple years *(27, 39, 44, 46, 47, 48, 55)*, active strategies, such as the Seek and Destroy strategy outlined by Butts *(7)*, are necessary to control persistent *L. monocytogenes* contamination.

The concern with persistent *L. monocytogenes* contamination is that the failure to eliminate *L. monocytogenes* niches (locations where pathogenic microbes survive or grow) in RTE food processing plants *(65)* may increase the probability of food contamination and subsequent human infection. In the context of *L. monocytogenes* contamination in food processing plants, the term *persistent* has been described as "loosely defined" in previous literature *(8)*. As *L. monocytogenes* persistence contributes significantly to the risk of food contamination *(14)*, the identification of persistence carries regulatory impacts. Isolation of a subtype of *L. monocytogenes* in a

food product which matches a subtype previously isolated from the environment in the same production facility can bring about regulatory consequences including warning letters, production injunctions, product holds, product seizures, and product recalls *(67)*. As persistent *L. monocytogenes* contamination affects both regulatory measures and consumer risk, we applied objective statistical tests to environmental sample results to identify and guide responses to persistent contamination.

The ecology and prevalence of *Listeria* in seafood plants has been investigated in multiple seafood production environments including mussels*(10)*, crawfish *(32, 63)*, gravad salmon *(1)*, and smoked fish *(3, 11, 12, 22, 23, 24, 31, 37, 38, 40, 46, 58)*. We investigated *L. monocytogenes* environmental contamination and persistence in two smoked fish processing plants. Raw fish entering the production process were swabbed to assess inbound *L. monocytogenes* contamination. Environmental sampling with *L. monocytogenes* isolation and ribotyping was used to develop quantitative measures of persistent contamination and to apply the results to *L. monocytogenes* control. Samples were collected monthly as a repeated set of samples focusing on potential high risk sites. After four months, results were analyzed to identify plant-level persistent ribotypes and putative persistence sites (niches) within the plants. Adaptive sampling was applied to putative persistence sites to localize and refine persistence assessments. Interventions were directed toward specific sampling sites that exhibited high prevalence, and intervention impacts were assessed in terms of *L. monocytogenes* prevalence before and after interventions.

Materials and Methods

Description of participating plants. Two plants (designated plants D and N) that predominantly produce cold-smoked seafood were enrolled in the study; both of these plants also produce some hot-smoked and other products. Both plants primarily process salmon, whitefish, sablefish, and chubs, while plant D also processes sturgeon,

bluefish, mackerel, tuna, rainbow trout, marlin, cisco, whiting, and mahi-mahi, and plant N also processes sea bass and brook trout. Plant D has an area roughly seven times the area of plant N, with approximately four times the production (by weight) and six times the number of employees. Plant D is in a building that was built over 100 years ago, and numerous upgrades and renovations have taken place. Plant N underwent significant expansion in 1994, more than tripling the overall area of the plant. Further details on the plants cannot be disclosed to assure anonymity.

Raw fish surface samples. To assess *L. monocytogenes* presence and subtype diversity on exterior surfaces of inbound raw fish or fillets, surface samples were obtained by swabbing the surfaces of untreated salmon or whitefish. Fish were thawed if necessary and sampled as they entered the production chain; fish did not undergo in-plant acid, alkaline, or antimicrobial treatment prior to sampling. A single sterile sample sponge was used to sample the surfaces of 10 to 15 whole eviscerated fish or fillets (both sides). Samples were initially taken using 18-oz. Whirl-Pak® Hydrated Speci-Sponge® Bag with Sterile Glove (Nasco, Fort Atkinson, WI). Due to limited availability of the sponges, starting in December for plant D and October for plant N, Hydra-Sponge with neutralizing buffer (3M, St. Paul, MN) was used. Samples were put on cold packs and were shipped overnight or hand-carried to the laboratory.

Logistic regression was used to assess the effect of fish species, pre-processing (e.g. fillet, whole-eviscerated), country of origin, growth environment (i.e., wild or farmed) or history of freezing (i.e. fresh or frozen) on *L. monocytogenes* isolation.

Baseline environmental samples. For each participating plant, an initial plant visit and evaluation were used to designate environmental sampling sites; sites were selected to (i) span the whole smoked fish processing chain from raw (prior to smoking) to finished (after smoking) product areas; (ii) span zones 1 through 4, as defined by International Commission on Microbiological Specifications for Foods *(25,*

59); and (iii) include likely sites of persistence. Due to plant D's larger area and product throughput, 48 sampling sites were designated for plant D, while 32 sampling sites were designated for plant N. Samples at designated sites were collected at least 2 h after the start of processing and before end-of-shift cleaning and disinfection. Samples were collected using the sponges detailed above, swabbing an area as close as practical to 2.0 ft^2 (0.19 m^2).

The overall study reported here encompassed 12 months (March 2011 through February 2012). All designated sampling sites were sampled for six months; because this study focused on sites of persistent contamination, after six months, sampling was discontinued at selected sites that had not yielded any *L. monocytogenes* isolates (i.e., 29 and 19 sampling sites at plants D and N, respectively). The sampling sites that were sampled over the initial six months were designated as "6-months baseline sites" (i.e., 48 and 32 sites at plants D and N, respectively), while the sites that were sampled monthly over the full 12 months were designated as "12-months baseline sites" (19 and 13 sites at plants D and N, respectively).

Adaptive sampling strategies. In addition to the baseline environmental sampling detailed above, we also performed additional sampling before and during processing, as well as vector swabbing to confirm persistence and identify niches. In plant N, in order to confirm suspected persistence on a finished product food contact surface, sequential samples were collected at a few selected sampling sites before processing start-up and throughout a processing shift in months 3 and 4. In addition to collecting sequential samples, vector swabbing was performed in months 5, 7, and 8, at sampling sites that were identified as persistently contaminated based on four months data. At a given sampling site, additional samples were collected at locations offset from the given site in different directions, or "vectors"*(2)*. The specific directions for the vector samples were not fixed; the goal was to acquire samples offset in different directions

from a site of contamination. Sampling sites to undergo vector swabbing were chosen to help identify niches that may harbor a persistent subtype (e.g. flexible, absorbent, or wet material, cracks, or accumulated organic material). For food contact surfaces, vector swabbing was performed during "deep cleaning"; equipment which had already been cleaned and sanitized according to SSOPs was disassembled as much as practical, and samples were taken within the equipment to identify niches. The goal in vector swabbing is (i) to acquire better spatial resolution of positive or negative samples at or near niches; (ii) confirm contaminated areas; and (iii) guide efforts towards eradicating persistent contamination.

L. monocytogenes detection and isolation from environmental and raw fish surface samples. Enrichment commenced within 24 h after sampling. A total of 90 ml of Buffered Listeria Enrichment Broth (Becton Dickinson Difco, Franklin Lakes, NJ) was added to each sponge, followed by homogenization for 60 s at 2 Hz (in a Seward Stomacher 400 circulator). After initial incubation at 30°C for 4 h, Listeria Selective Enrichment Supplement (LSES, Oxoid, Basingstoke, Hampshire, UK) was added as 4 µl of LSES per 1 ml of BLEB. After overall incubation, at 30°C, of 24 and 48 h, 100 µl of enrichment media was streaked onto both *Listeria monocytogenes* plating medium (LMPM, R&F Laboratories West Chicago, IL) and Modified Oxford (MOX, Difco Oxford Medium Base and Oxoid MOX Supplement, Oxoid, Basingstoke, Hampshire, UK) plates. LMPM plates were incubated at 30°C and MOX plates were incubated at 35°C. If putative *L. monocytogenes* colonies were identified on LMPM plates (based on colony morphology and color), up to four of these colonies were streaked onto LMPM and incubated at 30°C for 24 h. If no putative *L. monocytogenes* colonies were identified on LMPM plates, but *Listeria*-like colonies were present on MOX, two of these *Listeria*-like colonies were streaked onto LMPM. Putative *L. monocytogenes* identified by this approach (i.e., colonies that were blue on LMPM)

were further subcultured onto BHI agar plates and a single putative *L. monocytogenes* isolate for each sample was confirmed and further characterized with *Eco*RI ribotyping as detailed below. Colonies for ribotyping were selected preferentially from 24 h enrichment plates (rather than 48 h enrichment plates). All samples that did not yield *L. monocytogenes* using the procedures described above were classified as "*L. monocytogenes*-negative."

Ribotyping. A single putative *L. monocytogenes* colony from each sample was selected for characterization by *Eco*RI ribotyping, performed using a Dupont Qualicon Riboprinter *(6)*. Automated ribotype assignments (e.g., DUP-1039) were manually reviewed for quality assurance. As previously described *(56)*, a "DUP-" ribotype designation sometimes is assigned to multiple ribotype patterns that differ by the presence and location of a single minor band; these patterns were clarified by adding a single letter suffix to the "DUP" ribotype designation (e.g., DUP-1039C, DUP-1039E). All putative *L. monocytogenes* isolates were assigned *L. monocytogenes* specific ribotypes, thus confirming these isolates as *L. monocytogenes*. Automated ribotyping was chosen as a subtype method for this study because (i) a large database of ribotype data for > 4,000 of *L. monocytogenes* is publicly available (www.pathogentracker.net; see below) and (ii) as *L. monocytogenes Eco*RI ribotypes are highly stable, so subtypes with 1-3 band differences do not require consideration as closely related clonal groups with a possible recent common ancestor; this later point was critical as it facilitated statistical data analyses, which included the implicit assumption that transitions between ribotypes are rare events.

Identification of ribotypes that show evidence of persistence in a given plant. After four months of sampling, two distinct statistical approaches were employed to identify ribotypes that showed evidence of persistence within a given plant. The first was a binomial test based on ribotype frequencies *(51)*, comparing the frequency of a

specific recurrent ribotype among *L. monocytogenes* positive samples in a given plant to the frequency of the same ribotype in a reference distribution. This approach is based on the premise that persistent ribotypes will be observed at a frequency significantly above the frequency expected in an appropriate reference distribution. Thus, in the context of the binomial test based on ribotype frequencies, we define persistence as the isolation of a given ribotype at a frequency greater than expected based on the reference distribution. One key challenge in this approach is to develop or acquire an appropriate reference distribution that can be used to calculate the "baseline" frequency of a specific ribotype. This is particularly true as it is well established that the prevalence of different *L. monocytogenes* ribotypes can differ significantly between different source populations; e.g. *L. monocytogenes* isolates from humans have different ribotype distributions in contrast to isolates from animals *(26)*. We selected two reference distributions for our analysis, but we were *a priori* acutely aware of the fact that the choice of reference distributions can affect the outcomes of these analyses, as discussed in detail in the "Discussion" section. One reference distribution used a large data set of ribotype data for 4,397 *L. monocytogenes* isolates, publicly available in the Pathogen Tracker (www.pathogentracker.net) database *(9)*, which was accessed on 06/20/2011; this data set included 1,302 human, 2,102 food and food environment, and 532 animal isolates, as well as 461 isolates from "other sources." In an attempt to use a dataset that represents the specific food production systems evaluated here, the second reference distribution used here utilized ribotype data available in Pathogen Tracker for isolates from other smoked fish plants; this data set included 159 isolates with ribotype data, representing largely isolates from environmental (123) and food (36) samples. Ribotypes absent from the reference distributions were assigned a probability of 1 / (sample size of the reference distribution), representing a conservative approach. Both

reference distribution datasets are available as spreadsheets from the author, upon request. The binomial statistics for each ribotype used a likelihood of 1 for the first occurrence of each ribotype, as this approach conceptually evaluates conditional probabilities, i.e., the probability that subtype persists, given that it has been introduced; probabilities of independent subsequent isolation were derived from the two reference distributions.

The second statistic is a binomial test based on previous positive results, with the goal to evaluate ribotype results as a series of events *(61)*; this test does not require a ribotype reference distribution. In the context of this statistical test, we define that persistence is present among a set of samples when the samples show that a given ribotype repeats in consecutive samples from a given sampling site more often than it would show consecutive repeats from random occurrences (see Fig. 1 for an example of how this test is implemented). As sampling sites undergo multiple cycles of cleaning and sanitation between samplings, the outcome of sampling in the absence of persistence should be random; significance of a prior positive result as a risk factor is interpreted as an indication of persistence. This approach was applied to evaluate the distribution of the three most common ribotypes in each plant D and N. For each of these ribotypes, we determined the number of instances where (i) a sampling site was positive for a given ribotype twice in a row (designated as "++"), (ii) a sampling site was positive for a given ribotype, followed by a negative at the same site at the next sampling (designated as "+-"), as well as (iii) the reverse (designated as "-+"), and (iv) a sampling site was negative for a given ribotype twice in a row (designated as "--", see Fig. 1). For each plant, these measures were determined using data for all sampling sites combined, rather than for a single sampling site (as four sample months' data do not provide sufficient samples for individual sampling site analysis).

Figure 1. A hypothetical application of the binomial test based on previous positives. 'A' and 'B' ribotypes were intermittently isolated from sampling sites I through V at times 1 through 5. 'A Results' and 'B Results' show the results of isolating the specific ribotypes. 'A Transitions' and 'B Transitions' show the transitions between positive and negative results at each sampling site. 'A Totals' and 'B Totals' are the sums of the transitions for each ribotype. 'A Contingency Table' and 'B Contingency Table' show the results of relative risk calculations and Fisher's exact test (the p values for Fisher's exact tests for each contingency table are shown on the bottom). Positive results for ribotype A more consistently come immediately following other positive results for ribotype A, so the previous result is a significant risk factor for the recurrence of ribotype A. Positive results for ribotype B are not significantly dependent on the previous occurrences of ribotype B. Numbers in parentheses represent the lower and upper bounds of the 95% confidence interval for the relative risk.

Overall Results

site	time 1	2	3	4	5
I	A	A	-	A	A
II	-	A	A	A	-
III	-	A	A	A	A
IV	-	B	-	B	B
V	B	-	B	-	-

A Results

site	time 1	2	3	4	5
I	+	+	-	+	+
II	-	+	+	+	-
III	-	+	+	+	+
IV	-	-	-	-	-
V	-	-	-	-	-

B Results

site	time 1	2	3	4	5
I	-	-	-	-	-
II	-	-	-	-	-
III	-	-	-	-	-
IV	-	+	-	+	+
V	+	-	+	-	-

A Transitions

site	time 1	2	3	4	5
I		++	+-	-+	++
II		-+	++	++	+-
III		-+	++	++	++
IV		--	--	--	--
V		--	--	--	--

B Transitions

site	time 1	2	3	4	5
I		--	--	--	--
II		--	--	--	--
III		--	--	--	--
IV		-+	+-	-+	++
V		+-	-+	+-	--

A Totals

	count
++	7
+-	2
-+	3
--	8

B Totals

	count
++	1
+-	3
-+	3
--	13

A Contingency Table

current	previous +	-
+	7	3
-	2	8

p-value 0.03
relative risk 2.67 (0.98, 7.22)

B Contingency Table

current	previous +	-
+	1	3
-	3	13

p-value 0.6
relative risk 1.3 (0.18, 9.7)

Identification of persistence at specific sampling sites. After identification of persistent ribotypes at the plant level, persistence at specific sampling sites was identified using the binomial test based on ribotype frequencies described above. This test was used to evaluate each sampling site where a given ribotype was isolated more than once; these analyses were performed with both reference distributions detailed above.

L. monocytogenes prevalence assessment for individual sampling sites and combined sampling sites within each plant. In both plants, control strategies were implemented on rolling basis, informed by prior sampling results. At the level of specific sampling sites, we used Fisher's exact test to determine whether the number of samples positive for a given ribotype significantly decreased after implementation of an intervention.

Trends in *L. monocytogenes* prevalence among "12-months baseline samples" were evaluated using a regression analysis under a Poisson model with overdispersion and Firth adjusted maximum likelihood.

Bonferroni correction. As a multiple statistical tests were used for a number of analyses (e.g., multiple binomial statistics to evaluate persistence using one set of data for one plant), the chance of a false positive result is inflated. We thus reported actual p-values for these analyses, but only designated those comparisons that were significant after a Bonferroni correction *(50)* as significant in the results.

Results

Raw fish surface samples. Overall, 3 of 9 composite whitefish samples and 11 of 63 composite salmon samples, collected over the 12 months, were positive for *L. monocytogenes* (Table 1). Among the salmon samples, 4 of 25 fillets samples (3 from Chile and 1 from Norway) and 7 of 38 whole eviscerated fish (2 from Chile and 5 from Norway) were positive for *L. monocytogenes*. Logistic regression analysis

revealed no significant effects ($p<0.05$) on *L. monocytogenes* counts due to the covariates fish species, fillet versus whole eviscerated, country of origin, wild versus farmed, and fresh versus frozen.

Table 1. Ribotype results from inbound raw fish positive for L. monocytogenes.

Plant	Study Month	Ribotype[a]	Fish Species	Form[b]	Country of Origin	Source
D	1	1045A	salmon	whole	Norway	farm
D	2	1062D	salmon	fillet	Norway	farm
D	3	1045A	salmon	whole	Norway	farm
D	3	1045A	salmon	whole	Chile	farm
D	4	1044A	whitefish	whole	USA	wild
D	5	1045A	salmon	whole	Chile	farm
D	9	1027B	salmon	whole	Norway	farm
D	10	1042B	whitefish	whole	USA	wild
D	11	1039C	whitefish	whole	USA	wild
N	5	1045A	salmon	fillet	Chile	farm
N	5	1045A	salmon	whole	Norway	farm
N	6	1042A	salmon	fillet	Chile	farm
N	10	1039A	salmon	fillet	Chile	farm
N	11	1027B	salmon	whole	Norway	farm

[a]Ribotypes are abbreviated without the "DUP-" prefix.
[b]Whole eviscerated fish are abbreviated as "whole."

At Plant D, DUP-1045A was identified in 4 of 16 composite inbound raw fish surface samples in months 1, 3, and 5; DUP-1045 isolates were obtained from both whole eviscerated salmon and fillets, as well as from different source countries (Table 1). DUP-1045A was also identified in four environmental samples from the raw processing area in months 3 through 6. Notably, Plant D changed their source of salmon fillets in month 5; DUP-1045A was not isolated in Plant D raw fish or

environmental samples after month 6, possibly suggesting introduction of this ribotype with raw fillets and subsequent cross contamination of the raw area environment and other raw materials in plant D.

Statistical analysis provides for quantitative assessment of persistence. After sampling for the first four months, data were analyzed to identify ribotypes that show evidence for persistence in either plant, in order to allow for timely implementation of initial control strategies (Figs. 2 and 3) . The binomial test based on ribotype frequencies identified significant evidence (after Bonferroni correction) for persistence of (i) two ribotypes in plant D (DUP-1039C and DUP-1043A, see Table 2); and (ii) two ribotypes in plant N (DUP-1042A and 116-1451-S2; see Table 2) when the reference distribution using ribotype data for 4,397 *L. monocytogenes* isolates from diverse sources was used. When the reference distribution using ribotype data for 159 *L. monocytogenes* isolates from other fish plants was used, the same two ribotypes showed evidence for persistence in plant D, while only ribotype DUP-1042A showed evidence for persistence in plant N (Table 2).

Figure 2. Plant D process flow and sample results. Arrows represent the process flow, from raw materials progressing to finished products. The largest boxes represent categories of rooms, while the nested boxes (shown in dashed lines where appropriate) represent separate rooms. The numbers in circles are sampling site numbers. The results of ribotype analysis on L. monocytogenes isolates from regular monthly samples are color-coded in the rows of squares extending to the right of the number, going chronologically from left to right (one box per month, month 1 to month 12). Strings of circles extending down through a sample box represent sequential samples from the sampling site, while rows of rectangles below a sample box represent vector swabbing results for the sampling site. Figure A1 lists sampling sites and provides detailed results in tabular format.

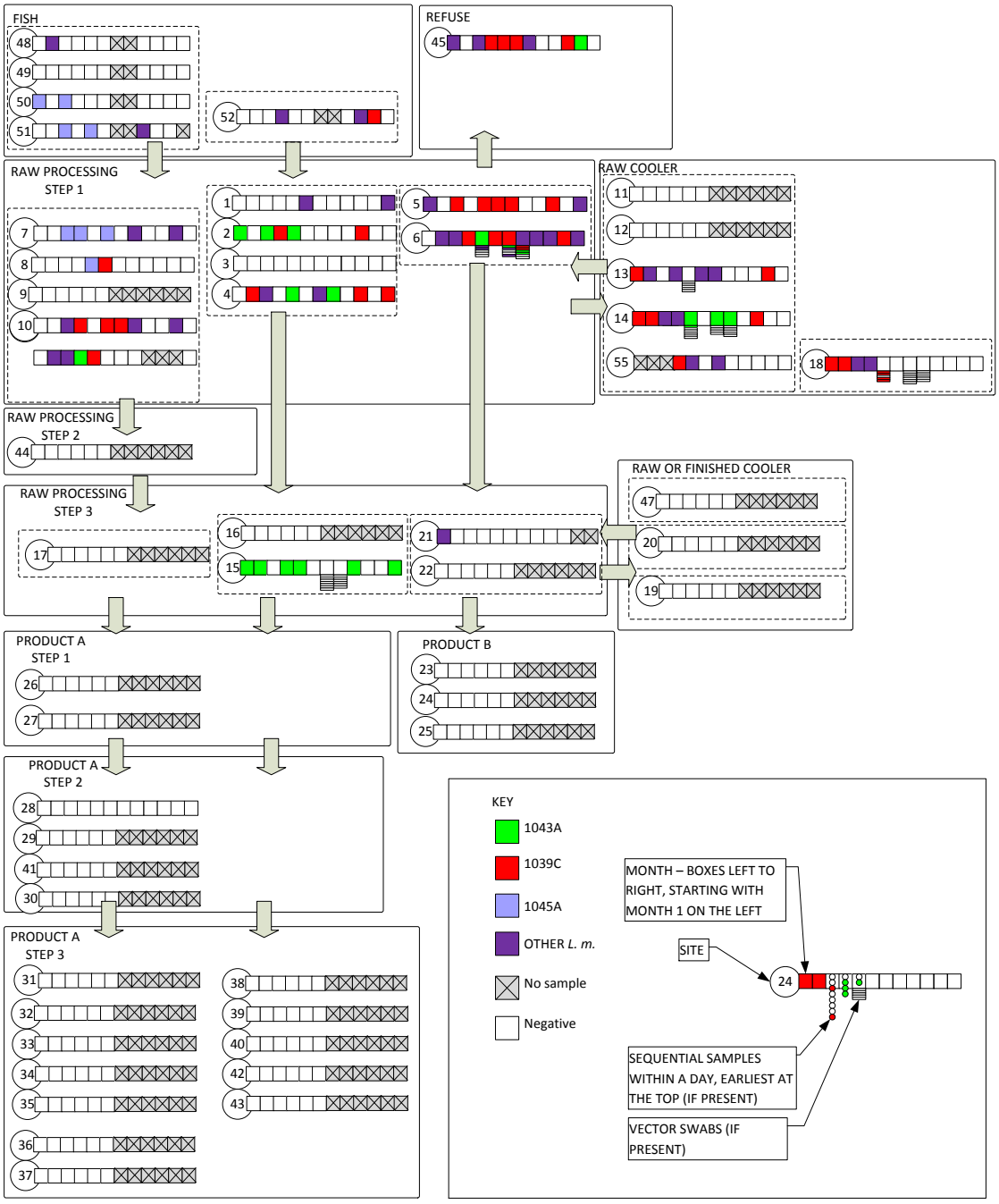
FISH
48
49
50
51
52
REFUSE
45
RAW PROCESSING
STEP 1
7
8
9
10
1
2
3
4
5
6
RAW COOLER
11
12
13
14
55
18
RAW PROCESSING
STEP 2
44
RAW PROCESSING
STEP 3
17
16
15
21
22
RAW OR FINISHED COOLER
47
20
19
PRODUCT A
STEP 1
26
27
PRODUCT B
23
24
25
PRODUCT A
STEP 2
28
29
41
30
PRODUCT A
STEP 3
31
32
33
34
35
36
37
38
39
40
42
43
KEY
1043A
1039C
1045A
OTHER L. m.
No sample
Negative
MONTH – BOXES LEFT TO RIGHT, STARTING WITH MONTH 1 ON THE LEFT
SITE
24
SEQUENTIAL SAMPLES WITHIN A DAY, EARLIEST AT THE TOP (IF PRESENT)
VECTOR SWABS (IF PRESENT)

Figure 3. Plant N process flow and sample results. See caption for Figure 2. Figure A2 lists sampling sites and provides detailed results in tabular format.

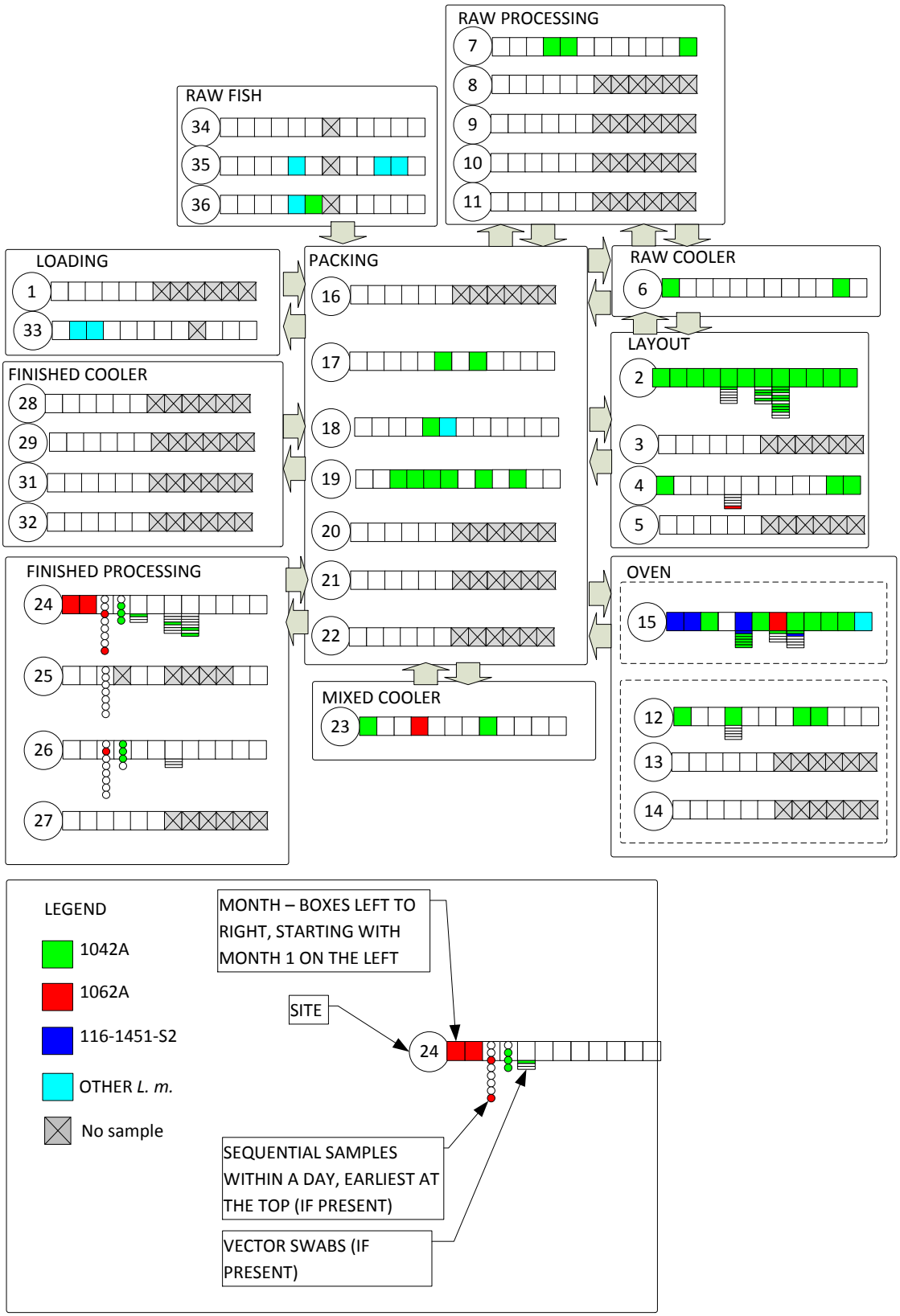
RAW PROCESSING
7
8
9
10
11
RAW FISH
34
35
36
LOADING
1
33
PACKING
16
17
18
19
20
21
22
RAW COOLER
6
LAYOUT
2
3
4
5
FINISHED COOLER
28
29
31
32
FINISHED PROCESSING
24
25
26
27
MIXED COOLER
23
OVEN
15
12
13
14
LEGEND
1042A
1062A
116-1451-S2
OTHER L. m.
No sample
MONTH – BOXES LEFT TO RIGHT, STARTING WITH MONTH 1 ON THE LEFT
SITE
24
SEQUENTIAL SAMPLES WITHIN A DAY, EARLIEST AT THE TOP (IF PRESENT)
VECTOR SWABS (IF PRESENT)

Table 2. Plant-level persistence metrics, after four months, using a binomial test based on ribotype frequencies.

Ribotype[a]	Plant-level fre-quency[b]	Ribotype reference frequency (all ribotypes) [binomial 99.5% CI][c]	Signif-icance (all ribotypes, p-value)[d]	Ribotype reference frequency (other fish plants) [binomial 99.5% CI][e]	Significance (other fish plants, p-value)[d]
Plant D					
1039C	3.14×10^{-1} (11/35)	1.11×10^{-1} (488/4,397) [9.81×10^{-2}, 1.25×10^{-1}]	1.03×10^{-3}*	6.29×10^{-2} (10/159) [2.15×10^{-2}, 1.37×10^{-1}]	6.16×10^{-6}*
1043A	1.43×10^{-1} (5/35)	2.27×10^{-2} (100/4,397) [1.69×10^{-2}, 2.98×10^{-2}]	1.12×10^{-3}*	6.29×10^{-3} (0/159) [1.57×10^{-5}, 5.05×10^{-2}]	2.73×10^{-6}*
1039A	5.71×10^{-2} (2/35)	3.07×10^{-2} (135/4,397) [2.39×10^{-2}, 3.87×10^{-2}]	2.92×10^{-1}	2.77×10^{-1} (44/159) [1.83×10^{-1}, 3.86×10^{-1}]	9.98×10^{-1}
1062D	2.86×10^{-2} (1/35)	1.32×10^{-2} (58/4,397) [8.87×10^{-3}, 1.88×10^{-2}]	3.72×10^{-1}	2.52×10^{-2} (4/159) [3.50×10^{-3}, 8.27×10^{-2}]	5.90×10^{-1}
1045A	2.86×10^{-2} (1/35)	1.59×10^{-2} (70/4,397) [1.11×10^{-2}, 2.20×10^{-2}]	4.30×10^{-1}	6.29×10^{-3} (1/159) [1.57×10^{-5}, 5.05×10^{-2}]	1.98×10^{-1}
1052A	2.86×10^{-2} (1/35)	7.05×10^{-2} (310/4,397) [6.01×10^{-2}, 8.20×10^{-2}]	9.23×10^{-1}	6.29×10^{-3} (0/159) [1.57×10^{-5}, 5.05×10^{-2}]	1.98×10^{-1}
1062A	2.86×10^{-2} (1/35)	6.48×10^{-2} (285/4,397) [5.48×10^{-2}, 7.59×10^{-2}]	9.04×10^{-1}	1.26×10^{-2} (2/159) [4.57×10^{-4}, 6.21×10^{-2}]	3.58×10^{-1}
Plant N					
1042A	6.50×10^{-1} (13/20)	2.02×10^{-2} (89/4,397) [1.48×10^{-2}, 2.70×10^{-2}]	$< 2.2 \times 10^{-16}$*	6.29×10^{-3} (0/159) [1.57×10^{-5}, 5.05×10^{-2}]	$< 2.2 \times 10^{-16}$*
1062A	1.00×10^{-1} (2/20)	6.48×10^{-2} (285/4,397) [5.48×10^{-2}, 7.59×10^{-2}]	3.75×10^{-1}	1.26×10^{-2} (2/159) [4.57×10^{-4}, 6.21×10^{-2}]	2.59×10^{-2}
116-1451-S2	5.00×10^{-1} (1/20)	2.27×10^{-4} (0/4,397) [5.69×10^{-7}, 1.87×10^{-3}]	4.54×10^{-3}*	6.29×10^{-3} (0/159) [1.57×10^{-5}, 5.05×10^{-2}]	1.19×10^{-1}

[a] Ribotypes are abbreviated without the "DUP-" prefix.

[b] Plant-level frequency represents the fraction of samples positive for a given *L. monocytogenes* ribotype (excluding the first positive sample) divided by all *L. monocytogenes* positive samples in a plant (excluding the first positive sample) for the first four months only; this approach was used to calculate frequencies as the statistics used assess conditional probabilities, i.e. the probability that a sample is positive for a ribotype given that this ribotype was introduced.

[c] Ribotype reference frequency (all ribotypes) is calculated as the fraction of the given ribotype out of all ribotypes in the reference distribution (count of given ribotype in reference distribution / reference distribution sample size [4,397]). If the given ribotype was not present in reference distributions, then a single occurrence of the ribotype was used as the prior frequency for the ribotype ([1/4,397]).

[d] * indicates statistical significance (the frequency of the given ribotype among positive results exceeds the frequency of the given ribotype in the reference distribution). Bonferroni's correction was applied to measures from each plant. Significance thresholds for plants D and N were 0.0071 (0.05/7) and 0.017 (0.05/3), respectively.

[e] Ribotype reference frequency (other fish plants) is calculated as the fraction of the given ribotype out of all ribotypes in other fish plants in the reference distribution (count of given ribotype in reference distribution / reference distribution sample size [159]). If the given ribotype was not present in reference distributions, then a single occurrence of the subtype was used as the prior frequency for the ribotype ([1/159]).

Using the data collected over the initial 4 months, the second statistical approach, a binomial test based on previous positives, (see "Materials and Methods" for details) identified evidence for persistence of (i) DUP-1045A in plant D and (ii) ribotype DUP-1042A in plant N, both significant after Bonferroni correction (Table 3). For plant N, ribotype 116-1451-S2, which showed significant evidence for persistence with the binomial test based on ribotype frequencies (using the reference distribution based on 4,397 ribotypes), had a p=0.02 for the binomial test based on previous positives, which was not significant after the Bonferroni correction (Table 3).

Table 3. Plant-level persistence metrics resulting from binomial tests based on previous positive results as a risk factor.

Ribotype[a]	++ count[b]	+- count[b]	-+ count[b]	-- count[b]	Relative risk (95% confidence interval)[c]	Fisher's exact test (right, one-tail) significance[d]
Plant D						
1039C	2	5	6	127	6.33 (1.55, 25.9)	5.2×10^{-2}
1045A	1	0	1	138	139 (19.7, 980)	1.4×10^{-2}*
1043A	1	3	3	133	11.3 (1.48, 86.6)	1.1×10^{-1}
Plant N						
1042A	4	5	5	82	7.73 (2.51, 23.7)	3.8×10^{-3}*
1062A	1	1	1	93	47 (4.30, 513)	4.1×10^{-2}
116-1451-S2	1	1	0	94	cannot be calculated	2.1×10^{-2}

[a] Ribotypes are abbreviated without the "DUP-" prefix.

[b] For the specific ribotype at every site, these are the number of transitions from one status (positive or negative) to another status (positive or negative). For example, '++' indicates the number of times a positive result was followed by a positive result, while '+-' indicates the number of times a positive result was followed by a negative result (see Fig. 1 for a detailed illustration).

[c] Relative risk in this case is the probability of a positive (given a previous positive) divided by the probability of a positive (given a previous negative).

[d] * indicates statistical significance. Bonferroni's correction was applied to measures from each plant; significance threshold for both plants was 0.017 (0.05/3).

Overall, these analyses provide strong support for persistence of ribotype DUP-1042A in plant N (which showed significant support for persistence with two distinct statistical methods). Persistence of ribotypes DUP-1039C and DUP-1043A in plant D and ribotype 116-1451-S2 in plant N was supported by only the binomial test based on ribotype frequencies, while persistence of DUP-1045A in plant D was supported only by the binomial test based on previous positives.

Ribotype results from the initial four months suggested potential sites and areas of persistence. As plant-level analyses provided evidence for persistence of specific ribotypes in each of the two plants, we also used the binomial test based on ribotype frequencies to identify specific sampling sites that show evidence for persistent isolation of a given ribotype; these data may provide initial evidence on an approximate location of a specific sampling site where a given ribotype persists (i.e., a niche). In plant D, a given ribotype was isolated more than once at five sampling sites (D-2, D-7, D-14, D-15 and D-18; see Fig. 2 and Table 4), providing initial descriptive evidence for persistence. Among these sampling sites, binomial tests (with a $P<0.01$, i.e., after Bonferroni's correction) found significant evidence for persistence of DUP-1043A at sampling site D-15 (under both reference distributions, see Table 4); ribotype DUP-1043A was isolated from this site at 3 of the 4 sampling dates. In addition, these tests found significant evidence for persistence of DUP-1045A at sampling site D-7 (this site was only significant at $p<0.01$ under the reference distribution that used ribotype data for 159 isolates from other fish plants; see Table 4). Ribotype DUP-1043A was isolated from sampling site D-7 on two of the first four sampling dates.

Table 4. Sampling site-level persistence metrics, after four months, using a binomial test based on ribotype frequencies.

Site	Ribotype	Site-level frequency[a]	Significance (all ribotypes, p-value)[b,c]	Significance (other fish plants, p-value)[b,d]
Plant D				
D-15 Oven Floor Drain	DUP-1043A	6.7×10^{-1} (2/3)	1.53×10^{-3}*	1.18×10^{-4}*
D-7 Cutting Table	DUP-1045A	1.0 (1/1)	1.59×10^{-2}	6.29×10^{-3}*
D-2 Fork Truck Bars	DUP-1043A	5.0×10^{-1} (1/2)	4.50×10^{-2}	1.25×10^{-2}
D-18 Cooler Drain	DUP-1039C	3.3×10^{-1} (1/3)	2.97×10^{-1}	1.77×10^{-1}
D-14 Cooler Floor Drain	DUP-1039C	3.3×10^{-1} (1/3)	2.97×10^{-1}	1.77×10^{-1}
Plant N				
N-2 Trench Drain	DUP-1042A	1.0 (3/3)	8.29×10^{-6}*	2.49×10^{-7}*
N-15 Oven Drain	116-1451-S2	5.0×10^{-1} (1/2)	4.59×10^{-4}*	1.25×10^{-2}
N-12 Trench Drain	DUP-1042A	1.0 (1/1)	2.02×10^{-2}	6.29×10^{-3}*
N-19 Trash Can Bottoms	DUP-1042A	1.0 (1/1)	2.02×10^{-2}	6.29×10^{-3}*
N-24 Slicer Outbound Belt	DUP-1062A	1.0 (1/1)	6.48×10^{-2}	6.29×10^{-3}*

[a] Sampling site-level frequency is the number of isolates of the given ribotype at the sampling site excluding the first, divided by the number of *L. monocytogenes* isolates at the sampling site excluding the first.
[b] * indicates statistical significance. Bonferroni's correction was applied to measures from each plant; significance level 0.01 (0.05/5).
[c] Significance indicates that the observed frequency of the given ribotype among *L. monocytogenes* positive samples exceeds the ribotype frequency from the reference distribution of all ribotypes in the Pathogen Tracker database.
[d] Significance indicates that the observed frequency of the given ribotype among *L. monocytogenes* positive samples exceeds the ribotype frequency from the reference distribution of other fish plants in the Pathogen Tracker database.

In plant N, a given ribotype was isolated more than once at five sampling sites (sites N-2, N-12, N-15, N-19 and N-24; see Fig. 3 and Table 4). Among these sampling sites, the binomial test based on ribotype frequencies (with a $P<0.01$, i.e., after Bonferroni's correction) indicated significant evidence for persistence of DUP-1042A at sampling site N-2 (under both reference distributions, see Table 4); ribotype DUP-1042A was isolated from this sampling site on all four sampling dates. In addition, the binomial test based on reference distributions found significant evidence for persistence of 116-1451-S2 at sampling site N-15 (this site was significant at $p<0.01$ only under the reference distribution that used ribotype data for all 4,398 isolates). This binomial test also found significant evidence for persistence of DUP-1042A at sites N-12 and N-19, and DUP-1062A at site N-24; these sites were significant at $p<0.01$ only under the reference distribution that used ribotype data for 159 isolates from other fish plants.

Sequential sampling confirmed *L. monocytogenes* persistence on a food contact surface. *L. monocytogenes* ribotype DUP-1062A was isolated from a food contact surface in plant N (sampling site N-24) in both months 1 and 2 (Fig. 3). Thus, in month 3, we sampled site N-24 as well as two sampling sites in close proximity (sampling sites N-25 and N-26), at multiple times, including (i) before operations (after cleaning and sanitation); (ii) after the equipment was run without food products; and (iii) subsequently, hourly throughout the processing shift. In order of process flow, sampling site N-25 is a table, followed by N-26 which represents an incoming belt and a central belt in the equipment; N-24 is the subsequent outbound belt. An isolate with DUP-1062A was obtained from the sample collected from site N-26 after the equipment was run for 15 min without product. In addition, DUP-1062A was isolated from two of the eight site N-24 samples collected during the processing shift. As these

results suggested *L. monocytogenes* persistence at this sampling site, parts of the equipment were subsequently replaced, and DUP-1062A was no longer isolated from sites N-24, N-25 or N-26.

Vector swabbing led to repeated positive samples below a cooler door in plant N. The analyses of the four months sampling data (Figs. 2 and 3) were also used to identify sampling sites for vector swabbing in months 5, 7, and 8; sites selected included four sites in plant D (D-6, D-14, D-15, and D-18) and three sites in plant N (N-2, N-15, and N-24, Table 5). Samples from site D-6 were positive for ribotypes DUP-1052A, DUP-1041A, and DUP-1039C in months 2, 3, and 4; this site was included in vector swabbing due to high *L. monocytogenes* prevalence rather than evidence of persistence. Vector swabs from sampling site D-6 were positive for *L. monocytogenes* in all three months of vector swabbing (for a total of 9 positive samples among 16 samples); however, positive samples resulted in six different ribotypes, and no clear pattern of persistent contamination was evident. Drain D-18 was positive for DUP-1039C in the drain and in three of four floor samples (representing vector samples) in month 5. Consequently, the sanitation staff was retrained and weekly drain and floor cleaning was intensified; in months 7 and 8, all vector swab results around D-18 were negative. Vector swabs from D-14 and D-15 were all negative with a single exception at D-14 (Fig. 2).

Table 5. Vector swabbing results for plants D and N in months 5, 7 and 8.

	Sub-site(s) (number of samples): result ribotype [a]		
Site	Month 5	Month 7	Month 8
D-6	Adjacent floor (2): 1062A Adjacent floor (3): neg.	Drain (1): 1043A Cart (1): 1451 Floor (1): 1062A Other vector swabs (2): neg.	Drain (1): 1039C Floor (1): 1043A White tub (1): 1039C Wall (1): 1043A Other vector swabs (2): neg.
D-14	Adjacent floor (1): 1043A Adjacent floor (4): neg. Floor adjacent to D-13 (5): neg.	Vector swabs (4): neg.	Vector swabs (5): neg.
D-18	Adjacent floor (4): 1039C Adjacent floor (1): neg.	Vector swabs (6): neg.	Vector swabs (5): neg.
D-15	(unavailable)	Vector swabs (7): neg.	Vector swabs (6): neg.
N-2	Adjacent floor (1): 1042A Adjacent floor (4): neg. Floor adjacent to N-4 (1): 1062A Floor adjacent to N-4 (4): neg.	Under cooler door (1): 1042A Floor (1): 1042A White tub (1): 1042A Other vector swabs (3): neg.	Drain (pre-clean) (1): 1042A Drain (post clean) (1): 1042A Under cooler door (5): 1042A Under cooler door (1): neg. Other vector swabs (4): neg.
N-15	Adjacent floor (1): 1451[b] Adjacent floor (4): 1042A	Floor (1): 1042A Other vector swabs (3): neg.	Drain (1): 1451[b] Rack wheels (1): 1042A Other vector swabs (4): neg.
N-24	Equipment parts (1): 1042A Other vector swabs (2): neg.	Floor (1): 1042A Other vector swabs (6): neg.	Drain (1): 1042A N-26 (1): 1042A Other vector swabs (6): neg.

[a] "Sub-sites" are sites near the baseline sampling site which were sampled during vector swabbing. Numbers in parentheses indicate the number of samples. "Vector swabs" encompass multiple sampling results when all the vector swabbing samples were *L. monocytogenes* negative ("neg."). For positive samples, ribotypes are shown (displayed without "DUP-" prefix); negative samples are indicated by "-."
[b] Ribotype 116-1451-S2 is abbreviated as "1451."

At plant N, vector swabbing at sampling site N-2, a trench drain in the raw processing area, isolated ribotype DUP-1042A in 1 of 5 samples, 3 of 6 samples, and 7 of 14 samples in months 5, 7 and 8, respectively (Table 5). One of the positive vector swabs from N-2 in month 7 was taken from flexible skirt at the base of a nearby cooler door. This cooler door is located within three feet of the drain N-2, and the flexible skirt at the base of the door helps seal the door when it is closed. After a positive result from the base of the cooler door in month 7, six samples were taken from the base of the cooler door in month 8; five of these were positive for ribotype DUP-1042A. Subsequent inspection of the cooler door revealed wet plywood clad with stainless steel on the lower part of the door, which was not hermetically sealed. As this was identified as a possible niche, the plywood was replaced with nonabsorbent synthetic material; however tests on the plywood removed from the door and three similar cooler doors in plant N were negative for *L. monocytogenes.* Based on the occurrence of *L. monocytogenes* on the skirt at the base of the cooler door in plant N, similarly-constructed doors were also sampled at plant D: three of the six samples were positive for *L. monocytogenes*.

Vector swabbing was also performed at oven drain N-15 in month 5; ribotypes DUP-1042A and 116-1451-S2 were isolated in 4/5 and 1/5 samples, respectively. In month 7, DUP-1042A was isolated from a swab of the adjacent floor, and in month 8, 116-1451-S2 was isolated from the drain and DUP-1042 was isolated from a cart wheel. Though subsequent samples from site N-15 collected in months 9 through 12 were positive for *L. monocytogenes*, including isolates of DUP-1042A, the most prevalent ribotype in the plant, *L. monocytogenes* was not eliminated at this sampling site. Ribotype 116-1451-S2 was not isolated in plant N after month 8.

Descriptive analysis suggests short- and long-term persistence of *L. monocytogenes* in both plants. Over the twelve months of the study, the selected high-

risk sampling sites at plant D exhibited presence of *L. monocytogenes* in 85 of 395 baseline samples (i.e., both 6- and 12-months baseline samples, excluding adaptive sampling; see Figures 2 and A1). Ribotypes DUP-1039C, DUP-1043A and DUP-1062A were isolated over periods of 12, 12, and 10 months, respectively. Among these ribotypes, DUP-1039C and DUP-1043A had been identified previously at this plant *(22, 23, 31, 46, 62)*, suggesting long term persistence. When sampling results were analyzed according to their location with respect to product flow (from raw material to finished product), plant D had only a single positive sample in the final 31 sampling sites of the process, and zero positive samples among the final 24 sampling sites in the process (Fig. 2).

Over twelve months, *L. monocytogenes* was isolated from in 51 of 267 plant N baseline samples (i.e. both 6- and 12-months baseline samples, excluding adaptive sampling; see Figures 3 and A2). Ribotype DUP-1042A was identified every month, while DUP-1062A and 116-1451-S2 were found over seven and five month intervals, respectively; these ribotypes were the most frequently isolated ribotypes among the total of eight different ribotypes isolated at plant N. Ribotype DUP-1042A alone represented 78% (40 of 51) of *L. monocytogenes* isolates from plant N that were ribotyped.

Persistent ribotypes represent the majority of *L. monocytogenes* positive samples in both plants. In plant D, *L. monocytogenes* ribotypes DUP-1039C (31 of 85 isolates), and DUP-1043A (17 of 85 isolates), and DUP-1045A (4 of 85 isolates) were identified as persistent based on one or both binomial tests; thus 61% (52 of 85) of *L. monocytogenes* positive baseline samples were due to these persistent ribotypes. Among all samples positive for ribotypes DUP-1039C, DUP-1043A, and DUP-1045A, no samples were from food contact surfaces that are exposed to RTE products after the smoking process.

For plant N, ribotypes DUP-1042A and 116-1451-S2, representing 40 and 3 of 51 positive baseline samples, respectively, were identified as persistent, representing 84% (43 of 51) of the *L. monocytogenes* positive baseline samples. Among all baseline samples positive for ribotypes DUP-1042A and 116-1452-S2, there were no positive samples from food contact surfaces that are exposed to RTE products after the smoking process. However, on two of twelve sampling dates, sequential sampling of finished product food contact surfaces resulted in isolation of DUP-1042A, the most prevalent ribotype identified in plant N. While isolation of DUP-1062A on a food contact surface in two consecutive months suggested short term persistence, the binomial test based on ribotype frequencies and the binomial test based on previous positive results were not significant for DUP-1062A (Tables 2 and 3).

Prevalence reductions of individual ribotypes at individual sampling sites in response to interventions were not significant, but *L. monocytogenes* prevalence among 12-months baseline sampling sites showed borderline significant decreases. Interventions were implemented in both plants directed toward sampling sites associated with high *L. monocytogenes* prevalence and multiple occurrences of a specific ribotype (Table 6). Fisher's exact test was applied to the results at the sampling site level before and after interventions to assess reduction of persistent ribotypes. Due in part to comparatively few (12) samples per sampling site and many (20) sampling sites undergoing Fisher's exact test, no site prevalence reductions were significant after applying Bonferroni's correction. Based on a regression analysis of overall *L. monocytogenes* prevalence among 12-months baseline samples by month, Plant D showed a significant monthly change of -5.87% (95% confidence interval: -10.17%, 0.01%; p= 0.026) based on the 12-months baseline samples. Plant N showed a similar monthly change of -5.88% (95% confidence interval: -12.1%, 0.01%); however this change was not significant (p=0.076).

Table 6. Listeria monocytogenes control measures implemented in Plants D and N.

Control Measures	Month[a]	Key Sites	Key Subtype(s) of Interest	Ribo-type pre-valence before[b]	Ribo-type pre-valence after[c]	Fisher's Exact Test (p-value)[d]
Plant D						
Dry quaternary ammonium granules used on floors	1	D-21	116-1612-S1	1/1	0/11	0.083
Start treating drains with peracetic acid foam	2	D-6	DUP-1052A	1/2	0/10	0.17
		D-13	DUP-1039C	1/2	1/10	0.32
		D-14	DUP-1039C	2/2	1/10	0.046
		D-15	DUP-1043A	2/2	3/10	0.15
		D-18	DUP-1039C	2/2	0/10	0.015
Initiate weekly cleaning of drains with foamers, detergent and water	3	D-6	DUP 1052A	1/3	0/9	0.25
		D-13	DUP 1039C	1/3	1/9	0.45
		D-14	DUP-1039C	2/3	1/9	0.13
		D-15	DUP-1043A	2/3	3/9	0.36
		D-18	DUP-1039C	2/3	0/9	0.046
Changed temperature control in raw processing (wet) room from ambient to 50 °F (10 °C)	4	D-2	DUP-1043A	2/4	1/8	0.24
		D-4	DUP-1039C	1/4	1/8	0.58
		D-7	DUP-1045A	2/4	0/8	0.091
		D-10	DUP-1039C	1/4	2/8	0.75
		D-46	DUP-1043A	1/4	0/5	0.44
Improved forklift fork sanitation	5	D-2	DUP-1043A	3/5	0/7	0.046
Intensified room cleaning and sanitation, raw area #3	9	D-6	DUP-1039C	4/9	1/3	0.63
		D-5	DUP-1039C	2/9	1/3	0.87
Daily drain foaming instead of weekly	10	D-6	DUP-1039C	3/10	1/2	0.91
		D-14	DUP-1039C	3/10	0/2	0.55
		D-15	DUP-1043A	5/10	0/2	0.32
		D-18	DUP-1039C	2/10	0/2	0.68
Plant N						
Defunct machine removed, next to drain N-2	3	N-2	DUP-1042A	3/3	9/9	1.0
		N-4	DUP-1042A	1/3	0/9	0.25
Acquired smaller brushes for cleaning	3	N-24	DUP-1062A	2/3	0/9	0.046
Replaced parts at food contact surfaces	4	N-24	DUP-1062A	2/4	0/8	0.091
Change drain cleaning from weekly to daily	9	N-18	DUP-1042A	1/9	0/3	0.75
Cleaned door base	9	N-2	DUP-1042A	9/9	3/3	1.0
Switch to dry cure	10	N-15	DUP-1042A	5/10	0/2	0.32
			116-1451-S2	3/10	0/2	0.55
Replaced wood from cooler door bases with plastic	10	N-2	DUP-1042A	10/10	2/2	1
		N-23	DUP-1042A	2/10	0/2	0.68

[a] The month indicated is the last month sampled prior to intervention (*L. monocytogenes* control measure).
[b] "Ribotype prevalence before" is the number of times the isolate was of the given ribotype out of the number of samples, for the period before to the intervention.
[c] "Ribotype prevalence after" is the number of times the isolate was of the given ribotype out of the number of samples, for the period after the intervention.
[d] No tests significant after Bonferroni correction (23 tests at plant D [p-value 0.002] , 10 tests at plant N [p-value 0.005]).

Discussion

Post-processing cross-contamination from environmental sites is well-established as the key source of *L. monocytogenes* in RTE foods *(3, 15, 21, 22, 31, 42, 43, 53, 62, 66, 69).* Persistence of *L. monocytogenes*, over months to years, in food processing environments has been identified as one of the most important sources of *L. monocytogenes* that cross contaminate finished products *(5, 34, 40)*. Industry has taken multiple approaches to address this issue, including strategies for environmental *Listeria* and *L. monocytogenes* monitoring of production environments as well as specific strategies to detect and identify *L. monocytogenes* niches (e.g., the "Seek and Destroy" strategy). While a number of published studies have reported identification of persistent *L. monocytogenes* populations in processing plants *(4, 5, 19, 20, 35, 49, 52, 62),* limited peer reviewed data are available on the evaluation of sampling plans for their ability to identify niches that can be successfully targeted to eliminate persistent *L. monocytogenes*, particularly in the context of small to medium plants. To address this knowledge gap, environmental sampling plans (with monthly sampling over twelve months) were implemented in two smoked fish plants to identify persistent contamination and to develop interventions. Ribotyping of *L. monocytogenes* was performed to identify persistent populations, and these data were used to identify sampling sites for additional sequential sampling and vector swabbing aimed at identifying niches. Statistical evaluation of these data with both a binomial test based on ribotype frequencies and a binomial test based on previous positives allowed for statistically-based identification of persistent *L. monocytogenes* subtypes. At a plant level, our data specifically showed that (i) continuous improvement and implementation of controls over the sampling period reduced *L. monocytogenes* prevalence at 12-months baseline sites over time; (ii) while a site of persistence could be identified and eliminated on a food contact surface in one plant,

persistence sites on non-food contact surfaces were difficult to identify and eliminate; (iii) despite presence of persistent *L. monocytogenes* ribotypes in non-food contact surface areas, these ribotypes were rarely found on finished product food contact surfaces. Our data further show the continued challenge of implementing and developing economically feasible sampling plans and control strategies that can eliminate *L. monocytogenes* niches, particularly in non-food contact surface sites in small and medium size plants. Preventing (i) persistence on food contact surfaces and (ii) transfer of persistent ribotypes from non-food contact surfaces to food contact surfaces, in contrast, appears more easily achievable.

Both the binomial test based on ribotype frequencies and the binomial test based on previous positives offer viable statistical test strategies to identify persistent *L. monocytogenes* subtypes. *L. monocytogenes* persistence as a concept and the specific issue of *L. monocytogenes* persistence in food processing plants have been well described by a number of authors *(33, 36, 46, 57)*. Previous studies typically used ad hoc, non-statistically-based approaches to identify a certain subtype as persistent *(4, 8, 13, 35, 41)*; commonly used criteria to classify a subtype as persistent include re-isolation of a given subtype in a certain plant or other food associated environment at least two or three times over a certain time period (often the time period over which a study was conducted). These approaches do not consider that re-isolation of a subtype, particularly a common subtype, with long intervening times, may also represent re-introduction. To overcome this issue, we applied two statistical tests, a binomial test based on ribotype frequencies and a binomial test based on previous positives, to identify persistent ribotypes. For the binomial test based on ribotype frequencies, two ribotype reference distributions from the Pathogen Tracker database were used, one based on all ribotypes in the database and one based on ribotypes from other fish processing plants. Both of the statistical methods identified ribotype DUP-1042A in

plant N as persistent. With the binomial test based on ribotype frequencies alone, additional ribotypes were identified as persistent, including (i) DUP-1039C and DUP-1043A in plant D, and (ii) 116-1451-S2 in plant N (Table 2). The binomial test based on previous positive results also identified DUP-1045A as persistent in plant D. Among these, ribotype DUP-1039C in plant D, and ribotypes DUP-1043A and DUP-1042A in plant N were found over the entire 12 months, supporting initial statistical identification of these ribotypes as persistent.

While our data support that both a binomial test based on ribotype frequencies and a binomial test based on previous positives offer viable statistical test strategies to identify persistent ribotypes, these methods differ conceptually and hence may differ with regard to situations where they are appropriate. Conceptually, the binomial test based on ribotype frequencies determines whether the frequency of isolation of a given ribotype in a plant or at a given site is significantly higher than expected based on the reference distribution. While the binomial test based on ribotype frequencies takes into account whether a particular ribotype is rare or common, it requires a reference distribution for the ribotypes of interest, which may be difficult to create or assess. In addition, ribotype frequencies may differ considerably between source populations, for example ribotype frequencies among isolates from humans may differ from ribotype frequencies among isolates from different foods *(17)* and an inappropriate ribotype reference distribution may impact the outcomes of the test. On the other hand, the binomial test based on previous positives evaluates whether previous isolation of a given ribotype represents a risk factor for a subsequent isolation of the same ribotype. The binomial test based on previous positives (i) is likely to require a larger sample size to provide meaningful results for a single site*(16),* and (ii) does not weigh prevalence directly as an indication of persistence. Hence, the binomial test based on ribotype frequencies may be more appropriate when few

positive samples were obtained, and a reference distribution, which is representative of the environment under study, is available. On the other hand, the binomial test based on previous positives may be more appropriate when (i) larger numbers of samples are available, (ii) repeated ribotype positives are present, and (iii) no appropriate reference distribution is available and suited to the source of the samples under study.

Importantly, the tests detailed above start to address the issue of differentiating between persistence in a given facility and repeated re-introduction. Significant evidence for persistence, by the tests described here, suggests that re-isolation of a given subtype was not a random event, such as random re-introduction from outside sources. On the other hand, a significant test statistic in either of the two tests described here could represent repeated reintroduction from an outside source where a given subtype persists. Use of sampling strategies that can differentiate between these two possibilities is thus required to determine whether repeat isolation truly reflects persistence in a processing facility. In particular, sampling after completion of cleaning and sanitation (including possible intensive cleaning) and before start-up of production can provide clear evidence for in-plant persistence, i.e., if a persistent subtype is isolated after sanitation and before production start-up. The importance of this is illustrated by our data for plant D, which suggested frequent reintroduction of ribotype DUP-1045A from raw materials, even though this could not be unequivocally proven.

Continuous improvement and implementation of controls over the 12-months sampling period reduced *L. monocytogenes* prevalence over time. Analyses of *L. monocytogenes* prevalence among sites that were sampled over the complete 12 months (representing high risk sampling sites, as sampling sites that were consistently negative after the initial 6 months were not sampled over the reminder of the study) showed an average monthly prevalence reduction of about 6% in both plants; this

reduction was significant in plant D. These findings are consistent with previous studies, which also reported significant reductions in *L. monocytogenes* prevalences among environmental samples in smoked fish processing plants after implementation of stringent environmental sampling protocols that employed molecular subtyping tools to identify persistent *L. monocytogenes (3, 31)*. Reductions of *L. monocytogenes* prevalence in environmental samples after implementation of intensive sampling protocols incorporating molecular subtyping have also been reported for ready-to-eat meat *(64, 69)* and dairy processing facilities *(21, 45)*. For example, in a dairy processing facility, increased ribotype prevalence in proximity to a footbath was remedied by checking the footbath's sanitizer concentration and changing the footbath sanitizer more often *(45)*. As a result, the specific ribotype was not isolated from future samples from sites which were previously positive for that ribotype.

While our data as well as previous data clearly demonstrate that intensive environmental sampling plans and concomitant implementation of improved *L. monocytogenes* controls can facilitate reduction of *L. monocytogenes* prevalence in food processing environments, identification of specific practices that are responsible for improved control of *L. monocytogenes* is, in most cases, challenging. For example, both plants studied here implemented a large number of practices aimed at reducing *L. monocytogenes* prevalence in general and aimed at eliminating potential niches. Except for apparent elimination of a specific *L. monocytogenes* niche in a food contact surface in plant N (discussed below), we were not able to definitively link specific practices to the reduction in *L. monocytogenes* prevalences observed in both plants. In contrast, some studies linked specific interventions directly to reduced *L. monocytogenes* prevalence in a plant or at specific sampling sites. For example, Lappi et al. *(31)* reported reduced *L. monocytogenes* prevalence on the wheels of

rolling carts in a plant after a door foamer was installed at the entrance to a finished product area.

While a site of persistence could be identified and eliminated on a food contact surface in one plant, persistence sites on non-food contact surfaces were difficult to identify and eliminate. Overall, we not only identified persistent *L. monocytogenes* ribotypes in both plants D and N, but also identified potential sites or areas where a given ribotype appeared to persist. Differentiation of niches (sites which are continuously contaminated) from transfer sites (surfaces which are regularly, but not continuously, contaminated) is challenging if samples are collected during plant operation, as in this case repeat isolation of the same subtype at a site may not necessarily indicate that the subtype persists at this given sampling site. Rather, repeat isolation of a given subtype, e.g. in a drain that is sampled during mid-operation, may indicate re-introduction during processing from a nearby site. Differentiation of transfer sites and growth niches can be achieved by testing of samples collected pre-operationally as well as by vector swabbing. For example, at Plant N, pre-operational sampling identified the same ribotype that we had previously isolated twice on a food contact surface (DUP-1062A), suggesting persistence at this site. Following replacement of food contact surface belts identified as likely growth niche based on pre-operational sampling, ribotype DUP-1062A was not isolated anymore from food contact surfaces, suggesting elimination of the niche harboring this subtype. This subtype was isolated from two non-food contact surfaces in two different rooms in months 4 and 7 though, which is likely to be unrelated as this ribotype is one of the most common ribotypes, representing 6.5% of 4,397 *L. monocytogenes* isolates in our reference distribution. Similarly, other studies have reported elimination of *L. monocytogenes* niches based on environmental sampling and subtype data *(3, 28).* For example, Keto-Timonen et al. *(28)* reported strain persistence based on amplified

fragment length polymorphism (AFLP) data in a chilled food processing plant; production line reconstruction decreased *L. monocytogenes* prevalence and eliminated two persistent AFLP types.

In our analysis of the initial four months sampling data, we also identified nine non-food contact surface sites where the same ribotype was isolated at least twice (five and four sampling sites in plants D and N, respectively); six of these sampling sites were drains, the other sampling sites were a raw material cutting table, forklift forks, and trash can bottoms. Based on pre-operational sampling, four of these sampling sites were identified as likely sites of persistence. For example, in plant N, a trench drain that was positive for ribotype DUP-1042A was also positive for this subtype in one pre-operational sampling. In addition, vector swabbing identified the bottom of a nearby sliding cooler door as a potential niche. Despite a number of control strategies implemented in order to eliminate ribotype DUP-1042A in plant N, this subtype was isolated throughout the full 12-months duration of this study. Specific interventions targeting this subtype included intensified cleaning and sanitation schedules for drains (including the trench drain where DUP-1042A was isolated consistently), as well removal of defunct equipment close to this drain (representing another potential niche) and remodeling a cooler door near the trench drain to eliminate potential sites on this door that could represent growth niches. These data not only illustrate how environmental testing results and subtyping data can facilitate identification of potential niches where *L. monocytogenes* persists, but also show the difficulties associated with eliminating niches, particularly those associated with floor drains or other facility features that cannot easily be removed or replaced. Consistent with our observations, others *(18, 20, 30)* have reported situations where implementation of multiple controls and changes has not eliminated an apparent *L. monocytogenes* niche.

Previous studies have identified drains as high prevalence sites and potential niches *(22, 31, 56, 69)*. Similarly, our data show relatively high *L. monocytogenes* prevalence from drains, and repeated isolation of the same ribotype from some drains in both plants. For example, in addition to a drain in plant N where the same ribotype was isolated in 12 of 12 samplings, plant D included two drains where ribotype 1039C was isolated in 4 of the 12 samplings as well as 3 drains where ribotype DUP-1039C was isolated in 3 of the 12 samplings. While this is similar to a number of previous studies *(22, 31, 62)*, we were unable to definitively eliminate any ribotype that was repeatedly isolated from drains. One study reported successful elimination of a persistent ribotype which was found regularly in a drain; this study used ovens, hot water, gas flame, and steam to eliminate niches *(3)*. Overall, defining the precise role of drains in persistence of *L. monocytogenes* in different processing plants remains a challenge. One model is that drains predominantly act as sinks (recipients), suggesting that repeat isolation of a given subtype from a drain typically is due to frequent (or almost constant) re-introduction of this subtype from a niche in the surrounding environment. If reintroduction is frequent enough and also occurs outside of regular processing (e.g., through condensation), re-isolation of the same subtype can even occur in pre-op samples, a strategy that typically is used to differentiate transfer points and niches *(60)*. In an alternative model, drains represent the actual growth niches and thus are the sources (donors) of the persistent subtypes. Testing this hypothesis in commercial operations is virtually impossible as it is difficult to clean and sanitize a drain and all subsequent drain components (i.e., drainpipes all the way to final barrier separating the plant from the sewer system). While improved cleaning and sanitation solutions for drains may facilitate elimination or at least control of persistent *L. monocytogenes*, sanitary design of drain systems will be critical to offer a long term solution for improved control of *L. monocytogenes*.

Consistent with previous studies *(19, 20, 29, 31)*, we found elimination of persistent *L. monocytogenes* to be extremely challenging. Nevertheless, we found a reduction in the number of positive samples for a given, presumably persistent, subtype can also often be observed despite the fact that this subtype has not been completely eliminated from a plant. These types of data suggest that a given plant may be able to manage, but not eliminate, a persistent subtype.

Despite presence of persistent *L. monocytogenes* ribotypes in non-food contact surface areas, these ribotypes were rarely found on finished product food contact surfaces. While both plants showed persistence of specific *L. monocytogenes* strains on non-food contact surfaces, the persistent ribotypes were rarely present in samples from food contact surfaces subsequent to the smoking process in the product flow. At one plant, no samples from food contact surfaces subsequent to smoking were positive for *L. monocytogenes*; at the other plant, ribotypes associated with persistent contamination were isolated from food contact surfaces subsequent to smoking in the product flow on two sampling dates. This is consistent with a study of three Latin-style fresh cheese processing plants, where Kabuki et al. *(27)* found *L. monocytogenes* positive finished products and food contact surfaces in only one of three plants, despite the fact that all three plants isolated *L. monocytogenes* from non-food contact environmental samples. In a study of 21 dairy processing plants, Pritchard et al. *(54)* found a significantly higher *L. monocytogenes* prevalence among "environmental" samples in contrast to "equipment" samples. Overall, these data suggest that at least some plants can successfully compartmentalize product processing steps and implement appropriate GMPs and other control strategies to minimize transfer of persistent *L. monocytogenes* from non-food contact surfaces to food contact surfaces and/or RTE foods.

Conclusions. We implemented statistical approaches that can facilitate objective and quantitative assessments of bacterial persistence, using subtyping data. Use of these approaches on an initial sample data set, i.e., monthly sampling data for four months, corresponded well with persistence assessment based on data for the full study duration of 12 months. The initial statistical approaches described here hopefully will give rise to additional efforts to use enhanced data analyses, potentially incorporating spatial as well as temporal parameters, to assess evidence for persistence and to also help with identification of sites or areas where these strains are most likely to persist. Integration of these approaches into sampling plan and "seek-and-destroy" strategies *(7)* should further facilitate detection and eradication of persistent *L. monocytogenes* contamination and may provide blue prints for sampling and analytic strategies to detect persistence of other foodborne pathogens (e.g., *Salmonella)* and spoilage organisms.

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REFERENCES

1. Aarnisalo, K., S. Sheen, L. Raaska, and M. Tamplin. 2007. Modelling transfer of *Listeria monocytogenes* during slicing of 'gravad' salmon. *Int. J. Food Microbiol.* 118:69-78.

2. Almond Board of California. 2010. Pathogen environmental monitoring program (PEM). Available at: http://ucfoodsafety.ucdavis.edu/files/26483.pdf. Accessed 10 April 2012.

3. Autio, T., S. Hielm, M. Miettinen, A. M. Sjöberg, K. Aarnisalo, J. Björkroth, T. Mattila-Sandholm, and H. Korkeala. 1999. Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. *Appl. Environ. Microbiol.* 65:150-155.

4. Autio, T., R. Keto-Timonen, J. Lundén, J. Björkroth, and H. Korkeala. 2003. Characterisation of persistent and sporadic *Listeria monocytogenes* strains by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). *Syst. Appl. Microbiol.* 26:539-545.

5. Berrang, M. E., R. J. Meinersmann, J. F. Frank, and S. R. Ladely. 2010. Colonization of a newly constructed commercial chicken further processing plant with *Listeria monocytogenes*. *J. Food Prot.* 73:286-286.

6. Bruce, J. 1996. Automated system rapidly identifies and characterizes microorganisms in food. *Food Technol.* 50:77-81.

7. Butts, J. 2003. Seek & destroy: identifying and controlling *Listeria monocytogenes* growth niches. Available at: http://www.foodsafetymagazine.com/articlePF.asp?id=3284&sub=sub1. Accessed 26 May 2012.

8. Carpentier, B., O. Cerf. 2011. Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *Int. J. Food Microbiol.* 145:1-8.

9. Cornell University Food Safety Laboratory. 2011. Pathogen Tracker. Available at: http://www.microbetracker.net. Accessed 20 June 2011.

10. Cruz, C. D., G. C. Fletcher. 2011. Prevalence and biofilm-forming ability of *Listeria monocytogenes* in New Zealand mussel (Perna canaliculus) processing plants. *Food Microbiol.* 28:1387-1393.

11. Dass, S. C., E. J. Cummins, and N. Abu-Ghannam. 2011. Prevalence and typing of *Listeria monocytogenes* strains in retail vacuum-packed cold-smoked salmon in the Republic of Ireland. *J. Food Saf.* 31:21-27.

12. Dass, S., N. Abu-Ghannam, S. Antony-Babu, and E. Cummins. 2010. Ecology and molecular typing of *L. monocytogenes* in a processing plant for cold-smoked salmon in the Republic of Ireland. *Food Res. Int.* 43:1529-1536.

13. Dauphin, G., C. Ragimbeau, and P. Malle. 2001. Use of PFGE typing for tracing contamination with *Listeria monocytogenes* in three cold-smoked salmon processing plants. *Int. J. Food Microbiol.* 64:51-61.

14. Elliot, E. 2000. Risk assessment used to evaluate the US position on *Listeria monocytogenes* in seafood. *Int. J. Food Microbiol.* 62:253-260.

15. Fenlon, D. R., J. Wilson, and W. Donachie. 1996. The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. *J. Appl. Microbiol.* 81:641-650.

16. Fu, Y., J. Arnold. 1992. A table of exact sample sizes for use with Fisher's exact test for 2 x 2 tables. *Biometrics* 48:1103-1112.

17. Gray, M. J., R. N. Zadoks, E. D. Fortes, B. Dogan, S. Cai, Y. Chen, V. N. Scott, D. E. Gombas, K. J. Boor, and M. Wiedmann. 2004. *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. *Appl. Environ. Microbiol.* 70:5833-5841.

18. Gudmundsdóttir, S., S. M. Roche, K. G. Kristinsson, and M. Kristjansson. 2006. Virulence of *Listeria monocytogenes* isolates from humans and smoked salmon, peeled shrimp, and their processing environments. *J. Food Prot.* 69:2157-2157.

19. Gudmundsdóttir, S., B. Gudbjörnsdóttir, H. Einarsson, K. G. Kristinsson, and M. Kristjansson. 2006. Contamination of cooked peeled shrimp (*Pandalus borealis*) by *Listeria monocytogenes* during processing at two processing plants. *J. Food Prot.* 69:1304-1304.

20. Gudmundsdóttir, S., B. Gudbjörnsdóttir, H. L. Lauzon, H. Einarsson, K. G. Kristinsson, and M. Kristjánsson. 2005. Tracing *Listeria monocytogenes* isolates from cold-smoked salmon and its processing environment in Iceland using pulsed-field gel electrophoresis. *Int. J. Food Microbiol.* 101:41-51.

21. Ho, A. J., V. R. Lappi, and M. Wiedmann. 2007. Longitudinal monitoring of *Listeria monocytogenes* contamination patterns in a farmstead dairy processing facility. *J. Dairy Sci.* 90:2517-2524.

22. Hoffman A.D, Gall K.L, Norton D.M, and Wiedmann M. 2003. *Listeria monocytogenes* contamination patterns for the smoked fish processing environment and for raw fish. *J. Food Prot.* 66:52-52.

23. Hu, Y., K. Gall, A. Ho, R. Ivanek, Y. T. Gröhn, and M. Wiedmann. 2006. Daily variability of *Listeria* contamination patterns in a cold-smoked salmon processing operation. *J. Food Prot.* 69:2123-2123.

24. Hudson, J. A., S. M. Avery. 1993. Presence and coincidence of *Listeria* spp., motile aeromonads and *Yersinia enterocolitica* in a cold-smoked salmon processing and packing plant. *N. Z. Vet. J.* 41:78-78.

25. ILSI Research Foundation, Risk Science Institute. 2005. Achieving continuous improvement in reductions in foodborne listeriosis--a risk-based approach *J. Food Prot.* 68:1932-1994.

26. Jeffers, G. T., J. L. Bruce, P. L. McDonough, J. Scarlett, K. J. Boor, and M. Wiedmann. 2001. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. *Microbiology* 147:1095-1104.

27. Kabuki, D. Y., A. Y. Kuaye, M. Wiedmann, and K. J. Boor. 2004. Molecular subtyping and tracking of *Listeria monocytogenes* in Latin-style fresh-cheese processing plants. *J. Dairy Sci.* 87:2803-2812.

28. Keto-Timonen, R., R. Tolvanen, J. Lunden, and H. Korkeala. 2007. An 8-year surveillance of the diversity and persistence of *Listeria monocytogenes* in a chilled food processing plant analyzed by amplified fragment length polymorphism. *J. Food Prot.* 70:1866-1866.

29. Klaeboe, H., B. Lunestad, K. Borlaug, A. Paulauskas, and O. Rosef. 2010. Persistence and diversity of *Listeria monocytogenes* isolates in Norwegian processing plants. *Veterinarija Ir Zootechnika* 50:42-47.

30. Klaeboe, H., O. Rosef, and M. Saebø. 2005. Longitudinal studies on *Listeria monocytogenes* and other *Listeria* species in two salmon processing plants. *Int. J. Environ. Health Res.* 15:71-77.

31. Lappi, V. R., J. Thimothe, K. K. Nightingale, K. Gall, V. N. Scott, and M. Wiedmann. 2004. Longitudinal studies on *Listeria* in smoked fish plants: impact of intervention strategies on contamination patterns. *J. Food Prot.* 67:2500-2500.

32. Lappi, V. R., J. Thimothe, J. Walker, J. Bell, K. Gall, M. W. Moody, and M. Wiedmann. 2004. Impact of intervention strategies on *Listeria* contamination patterns in crawfish processing plants: a longitudinal study. *J. Food Prot.* 67:1163-1163.

33. Lawrence, L. M., A. Gilmour. 1994. Incidence of *Listeria* spp. and *Listeria monocytogenes* in a poultry processing environment and in poultry products and their rapid confirmation by multiplex PCR. *Appl. Environ. Microbiol.* 60:4600-4604.

34. Lawrence, L., A. Gilmour. 1995. Characterization of *Listeria monocytogenes* isolated from poultry products and from the poultry-processing environment by random amplification of polymorphic DNA and multilocus enzyme electrophoresis. *Appl. Environ. Microbiol.* 61:2139-2144.

35. Lunden, J. M., T. J. Autio, A. M. Sjoberg, and H. J. Korkeala. 2003. Persistent and nonpersistent *Listeria monocytogenes* contamination in meat and poultry processing plants. *Journal of Food Protection&# 174;* 66:2062-2069.

36. Lunden, J., M. Miettinen, T. Autio, and H. Korkeala. 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. *J. Food Prot.* 63:1204-1207.

37. McCarthy, S. 1997. Incidence and survival of *Listeria monocytogenes* in ready-to-eat seafood products. *J. Food Prot.* 60:372-376.

38. Miettinen, H., K. Aarnisalo, S. Salo, and A. Sjoberg. 2001. Evaluation of surface contamination and the presence of *Listeria monocytogenes* in fish processing factories. *J. Food Prot.* 64:635-639.

39. Miettinen, M. K., K. J. Björkroth, and H. J. Korkeala. 1999. Characterization of *Listeria monocytogenes* from an ice cream plant by serotyping and pulsed-field gel electrophoresis. *Int. J. Food Microbiol.* 46:187-192.

40. Mirjana, D., R. Anderson, N. Karabasil, P. Natasa, S. Jovanovic, N. Jelena, V. Teodorovic, M. Maja, and S. Dojcinovic. 2011. Environmental prevalence and persistence of *Listeria monocytogenes* in cold-smoked trout processing plants. *Acta Veterinaria-Beograd* 61:429-442.

41. Muraoka W, Gay C, Knowles D, and Borucki M. 2003. Prevalence of *Listeria monocytogenes* subtypes in bulk milk of the Pacific Northwest. *J. Food Prot.* 66:1413-1413.

42. Nakamura, H., M. Hatanaka, K. Ochi, M. Nagao, J. Ogasawara, A. Hase, T. Kitase, K. Haruki, and Y. Nishikawa. 2004. *Listeria monocytogenes* isolated from cold-smoked fish products in Osaka City, Japan. *Int. J. Food Microbiol.* 94:323-328.

43. Nakamura, H., Y. Tokuda, A. Sono, T. Koyama, J. Ogasawara, A. Hase, K. Haruki, and Y. Nishikawa. 2006. Molecular typing to trace *Listeria monocytogenes* isolated from cold-smoked fish to a contamination source in a processing plant. *J. Food Prot.* 69:835-835.

44. Nesbakken, T., G. Kapperud, and D. A. Caugant. 1996. Pathways of *Listeria monocytogenes* contamination in the meat processing industry. *Int. J. Food Microbiol.* 31:161-171.

45. Nightingale, K. K., E. D. Fortes, A. J. Ho, Y. H. Schukken, Y. T. Grohn, and M. Wiedmann. 2005. Evaluation of farm management practices as risk factors for clinical listeriosis and fecal shedding of *Listeria monocytogenes* in ruminants. *J. Am. Vet. Med. Assoc.* 227:1808-1814.

46. Norton, D. M., M. A. McCamey, K. L. Gall, J. M. Scarlett, K. J. Boor, and M. Wiedmann. 2001. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Appl. Environ. Microbiol.* 67:198-205.

47. Ojeniyi, B., H. Wegener, N. Jensen, and M. Bisgaard. 1996. *Listeria monocytogenes* in poultry and poultry products: Epidemiological investigations in seven Danish abattoirs. *J. Appl. Bacteriol.* 80:395-401.

48. Orsi, R. H., M. L. Borowsky, P. Lauer, S. K. Young, C. Nusbaum, J. E. Galagan, B. W. Birren, R. A. Ivy, Q. Sun, and L. M. Graves. 2008. Short-term genome evolution of *Listeria monocytogenes* in a non-controlled environment. *BMC Genomics* 9:539.

49. Ortiz, S., V. López, D. Villatoro, P. López, J. C. Dávila, and J. N. V. Martínez-Suárez. 2010. A 3-year surveillance of the genetic diversity and persistence of *Listeria monocytogenes* in an Iberian pig slaughterhouse and processing plant. *Foodborne pathogens and disease* 7:1177-1184.

50. Ott, R. L., M. T. Longnecker, and J. Miller. 2010. An introduction to statistical methods and data analysis. Brooks/Cole/Cengage Learning, Boston.

51. Pearson, E. S. 1947. The choice of statistical tests illustrated on the interpretation of data classed in a 2 × 2 table. *Biometrika* 34:139-167.

52. Peccio, A., T. Autio, H. Korkeala, R. Rosmini, and M. Trevisani. 2003. *Listeria monocytogenes* occurrence and characterization in meat-producing plants. *Lett. Appl. Microbiol.* 37:234-238.

53. Prencipe, V. A., V. Rizzi, V. Acciari, L. Iannetti, A. Giovannini, A. Serraino, D. Calderone, A. Rossi, D. Morelli, L. Marino, G. Migliorati, and V. Caporale. 2012. Listeria monocytogenes prevalence, contamination levels and strains characterization throughout the Parma ham processing chain. *Food Control* 25:150-158.

54. Pritchard, T. J., K. J. Flanders, and C. W. Donnelly. 1995. Comparison of the incidence of *Listeria* on equipment versus environmental sites within dairy processing plants. *Int. J. Food Microbiol.* 26:375-384.

55. Rorvik, L. M., B. Aase, T. Alvestad, and D. A. Caugant. 2000. Molecular epidemiological survey of *Listeria monocytogenes* in seafoods and seafood-processing plants. *Appl. Environ. Microbiol.* 66:4779-4779.

56. Sauders, B. D., M. D. Sanchez, D. H. Rice, J. Corby, S. Stich, E. D. Fortes, S. E. Roof, and M. Wiedmann. 2009. Prevalence and molecular diversity of *Listeria monocytogenes* in retail establishments. *J. Food Prot.* 72:2337-2337.

57. Senczek, D., R. Stephan, and F. Untermann. 2000. Pulsed-field gel electrophoresis (PFGE) typing of *Listeria* strains isolated from a meat processing plant over a 2-year period. *Int. J. Food Microbiol.* 62:155-159.

58. Serio, A., C. Chaves-López, and A. Paparella. 2011. *Listeria monocytogenes* isolated from the smoked salmon industry: Growth potential under different environmental conditions. *Food Control* 22:2071-2075.

59. Skovgaard, N. 2003. Microorganisms in foods 7: microbiological testing in food safety management. *Int. J. Food Microbiol.* 89:291-292.

60. Smoked Seafood Working Group of the National Fisheries Institute & National Food Processors Association. 2006. *Listeria monocytogenes* control manual draft 9. Available at: http://foodscience.cornell.edu/cals/foodsci/research/labs/wiedmann/upload/SSWGLMManual.pdf. Accessed 25 April 2012.

61. Tavaré, S. 1983. Serial dependence of observations leading to contingency tables, and corrections to chi-squared statistics. *Biometrika* 70:139-144.

62. Thimothe J, Nightingale K.K, Gall K, Scott V.N, and Wiedmann M. 2004. Tracking of *Listeria monocytogenes* in smoked fish processing plants. *J. Food Prot.* 67:328-328.

63. Thimothe, J., J. Walker, V. Suvanich, K. Gall, M. Moody, and M. Wiedmann. 2002. Detection of *Listeria* in crawfish processing plants and in raw, whole crawfish and processed crawfish (*Procambarus* spp.). *J. Food Prot.* 65:1735-1739.

64. Tompkin, R., L. Christiansen, A. Shaparis, R. Baker, and J. Schroeder. 1992. Control of *Listeria monocytogenes* in processed meats. *Food Aust.* 44:370-370.

65. Tompkin, R. 2002. Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* 65:709-725.

66. Tompkin, R., V. N. Scott, D. T. Bernard, W. H. Sveum, and K. S. Gombas. 1999. Guidelines to prevent post-processing contamination from *Listeria monocytogenes*. *Dairy, Food, and Environmental Sanitation* 19:552-562.

67. U. S. Food and Drug Administration. 2011. Regulatory Procedures Manual Available at: http://www.fda.gov/ICECI/ComplianceManuals/RegulatoryProceduresManual/default.htm. Accessed 16 March 2012.

68. U. S. Food and Drug Administration. 2012. Quantitative assessment of relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. Available at: http://www.fda.gov/downloads/food/scienceresearch/researchareas/riskassessmentsafetyassessment/ucm197330.pdf. Accessed 14 April 2012.

69. Williams, S. K., S. Roof, E. A. Boyle, D. Burson, H. Thippareddi, I. Geornaras, J. N. Sofos, M. Wiedmann, and K. Nightingale. 2011. Molecular ecology of *Listeria monocytogenes* and other *Listeria* species in small and very small ready-to-eat meat processing plants. *J. Food Prot.* 74:63-77.

APPENDIX

Figure A1. Plant D *L. monocytogenes* ribotype results from raw fish surfaces and environmental samples. Sample results are presented from top to bottom in a progression from inbound raw product to outbound finished product. "RF Cat." (raw/finished category) indicates "raw" for areas designated for non-smoked fish, "finished" for areas designated for only smoked fish, and "both" for areas designated for non-smoked or smoked fish."Surface" column indicates categories of environmental samples: food contact surfaces (FCS, surfaces in contact with product), drain or floor (DF), floor transfer points (FTP, surfaces of movable objects that contact the floors), employee contact surfaces (ECS, surfaces employees come into contact with), fish, and other. Ribotype results are abbreviated without the "DUP-" prefix. Abbreviated ribotypes include 116-1451-S2, abbreviated "1451," and ribotype 116-1632-S5, abbreviated "1632." "NS" indicates no sample was taken, while '-' indicates an L. monocytogenes negative result. Ribotypes which occurred more than once are color-coded.

Site	Description	RF Cat.	Surface	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb
D-48	salmon fillets surface	raw	fish	-	1062D	-	-	-	-	NS	NS	-	-	NS	-
D-49	salmon fillets surface	raw	fish	-	-	NS	-	-	-	NS	NS	-	-	NS	-
D-50	whole salmon surface	raw	fish	1045A	-	1045A	NS	-	-	NS	NS	-	-	NS	-
D-51	whole salmon surface	raw	fish	-	-	1045A	NS	1045A	-	NS	NS	1027B	-	NS	NS
D-52	whitefish surface	raw	fish	-	NS	-	1044A	-	-	NS	NS	-	1042B	1039C	-
D-45	refuse drain	other	DF	1027B	-	1039A	1039C	1039C	1039C	1042B	-	-	1039C	1043A	-
D-1	cutting table	raw	FCS	-	-	-	-	-	1040A	-	-	-	-	-	1039E
D-2	forklift forks	raw	FTP	1043A	-	1043A	1039C	1043A	-	-	-	-	1039C	-	-
D-3	floor outside oven	raw	DF	-	-	-	-	-	-	-	-	-	-	-	-
D-4	raw processing drain	raw	DF	-	1039C	1039A	-	1043A	-	1062A	1043A	-	1039C	-	1039C
D-5	white plastic tank	raw	other	1052A	-	1039C	-	1039C	1039C	1039C	-	-	1039C	-	1451
D-6	raw processing drain	raw	DF	-	1052A	1041A	1039C	1043A	1039C	1039C	1451	1062A	1062A	1039C	1062A
D-7	raw cutting table	raw	FCS	-	-	1045A	1045A	-	1045A	-	1040A	-	-	1039A	-
D-8	blue hose	raw	FTP	-	-	-	-	1045A	1039C	-	-	-	-	-	-
D-9	white hose	raw	FTP	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-10	cutting table drain	raw	DF	-	-	1062A	1039C	-	1039C	1039C	1040A	-	-	1040A	-
D-46	grease trap	raw	other	-	18616	1030A	1043A	1039C	-	-	-	NS	NS	NS	-
D-18	cooler drain	raw	DF	1039C	1039C	1039A	1062A	-	-	-	-	-	-	-	-
D-11	rolling tank drain	raw	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-12	rolling tank top rim	raw	ECS	-	-	-	NS	-	-	NS	NS	NS	NS	NS	NS
D-13	cooler drain	raw	DF	1039C	1632	-	1053A	-	1041A	1052A	-	-	-	1039C	-
D-14	cooler drain	raw	DF	1039C	1039C	1027A	1062D	1043A	-	1043A	1043A	-	1039C	-	-
D-55	rolling tank wheel	raw	FTP	NS	NS	NS	1039C	1040A	-	1062A	-	-	-	-	-
D-44	brine station floor	raw	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-15	oven drain	both	DF	1043A	1043A	-	1043A	1043A	-	-	-	1043A	-	-	1043A
D-16	oven floor	both	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-17	oven floor	both	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-19	cooler drain	both	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-20	cooler drain	both	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-47	cooler drain	both	DF	-	-	-	-	-	-	-	-	-	-	-	-
D-21	oven-floor junction	both	DF	1054D	-	-	-	-	-	-	-	-	-	-	-
D-22	drain outside ovens	both	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-23	finish process site 1	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-24	finished processor gloves	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-25	finished drain	finished	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-27	cooler drain	finished	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-26	cooler drain	finished	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-28	cooler drain	finished	DF	-	-	-	-	-	-	-	-	-	-	-	-
D-41	finish 1 - gloves	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-29	finish process site 2	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-30	finishe process site 3	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-42	finished process table 3	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-40	finished process table 4	finished	other	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-38	finished process site 4	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-39	finished process site 5	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-32	finished process drain	finished	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-33	finished processor gloves	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-35	finish process site 6	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-37	finished process site 7	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-34	finishe process site 8	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-36	finished process site 9	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-31	finished process floor	finished	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
D-43	floor mat(s)	finished	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS

Figure A2. Plant N *L. monocytogenes* ribotype results from raw fish surfaces and environmental samples. Sample results are presented from top to bottom in a progression from inbound raw product to outbound finished product. "RF Cat." (raw/finished category) indicates "raw" for areas designated for non-smoked fish, "finished" for areas designated for only smoked fish, and "both" for areas designated for non-smoked or smoked fish. "Surface" column indicates categories of environmental samples: food contact surfaces (FCS, surfaces in contact with product), drain or floor (DF), floor transfer points (FTP, surfaces of movable objects that contact the floors), employee contact surfaces (ECS, surfaces employees come into contact with), fish, and other. Ribotype results are abbreviated without the "DUP-" prefix. Ribotype 116-1451-S2 is abbreviated "1451." "NS" indicates no sample was taken, while '-' indicates an L. monocytogenes negative result. Ribotypes which occurred more than once are color-coded.

Site	Description	RF Cat.	Surface	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb
N-34	whole salmon surface	raw	fish	-	-	-	-	-	-	NS	-	-	-	-	-
N-35	whole salmon surface	raw	fish	-	-	-	-	1045A	-	NS	-	-	1039A	1027B	-
N-36	salmon fillets	raw	fish	-	-	-	-	1045A	1042A	NS	-	-	-	-	-
N-1	dumpster wheels	Other	FT	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-33	drain	Other	DF	-	1039A	1052A	-	-	-	-	-	NS	-	-	-
N-7	raw area drain	raw	DF	-	-	-	1042A	1042A	-	-	-	-	-	-	1042A
N-8	table bracing	raw	Other	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-9	prep table	raw	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-10	pipes, insulation	raw	Other	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-11	wall and floor	raw	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-2	drain	raw	DF	1042A	1042A	1042A	1042A	1042A	1042A	1042A	1042A	1042A	1042A	1042A	1042A
N-3	floor near electrical	raw	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-4	floor wall junction	raw	DF	1042A	-	-	-	-	-	-	-	-	-	-	-
N-5	cleaning machine	raw	DF	-	-	-	-	NS	NS	NS	NS	NS	NS	NS	NS
N-6	cooler drain	raw	DF	1042A	-	-	-	-	-	-	-	-	-	1042A	-
N-12	drain	raw	DF	1042A	-	-	1042A	-	-	-	1042A	1042A	-	-	-
N-13	broom	raw	FT	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-14	door frame	raw	Other	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-15	oven drain	raw	DF	1451	1451	1042A	-	1451	1042A	1062A	1042A	1042A	1042A	1042A	1062E
N-23	cooler floor and wall	raw	DF	1042A	-	-	1062A	-	-	-	1042A	-	-	-	-
N-16	carts	both	ECS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-17	pallet jack	both	FT	-	-	-	-	-	1042A	-	1042A	-	-	-	-
N-18	floor drain	both	DF	-	-	-	-	1042A	1052A	-	-	-	-	-	-
N-19	trash can bottoms	both	FT	-	-	1042A	1042A	1042A	1042A	-	1042A	-	1042A	-	-
N-20	condensate pipe	both	Other	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-21	cart wheels	both	FT	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-22	hose	both	FT	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-25	table	finished	FCS	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-26	in, middle belts	finished	FCS	-	-	-	1042A	-	-	-	-	-	-	-	-
N-24	out belt	finished	FCS	1062A	1062A	-	-	-	-	-	-	-	-	-	-
N-27	drain	finished	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-28	trash can	finished	FT	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-29	cooler drains	finished	DF	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-31	cart wheels	finished	FT	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS
N-32	door frame	finished	Other	-	-	-	-	-	-	NS	NS	NS	NS	NS	NS