CENTER for PRODUCE SAFETY

CPS 2016 RFP
FINAL PROJECT REPORT

Project Title
Resolving postharvest harborage sites of Listeria protects Zone 1 surfaces

Project Period
January 1, 2017 – December 31, 2018

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Objectives
1. *Develop a detailed baseline spatial mapping profile, among confidentially enrolled handlers, toward the development of a model environmental monitoring program (EMP) and guidance in establishing an environmental-zone Master Sanitation Schedule linked to EMP outcomes for California fresh citrus packinghouses.*
2. *Evaluate the efficacy of emerging, registered hard-surface, nonporous- and porous-surface sanitizers on diverse non-contact surfaces that test positive for genus Listeria in enrolled citrus packinghouses.*

Funding for this project provided by the Center for Produce Safety through:
CDFA SCBGP grant# SCB16069

FINAL REPORT

Abstract
Fresh whole citrus has not experienced an incident of recall, illness, or outbreak, and California citrus production practices and regions appear to significantly limit the environmental risk of preharvest contamination. However, recent serious incidents associated with California apples, which involved the bacterial pathogen *Listeria monocytogenes*, have prompted proactive measures to more carefully assess postharvest risks and develop validated interventions for citrus system-wide. In this project, seven handlers were confidentially enrolled in a detailed survey involving facility sampling for indicator *Listeria* spp. (*Listeria*) and *L. monocytogenes* over a two-year period. In total, 1223 environmental sponges/swab samples were collected. The overall occurrence of *Listeria* among diverse non-food contact surfaces was 31%, suggesting that this indicator is a relatively common colonizer of citrus packing operations. The occurrence of *Listeria* fluctuated seasonally and decreased in some facilities over the course of the study, in part as a response to the real-time information of detection shared with each operation. The highest prevalence of repeated *Listeria* positives for an operational location, across enrolled facilities, was observed in the harvest bin handling areas: bin drying (81.0%), followed by incoming fruit-dumping area (58%), and bin washer (51%). Nevertheless, consistent occurrence of *Listeria* in pre-coolers and cold storage rooms, UV sorting chambers (proximate grading and sorting line), and areas near final packout, across packinghouses, was also observed. PFGE-subtyping and PCR serogrouping analysis of purified *L. monocytogenes* isolates identified serogroups IIb and IVb as the predominant subtypes. Additional subtyping and diversity analysis of a subset of *L. monocytogenes* strains are currently being conducted by whole genome sequencing (WGS). Analysis of the spatial and temporal pulsotypes in two regional packing facilities, ESJV-1 and ESJV-2, enabled an evaluation of clonal relationships and best opportunity to differentiate persistent from transient strains. In environmental swab areas determined to be repeatedly *Listeria* positive, the efficacy of in-house programs and selected emerging, registered hard-surface and nonporous- and porous-surface sanitizers was evaluated. Cleaning and sanitization procedures reduced the number of *Listeria*-positive spots, as compared to pre-cleaning and pre-sanitizer treatment, however some sanitizers did not seem to be very effective in on-site assessments. These data indicate that effective prevention management programs are essential to minimize persistence of *Listeria*, especially in routinely wet packing operation environments. This study provides a general overview and report card of the California citrus packing environment, and the identification of potential sources of *Listeria* related to citrus growing regions and harvest/postharvest practices, providing scientific baseline data required for developing industry guidelines and standards for environmental monitoring programs (EMPs).

Background
Listeria monocytogenes is a facultative anaerobic foodborne pathogen that can be found in a wide variety of natural and food processing environments [1]. *L. monocytogenes* has been estimated to cause 1455 hospitalizations and 255 deaths annually in the U.S., causing billions of dollars in productivity loss and medical costs [2, 3]. Fresh citrus is an important global commodity, a major specialty crop in the Pacific States, and remains important in California. The 2014 to 2015 California Agricultural Statistics Review places the combined value of oranges, lemons, and tangerines at more than $2 billion in farm gate value, and all three fruits are among the top 15 specialty crops by value. Recent large-scale recalls and serious outbreak incidents with other tree fruit have prompted proactive measures to more carefully assess postharvest risks and develop validated interventions [3, 4]. The systematic and thorough identification of prevalence, specific harborage sites, and source-tracking of transient and resident *L.*

monocytogenes rarely occurs in the absence of a "crisis" incident for a company and its associated commodity group. Through discussions, the California citrus leadership focused on the need to develop a detailed cross-seasonal baseline of *Listeria* incidence (among confidentially enrolled handlers) toward the development of a model EMP and guidance in establishing an environmental-zone Master Sanitation Schedule linked to EMP outcomes for California fresh citrus packinghouses. In this study, the Center for Produce Safety (CPS), California Citrus Research Board, and CA Citrus Quality Council partnered with University of California, Davis (UC Davis) to conduct multi-site investigative environmental sampling for *Listeria* in California citrus packinghouses. Confidentially enrolled handlers participated in a detailed survey involving swab sampling of multiple facilities for indicator *Listeria* spp. (*L*. spp.) and *L. monocytogenes* over two years. The key goals of this study included a general overview of the California citrus packing environment and the identification of potential sources of *Listeria* related to citrus growing regions and harvest/postharvest practices, and to evaluate the efficacy of current programs, among enrolled cooperators, and selected emerging, registered hard-surface and nonporous- and porous-surface sanitizers and disinfectants on diverse non-contact surfaces that consistently test positive for the genus *Listeria* in citrus packinghouses.

Research Methods

Swabbing – sample collection

Samples were collected from seven packing facilities. Information regarding physical layout, facility and equipment design, and their operating characteristics were collected on the first visit. A total of 1223 environmental sponges-swabs were collected from non-food contact surfaces (NFCS), predominantly in Zones 2 and 3, which included equipment surfaces, drains, floors, forklift wheels, sinks, and walls. At the mid-point of year 2 of the project, a limited number of food contact surface swabs (Zone 1) were taken with permission from two enrolled cooperators. Samples were collected using Whirl-Pak Speci Sponge® kits (Nasco, Modesto, CA), each containing a sterile sponge moistened with 10 ml of neutralizing buffer, sterile gloves, and sterile bag. Both sides of the sponge were passed over the sample site surface several times. Samples were placed into a sterile Whirl-Pak bag and kept on ice during transportation to the laboratory, and processed within 24 h. All sampling was performed while the facilities were operating, including fruit receiving, grade, sort, and pack lines, bin washing and short-term storage, pre-cooled pallet handling at loading docks, and cull transport.

Listeria detection and isolation

Environmental sponges and swabs were aseptically introduced into, and manually massaged with 30–100 ml of enrichment media ACTERO™ Listeria Enrichment Media (A-LEM) (Food Check, Calgary, Canada). Samples were incubated at 30 ± 2 °C for 24 h. After incubation 10 µl of each sample was streaked onto CHROMagar™ Listeria (CHROMagar Microbiology, Paris, France) and plates were incubated at 37°C for 48 h. Simultaneously, 12 µl of each enriched sample was used for *Listeria* molecular screening using the Atlas® Listeria LSP detection assay (Roka Bioscience). A second cultural assessment was performed on samples that were positive in the Atlas system but failed to yield colonies using Listeria Enrichment Broth Base (BLEB, Difco, Becton-Dickinson-BBL, Franklin Lakes, NJ) and anti-*Listeria* IMS Dynabeads® (Invitrogen, Dynal). Bacteria-bead suspensions were incubated, rinsed, and collected following the manufacturer's instructions (Invitrogen, Dynal). After collection, 30 µl from the suspension was inoculated onto CHROMagar Listeria plates and streaked to obtain isolated colonies; plates were incubated at 37°C for 48 h. Presumptive *Listeria* spp. (non-*L. monocytogenes*) and *L. monocytogenes* colonies were streaked onto CHROMagar Listeria to purify. Controls were processed in parallel with the cultural scheme. *L. monocytogenes* strain PTVS-335 and sterile

enrichment media were used as the positive and negative control, respectively. All presumptive *Listeria* spp. and *L. monocytogenes* colonies were purified through standard subculture transfers. For further confirmation, real-time PCR (RTi-PCR) was performed. DNA was isolated by enzymatic lysis as follows: purified colonies were re-suspended in 200 µl of Butterfield's phosphate buffer (BPB), and 1.5 µl of lysozyme (Qiagen, Maryland) was added. Colony suspensions were incubated at 37°C for 20 min followed by 10 min at 95°C. The supernatant was used as DNA template for RTi-PCR. A *Listeria* spp.–*L. monocytogenes* duplex RTi-PCR confirmation assay was carried out as described by Rodriguez-Lazaro et al. [5]. All *Listeria* spp. and *L. monocytogenes* strains were stored at −80°C in 20% glycerol for further analysis.

L. monocytogenes multiplex PCR-based serogrouping

Multiplex PCR was carried out to cluster *L. monocytogenes* strains into five molecular serogroups (IIa, IIb, IIc, IVa, and IVb) by targeting serotype-specific gene targets *lmo0737*, *lmo118*, *ORF2819*, and *ORF2110* [6]. Each molecular serogroup contains one of the four main serotypes associated with human disease (1/2a, 1/2b, 1/2c, and 4b). The multiplex PCR products were resolved and visualized by electrophoresis on 1.5% agarose gel in 1x TAE buffer (ThermoScientific) and visualized by SYBR® Safe (Invitrogen, Carlsbad, CA) staining.

Pulsed-field gel electrophoresis typing (PFGE)

A subset of *L. monocytogenes* isolates was characterized by PFGE typing, according to the Centers for Disease Control and Prevention (CDC) PulseNet protocol. Briefly, isolates were grown on brain heart infusion (BHI) agar plates at 37°C for 18 h. The optical density of each culture was adjusted to 1.0 at 600 nm, and cultures were embedded in 1% SeaKem Gold agarose plugs. Plugs were lysed, washed, and digested separately with *AscI* and *ApaI* (New England Biolabs, MA). The macrorestriction fragments were separated by electrophoresis on the CHEF-DRIII System (Bio-Rad) for 21 h at 6v/cm. *XbaI*-digested *Salmonella* ser. Braenderup (H9812) DNA was used as the reference size standard [7]. Restriction PFGE patterns were analyzed were generated using Applied Maths BioNumerics software (Saint-Matins-Latem, Belgium). The similarity analysis was carried out using Dice's correlation coefficient with 1.0% optimization and 1.5% tolerance. Clustering and construction of dendograms were performed by the unweighted pair group method with arithmetic mean (UPGMA).

Genome sequencing (analysis in progress)

A subset of 75 *L. monocytogenes* isolates was sequenced by WGS. Prior to DNA extraction, isolates were grown in BHI broth, incubated at 37°C for 18–24 h. DNA was purified using Qiagen Genomic-tip 20/G (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA was eluted in 50 µl of Tris-HCl (pH 8.0). DNA samples were processed using Nextera XT libraries and protocols (Illumina, San Diego, CA) and sequenced using the Illumina HiSeq system with minimum 80X coverage and average read length 150–250 bp. Prior to alignment, trimming was conducted on raw FASTQ sequences using a UrQt tool with a probabilistic model.

Assessment of sanitizers on non-food contact surfaces that test positive for *Listeria* in citrus packinghouses

Efficacy of EPA-registered disinfectants peroxyacetic acid (PAA, 200–300 ppm), Safe Zone™ (100 ppm), RelyOn™ (2 or 3%), and Decon7 (D7™) was assessed on floors directly beneath equipment that consistently and predictably tested positive, at each swab survey event, at different facilities. Sanitizer application was carried out post-cleaning based on commercial recommendations. Floor spots within an area previously positive for *Listeria* were swabbed before and after typical in-house cleaning procedures, and after the sanitizing treatment. Replicates of each treatment per area were included as the physical area of *Listeria* positive

detection at each location was sufficiently large. *Listeria* detection, isolation and characterization were carried out as described above.

In-vitro assessment of survival and removal of *Listeria* biofilms on packinghouse FCS and non-food contact surface (NFCS) materials using different sanitizers

Effectiveness of four commercial sanitizers against established *L. monocytogenes* biofilms was evaluated. Three strong biofilm-producing *L. monocytogenes* strains (PTVS 359, 360 and 361) isolated from postharvest environments handling tree fruit were used for the assays. Six used food or environmental contact surface materials (two conveyers, green tarp, crate, bin and curtain) obtained from fruit handling operations were cut in 4 × 4 cm pieces and sanitized in 5.3% sodium hypochlorite for 24 h. Materials were rinsed three times in sterile water to remove any reacted residues. Each material was placed into sterile specimen cups, and BHI broth was aseptically added, followed by inoculation with the *L. monocytogenes* cocktail. Cups were incubated at 37°C for 18–24 h. After incubation, all material pieces were aseptically rinsed twice with sterile deionized (DI) water to remove loose planktonic cells. Samples were placed into new sterile specimen cups, BHI was added, and cups were incubated at 37°C for 72 h. After the final incubation, all samples were aseptically removed, individually rinsed with sterile DI water and slow dried at room temperature. Four sanitizers were selected including chlorine (sodium hypochlorite, 200 ppm), PAA (200 ppm), Safe Zone™ (100 ppm), and RelyOn™ (1%). Sanitizer application was performed based on commercial recommendations; after 5 min contact time the samples were turned over and sprayed again for the same contact time. Samples were placed in Whirl-Pak bags holding 20 ml of neutralizing buffer, and were hand-massaged vigorously for 1 min. For *L. monocytogenes* quantification, 10-ml aliquots were removed, serially diluted and plated onto CHROMagar Listeria and incubated at 37°C for 48 h. For *L. monocytogenes* qualitative analysis, the remaining buffer was in 2X A-LEM and incubated at 30°C for 24 h. After incubation, three 33-µl aliquots per sample were pipetted onto CHROMagar Listeria and incubated at 37°C for 48 h.

Research Results

Objective 1.

A total of 1223 environmental sponges/swabs were collected from seven citrus packinghouses, and represented 954 (78.1%) zone 2 and 3 samples and 269 (21.9%) zone 1 food contact surfaces. Of the 1223 samples, 378 (30.9%) had a positive outcome for *Listeria* in the molecular test (Atlas Roka Bioscience) and 366 (29.9%) had a culture-positive outcome (**Figure 1**). In the initial culture assessment, 74 samples (19.6%) were molecular positive but culture negative and extra steps including secondary enrichment–IMS Listeria beads were required for *Listeria* recovery. Only 7 samples (1.6%) had a negative molecular outcome but were culture positive.

The overall *Listeria* occurrence at citrus packinghouses ranged from 8.0 to 56.9% (**Table 1**). From the 1223 enrichments tested, 105 samples (8.6%) were culture positive for *L. monocytogenes*, 158 (12.9%) were culture positive for *Listeria* spp., and in 104 samples (8.5%) *L. monocytogenes* and *Listeria* spp. were isolated concurrently. The overall *Listeria* prevalence in enrolled packinghouses was 30.0% (**Table 2**). ESJV-4, the last facility enrolled in the study, late in year 1, had the lowest percentage of samples *Listeria* positive (8.0%) among all the packinghouses and was also negative in all samples for *L. monocytogenes.*

In addition, of all analyzed samples, the recovery of *L. monocytogenes*, *L.* spp. and both concurrently varied, depending on the facility. Facilities SCY-5 and SPC-4B showed the highest occurrence of *L. monocytogenes* (17.6% and 12.5%, respectively). *L.* spp. were recovered from all seven citrus packinghouses. Specifically, packinghouse ESJV-2 showed the highest culture

recovery of *L*. spp. and *L. monocytogenes* concurrently with other *L*. spp. (21.9% for each) (**Figure 2**).

Among the 378 Listeria molecular-positive samples, during the culture recovery phase, *L. monocytogenes* was isolated in 27.8% of the samples, *L*. spp. in 41.8%, and both concurrently in 27.5% of samples. In total, 471 strains were isolated from the samples positive in the molecular screening: 209 *L. monocytogenes* and 262 *L*. spp. (**Table 3**). A reduction in the percentage of samples positive over time was observed in two facilities provided with survey results in near real-time (ESJV-1 and SPC-4B). However, an increase or the same level of *Listeria* occurrence was observed in the other packinghouses (**Figure 3**).

Based on the confirmed detection results, the effect of weather/meteorological conditions on the occurrence of *Listeria* previous to or during the sampling was evaluated. Specifically, precipitation or absence of rain among 5 to 10 days before the sampling was evaluated. In the typical rainy season, the amount of rain ranged from 0.51 to 3.92 inches, and in the dry season no measureable rain was observed between one and six months. In general, a decrease in the number of samples *Listeria* positive in the dry season and a rise in the number of sample positives during the flanking preceding and subsequent rainy season was observed. However, two facilities showed a slightly different pattern: in packinghouse SPC-4A, *Listeria* occurrence did not seem to be impacted by weather conditions, and in ESJV-4 a low prevalence of *Listeria* was found during the rainy period (**Figure 4**).

In general, the highest *Listeria* occurrence in almost all citrus packinghouses was from samples collected around the bin handling areas and operations. Most of the samples tested from the bin drying and staging area were positive (81.0%), followed by the incoming fruit dumping area (57.6% positive) and bin washer (50.8% positive) (**Figure 5**). Although bins areas seemed to be the most important hotspot for *Listeria* across all facilities (at least 50% samples were positive), other operational locations like the UV inspection room/sorting room in ESJV-2 and SPC-4B seemed to be a *Listeria* niche as well (**Figure 6**). The consistent occurrence of *Listeria* in the cold room and/or final packout area across packinghouses also was observed (Figure 6). Out of the 269 FCS (Zone 1) samples processed, only 14 (5.2%) and 8 (3.0%) of samples were molecular and culture positive, respectively. All FCS isolates corresponded to *L.* spp. and not *L. monocytogenes* (**Table 4**). The data collected from this study demonstrated that *L*. spp. and *L. monocytogenes* are commonly recovered from CA citrus packing operations and appear to include resident populations, including in readily accessed control areas. The uncharacterized potential for cross-contamination risk exists and should and can be managed with available systems and procedures.

A primary characterization of all 209 purified isolates was carried out by multiplex PCR. Multiplex PCR serogroup characterization of *L. monocytogenes* was performed, which allowed us to quickly and inexpensively differentiate, at a general and base low level of discrimination, *L. monocytogenes* isolates into four major serogroups: IIa (1/2a and 3a), IIb (1/2b and 3b) IVb (4b, 4d and 4e) and IVa (4c). Based on PCR results, most *L. monocytogenes* isolates were classified as serogroup IIb (n=90) and IVb (n=85); followed by serogroup IVa (n=27), serogroups IIa (n=6) and IVb variant 1(IVb-v1, n=3). The ESJV-2 facility was found to have more *L. monocytogenes* diversity (all five serogroups were identified) than the other facilities (**Figure 7**). Serogroups IIb and IVb were found to be predominant among facilities; nevertheless one appears to replace the other one over time in some facilities. In the case of ESJV-3, serogroup IVb (predominant in sampling 1) was replaced by IIb; in contrast, in SPC-4A and SPC-4B, serogroup IIb disappeared and was substituted by IVb (**Figure 8**). Monitoring *L. monocytogenes* PCR subtypes over time provided useful information regarding potential persistent strains, and could be useful to understand the ecology or niches of *L. monocytogenes*

associated with packing operations. Newly emerging technologies are a likely improvement in this high-level, low specificity pattern approach.

Genotypic subtyping of a subgroup of *L. monocytogenes* strains was carried out by PFGE. Isolates recovered from ESJV-1 and ESJV-2 in the first three sampling events were analyzed. Analysis of the 55 individual isolates at the similarity level of 98% resulted in discrimination of 18 pulsotypes (1–18). Eight pulsotypes were detected at ESJV-1, and the one predominant pulsotype (15) observed was also recovered from ESJV-2; pulsotypes 5 and 15 were found in both packinghouses (**Figures 9** and **10**). Based on PCR serogroup–PFGE data the predominant serogroup IVb in facility ESJV-1 consisted of three pulsotypes (14, 15 and 16), from which 15 was found to be predominant and was isolated from three different locations. In contrast, in facility ESJV-2, despite most of the strains isolated belonging to PCR serogroup IIb, they were not genetically related and none of the pulsotypes seemed to dominate; however, all isolates from serogroup IVa showed an indistinguishable pattern (pulsotype 17) and seemed to predominate in four operational locations of the facility (**Figure 11**).

Presence and distribution of *L.* spp., *L. monocytogenes* and both concurrently was evaluated in citrus packinghouses according to operational location and sampling. The type of isolate was plotted onto floor plans of each facility, and a spatial map by operational location section was performed (**Figure 12**). To evaluate the spatial and temporal distribution of *L. monocytogenes* pulsotypes in different operational locations of the packinghouses and reveal the dynamics of strain establishment or elimination, pulsotypes were mapped on the ESJV-1 and ESJV-2 packinghouse plans and spatial mapping was performed (**Figures 13** and **14**, respectively). In both packinghouses a specific pulsotype dominated: pulsotype 15 in ESJV-1 and pulsotype 17 in ESJV2. However, results from sampling 3 showed that both genotypes disappeared (or became less recoverable due to competition), and an introduction/emergence of a different pulsotype (5) was observed in both facilities. Also, almost all strains were isolated from samples collected around bins, perhaps identifying a revolving source of *Listeria* introduction.

Objective 2.

Efficacy of sanitizers was assessed in three different citrus packinghouses. The first assessment was performed at the ESJV-2 facility: PAA, Safe Zone and RelyOn (2%) were tested on floor surfaces, as described above. Five floor spots within a large area repeatedly positive for *Listeria* were sampled before and after a typical cleaning, and after the sanitizing procedure. In total, 15 sponges were tested for each sanitizer. All samples prior to cleaning (n=15) and after the normal cleaning (n=15) were positive for *Listeria*. Swabs from floors treated with PAA (n=5) and Safe Zone (n=5) for 5 min were molecular and culture positive for *Listeria*. Three of the five sponges from floors treated with RelyOn were molecular and culture negative for *Listeria.* Purification of the *Listeria* isolates recovered was carried out.

Overall, floors were colonized by both *L*. spp. and *L. monocytogenes*, although from three specific spots only either *L*. spp. or *L. monocytogenes* were isolated. After cleaning, both *L*. spp. and *L. monocytogenes* were recovered concurrently from 14 of the 15 spots. After treatment with RelyOn, all spots were negative for *L. monocytogenes* but one remained positive for *L*. spp. Although after PAA treatment all five replicates were still *Listeria* positive, only three were positive for *L. monocytogenes*. Four floor spots treated with Safe Zone remained positive for both *L*. spp. and *L. monocytogenes* and one for *L*. spp. (**Table 5**). Purified *L. monocytogenes* strains were characterized by multiplex PCR serogroup. *Listeria monocytogenes* serogroups IIb, IVa and IVb were found on the tested floor areas. Some of areas examined were colonized by a mix of *L. monocytogenes*, since different serogroups were observed before and after cleaning and after sanitizer treatment. The two more prevalent serogroups were IIb and IVa, which appeared to better survive the sanitizer treatments (**Table 6**).

In the second assessment the effectiveness of PAA and RelyOn (3%) on floor surfaces at SPC-4A facility was evaluated. In total, 30 samples were taken, and 15 sponges were tested for each sanitizer in the same manner as in the previous trial; five floor spots were sampled before and after cleaning, and after the sanitizing procedure. Unfortunately, for our studies, all sponges from PAA testing prior to cleaning were molecular and culture negative for *Listeria*. PAA and RelyOn were used for sanitizing procedures. Two sponges used for RelyOn testing were positive for *Listeria* before cleaning and after normal cleaning procedures, but negative after treatment with the sanitizer (**Table 7**). The third assessment was carried out at facility ESJV-3, where the efficacy of PAA, RelyOn (3%) and D7 was evaluated. In total, 18 samples were taken, six for each sanitizer. Three floor spots were sampled before and after sanitation treatment. All floor spots sampled before PAA and D7 treatments were molecular and culture positive for *Listeria*, and one of the three spots was negative before RelyOn. All sanitized surfaces were negative for presence of *Listeria* (**Table 8**).

In-vitro evaluation of the survival and removal of *Listeria* on packinghouse food contact (FCS) materials using different sanitizers was assessed by measuring the effectiveness of four commercially available sanitizers against *L. monocytogenes* biofilms. *L. monocytogenes* reduction after treatment with sanitizers was measured on 15-, 21- and 30-day-old biofilm growth on food contact materials (**Table 9**). The cell density in biofilms formed on different surfaces by *L. monocytogenes* was approximately $10^{4.5}$ CFU to 10^{6} CFU.

When 15-day-old biofilms were treated with PAA, Safe Zone and RelyOn,(1%) a 3–4.5 log reduction was observed for bin, conveyer and curtain parts, and 2.3–4.1 and 0.5–1.9 log reductions for black tarp and white crate pieces, respectively. Applying Safe Zone to green tarp achieved a 3 log reduction. In contrast, PAA and RelyOn treatments showed a 0.6 and 1.1 log reduction, respectively. Chlorine was only effective when applied to conveyer and curtain pieces (1.2 and 3 log reductions, respectively), and achieved a reduction of less than 0.7 for the other surfaces. Treated 21-day-old biofilms on green tarp parts showed log reductions of 1.5 when chlorine or Safe Zone were used, and 2.1 when PAA or RelyOn were applied. Similarly, Safe Zone and RelyOn showed similar log reductions of 0.7 to 0.9 for bin parts and 2.0 for conveyer parts, respectively. Curtain pieces treated with Safe Zone, PAA and RelyOn achieved 0.2, 1.6 and 3.6 log reductions, respectively. Black tarp treated with Safe Zone and RelyOn achieved 0.4 and 1.4 log reductions, respectively. None of the sanitizers applied to white crate parts were able to reduce *L. monocytogenes* populations. For 30-day-old biofilms, a 2 log reduction was achieved on green tarp parts with all sanitizers tested. PAA and Safe Zone were able to achieve a 0.9 and 1.2–1.5 log reductions when applied to conveyer and curtains parts, respectively. RelyOn applied to black tarp, curtain, or white crate and converter parts achieved 1.8, 2.5, 2.4 and 5.2 log reductions, respectively. In contrast, when chlorine was applied to white crate and conveyer parts only 0.2 and 0.4 log reductions were observed, respectively. None of the sanitizers applied to bin pieces were able to reduce *L. monocytogenes* populations (**Table 9**).

In the qualitative analysis of 15-day-old biofilms, PAA, Safe Zone and RelyOn treatments were able to completely eliminate *L. monocytogenes* on curtain pieces. Conversely, 60% of chlorine-treated curtain pieces remained positive. RelyOn eliminated *L. monocytogenes* from conveyer parts, whereas 25 and 60% remained positive when PAA and Safe Zone were used, respectively. *L. monocytogenes* was only eliminated from bin parts treated with Safe Zone and RelyOn. For 21- and 30-day-old biofilms, 40 and 20% of RelyOn–treated curtain pieces remained positive; moreover, 80% of curtain pieces treated with PAA and 40% treated with Safe Zone remained positive. When Safe Zone was used on conveyer parts, only 20% remained positive. Green tarp, white crate and black tarp were always qualitatively positive for samples treated after 15, 21 and 30 days. In addition, at 21 days the bin and conveyer parts were also all positive for *L. monocytogenes* presence (**Table 10**).

Outcomes and Accomplishments

Overall this project provided new insights into the *Listeria* occurrence and ecology in the California citrus packing environment. This two-year study showed the generally high prevalence of indicator *Listeria* (*L*. spp.) and *L. monocytogenes* in seven citrus packinghouses. Microbiological-analytical techniques applied and developed in this study allowed us to verify (culture confirmation) almost 100% of the *Listeria* molecular-positive samples, which provides the industry a real reference for "trusting" certified or Performance-Tested rapid *Listeria* indicator molecular screening. In this study, chronic and sporadic *Listeria* positive spots and niches were identified; this information has served as a guide for prioritization of cleaning and sanitation scheduling of Zones 2 and 3 that represent high-risk potential for non-direct contact transfer areas. Through PFGE-subtyping and PCR serogrouping analysis in this study, the diversity of *L. monocytogenes* in citrus packinghouses showed that serogroups IIb and IVb were predominant. Additional subtyping and diversity analysis of a subset of isolated *L. monocytogenes* strains is currently being conducted by whole genome sequencing (WGS). WGS analysis will provide a comprehensive insight into genomic diversity and increase the understanding of persistence and transient re-introduction in fresh produce packing environments. Considerable fluctuation in the frequency of *Listeria* related to the weather (dry/rainy seasons) was observed. Efficacy of the disinfectants peroxyacetic acid (PAA, 200–300 ppm), Safe Zone™ (100 ppm), RelyOn™ (1–3%) and Decon7 (D7™) was successfully evaluated, showing that effective cleaning sanitizing schedules can reduce contamination and limit *L. monocytogenes* occurrence in citrus packinghouses, and improve food safety. The partnerships and alliances developed with citrus industry collaborators were crucial for this study. This collaboration allowed us to successfully conduct the samplings and gather information about operations. Furthermore, this relationship will allow us to conduct important on-site projects in the future. The data described here was shared with the citrus collaborators on a regular basis. This study provides scientific reference/baseline data required for developing industry guidelines and standards for environmental monitoring programs (EMPs).

Summary of Findings and Recommendations

- This study provides an overview of *Listeria* occurrence in California citrus packinghouses. In total, 31% of tested samples were positive for *Listeria*, demonstrating that *Listeria* is a common colonizer of CA citrus packing operations, and the potential for cross-contamination risk is present.
- Despite considerable *Listeria* prevalence in Zones 2 and 3, in a more limited survey, only 5.2% of FCS (Zone 1) tested samples were positive for indicator *Listeria*, and only *Listeria* spp. were recovered from FCS; *L. monocytogenes* was not isolated from FCS.
- The bin area (bin dumping/washer/drying) in the packinghouses was demonstrated to be a *Listeria* hotspot. However the consistent occurrence of *Listeria* in the cold room, UV room/sorting room and final packout area across packing facilities was also observed, representing possible *Listeria* niches as well.
- Prevalence of *Listeria* in citrus packing facilities may be influenced by precipitation, including any standing water and flooded external drains around the facility at receiving and shipping areas, particularly in lift truck ramp and traffic areas. Specifically, increased occurrence of *Listeria* is likely to be associated with higher precipitation occurring over a period of time, suggesting that rain-facilitated processes, such as an increase in water or moisture that can get to harboring locations, could contribute to the survival and dissemination of *Listeria.* Soil and debris with higher moisture probably act as vehicles for introducing *Listeria* isolates from preharvest or general urban and rural environments.

- Analysis of the spatial and temporal pulsotypes in the ESJV-1 and ESJV-2 facilities enabled an evaluation of clonal relationships and also allowed us to determine whether persistent strains existed in the facilities.
- Surface cleaning, elevation above flooring and drainage channels, and drying (elimination the ponding of water) are highly recommended to facilitate *Listeria* reduction in facilities, especially in the rainy season.
- In general, cleaning-sanitization reduced the prevalence of *Listeria* positives; however, PAA and Safe Zone™ did not seem to be very effective in facility ESJV-2, with the procedures used and at the doses applied. These data confirm that ineffective cleaning and disinfection, by frequency and/or chemistry, is likely to result in widespread persistence of *Listeria* in packing and processing environments. These outcomes are complex phenomena that are likely the result of a combination of factors.
- Considerable decreases in *Listeria* and *L. monocytogenes* positive spots were observed when cleaning and sanitizer treatment was carried out, showing the importance of disruption of biofilms and/or any associated organic material before a sanitizer is used.
- The *L. monocytogenes* biofilm sanitization in-vitro assessments showed that older established biofilms on FCS and NFCS that are left untreated are more difficult to eradicate than fresh ones, which reflected the importance of periodical application of sanitizers to avoid establishment of aged biofilms.

APPENDICES

Publications and Presentations

(No publications to date)

Presentations:

Pinzon, J., Hill, D., Skots, M., and Suslow, T. 2018. Comparative Growth of Alternate Environmental Listeria Strains in Selective Enrichments and Competitive Effect on Detection and Recovery of *Listeria monocytogenes*. International Association for Food Protection Annual Meeting, Salt Lake City, Utah, July 8–11.

Suslow, T. 2018. Resolving Postharvest Harborage Sites of Listeria Protects Zone 1 Surfaces. CPS Research Symposium, Charlotte, North Carolina, June 20.

Suslow, T. 2017. Resolving Postharvest Harborage Sites of Listeria Protects Zone 1 Surfaces. CPS Research Symposium, Denver, Colorado, June 21.

Budget Summary

All project funds awarded ($359,523) will be expended.

Tables and Figures

(See below)

Tables 1–10 and Figures 1–14

Table 1. Molecular *Listeria* testing outcomes in citrus packinghouses (number and percentage per facility)

Facility	Negative		Positive	
ESJV-1	68	68.0%	32	32.0%
ESJV-2	112	43.1%	148	56.9%
ESJV-3	391	81.1%	91	18.9%
ESJV-4	23	92.0%	2	8.0%
SCY5	23	45.1%	28	54.9%
SPC-4A	129	74.1%	45	25.9%
SPC-4B	99	75.6%	32	24.4%
Total	845	69.1%	378	30.9%

Table 2. Distribution of samples culture positive from citrus packing facilities

Facility	Samples tested	No. of samples molecular positive		*L. monocytogenes*		*Listeria* spp.[a]		*L. monocytogenes* + other *Listeria*[b]		Total samples positive for *Listeria*	
ESJV-1	100	32	32.0%	8	8.0%	16	16.0%	9	9.0%	33	33.0%
ESJV-2	260	148	56.9%	31	11.9%	57	21.9%	57	21.9%	145	55.8%
ESJV-3	482	91	18.9%	30	6.2%	40	8.3%	15	3.1%	85	17.6%
ESJV-4	25	2	8.0%	0	0.0%	1	4.0%	1	4.0%	2	8.0%
SCY5	51	28	54.9%	9	17.6%	6	11.8%	8	15.7%	23	45.1%
SPC-4A	174	45	25.9%	11	6.3%	26	14.9%	11	6.3%	48	27.6%
SPC-4B	131	32	24.4%	16	12.2%	12	9.2%	3	2.3%	31	23.7%
Total	1223	378	**30.9%**	105	**8.6%**	158	**12.9%**	104	**8.5%**	367	**30.0%**

[a] Listeria other than *monocytogenes*

[b] Simultaneous presence of *Listeria monocytogenes* and other *Listeria*

Table 3. Occurrence of *L. monocytogenes* and *L.* spp. from enrichments *Listeria* molecular positive

Facility	No. of samples molecular positive	*L. monocytogenes*		*Listeria* spp.	
ESJV-1	32	17	53.1%	25	78.1%
ESJV-2	148	88	59.5%	114	77.0%
ESJV-3	91	45	49.5%	55	60.4%
ESJV-4	2	1	50.0%	2	100.0%
SCY5	28	17	60.7%	14	50.0%
SPC-4A	45	22	48.9%	37	82.2%
SPC-4B	32	19	59.4%	15	46.9%
Total	378	209	59.4%	262	69.3%

Table 4. *Listeria* results from Zone 1 food contact surfaces

Operational location FCS	Samples tested	Samples molecular positive		Samples culture positive*	
Final packout	221	11	5.0%	6	2.7%
Sorting grader	10	2	20.0%	2	20.0%
Sorting room	4	0	0.0%	0	0.0%
Washing/fungicide/drying/waxing	34	1	2.9%	0	0.0%
Total	269	**14**	**5.2%**	8	**3.0%**

*All strains isolated corresponded to *Listeria* spp.

Table 5. Efficacy of sanitizers on citrus packinghouse ESJV-2

Sanitizer	Replicate	Before cleaning		Cleaning		Sanitizer treatment 5 min	
		Molecular	Culture	Molecular	Culture	Molecular	Culture
RelyOn™ 2%	R1	Positive	L. spp./L. mono	Positive	L. spp./L. mono	Positive	Negative
	R2				L. spp.		L. spp.
	R3				L. spp./L. mono	Negative	Negative
	R4					Negative	Negative
	R5					Negative	Negative
PAA 200 ppm	P1	Positive	L. spp./L. mono	Positive	L. spp./L. mono	Positive	L. spp.
	P2						L. mono
	P3		L. mono				L. mono
	P4		L. spp.				L. spp .
	P5		L. spp./L. mono				L. spp.
Safe Zone™ 100 ppm	S1	Positive	L. spp./L. mono	Positive	L. spp./L. mono	Positive	L. spp/L. mono
	S2		L. spp.				L. spp
	S3		L. spp./L. mono				L. spp/L. mono
	S4						
	S5						

Table 6. PCR serogroups of *L. monocytogenes* isolated from sanitizer treatment

Sanitizer	Replicate	Before cleaning	After cleaning	After sanitizer
RelyOn™ 2%	R1	**IVa**	**IIb**	Negative
	R2	**IIb**	L. spp.	L. spp.
	R3	**IIb**	**IIb**	Negative
	R4	**IIb**	**IIb**	
	R5	**IIb**	**IIb**	
PAA 200 ppm	P1	**IIb**	**IVa**	L. spp.
	P2	**IVa**	**IVb**	**IIb**
	P3	**IVa**	**IVa**	**IVa**
	P4	L. spp.	**IIb**	L. spp.
	P5	**IIb**	**IVb**	L. spp.
Safe Zone™ 100 ppm	S1	**IVa**	**IVa**	**IVa**
	S2	L. spp.	**IIb**	L. spp.
	S3	**IVa**	**IVb**	**IIb**
	S4	**IVb**	**IVb**	**IVa**
	S5	**IIb**	**IVa**	**IVa**

Table 7. Efficacy of sanitizers on citrus packinghouse SPC-4A

Sanitizer	Replicate	Before cleaning		Detergent-water rinse		Sanitizer treatment 5 min	
		Molecular	Culture	Molecular	Culture	Molecular	Culture
RelyOn™ 3%	R1	Negative	Negative	Negative	Negative	Negative	
	R2	Positive	L. spp.	Positive	L. spp.		
	R3	Positive	L. spp.	Positive	L. spp.		
	R4	Negative	Negative	Negative	Negative		
	R5						
PAA 300 ppm	P1	Negative					
	P2						
	P3						
	P4						
	P5						

Table 8. Efficacy of sanitizers on citrus packinghouse ESJV-3

Sanitizer	Replicate	Before treatment		Sanitizer treatment 5 min	
		Molecular	Culture	Molecular	Culture
RelyOn™ 3%	R1	Negative	Negative	Negative	
	R2	Positive	L. spp.		
	R3	Positive	L. mono		
PAA 200 ppm	R1	Positive	L. mono	Negative	
	R2		L. mono		
	R3		L. mono		
D7	R1	Positive	L. mono	Negative	
	R2		L. spp./L. mono		
	R3		L. spp./L. mono		

Table 9. *L. monocytogenes* log reduction after in-vitro sanitizer treatment

	Sanitizer	Bin	Conveyer	Green tarp	Curtain	Black tarp	White crate
15-day-old Biofilm	Control	0.3	-0.7	-0.4	-0.2	-0.2	0.1
	Chlorine	0.7	1.2	-0.3	3.0	-0.1	0.0
	PAA	3.1	3.3	0.6	3.9	2.3	0.4
	Safe Zone™	4.5	3.0	3.0	3.9	3.5	1.7
	RelyOn™	4.5	3.8	1.1	3.9	4.2	1.9
21-day-old Biofilm	Control	-0.6	0.0	0.6	-0.1	-0.3	-1.0
	Chlorine	0.4	-0.1	1.6	0.1	-0.3	-0.5
	PAA	-0.2	0.1	2.0	1.6	-0.2	0.0
	Safe Zone™	0.7	2.1	1.5	0.2	0.4	-0.2
	RelyOn™	0.9	1.9	2.1	3.6	1.4	-0.4
30-day-old Biofilm	Control	-0.7	0.3	1.4	0.2	0.4	-0.3
	Chlorine	-0.9	0.4	1.9	0.1	-0.3	0.2
	PAA	-0.4	0.9	2.4	0.9	0.5	-0.1
	Safe Zone™	-0.2	1.2	2.2	1.5	0.5	0.6
	RelyOn™	-0.3	5.2	2.1	2.5	1.8	2.4

Table 10. *L. monocytogenes* qualitative results after sanitizer treatment

Biofilm age	Sanitizers	Bin	Conveyer	Green tarp	Curtain	Black tarp	White crate
15 days	Control	100%	100%	100%	100%	100%	100%
	Chlorine	100%	100%	100%	60%	100%	100%
	PAA	100%	25%	100%	0%	100%	100%
	Safe Zone™	0%	50%	100%	0%	100%	100%
	RelyOn™	0%	0%	100%	0%	100%	100%
21 days	Control	100%	100%	100%	100%	100%	100%
	Chlorine	100%	100%	100%	100%	100%	100%
	PAA	100%	100%	100%	80%	100%	100%
	Safe Zone™	100%	100%	100%	100%	100%	100%
	RelyOn™	100%	100%	100%	40%	100%	100%
30 days	Control	100%	100%	100%	100%	100%	100%
	Chlorine	100%	100%	100%	100%	100%	100%
	PAA	100%	100%	100%	100%	100%	100%
	Safe Zone™	100%	20%	100%	40%	100%	100%
	RelyOn™	100%	100%	100%	20%	100%	100%

Figure 1. Molecular and culture *Listeria* testing outcomes in citrus packinghouses

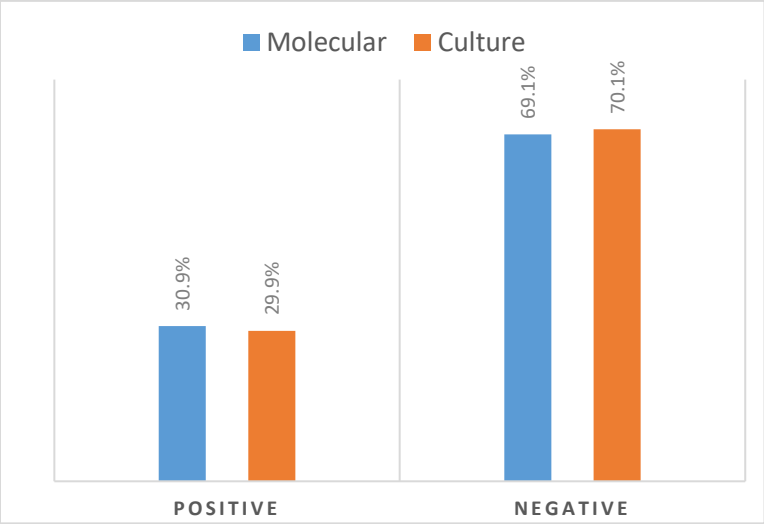


Figure 2. Percentage of *Listeria* isolated from different citrus packing facilities

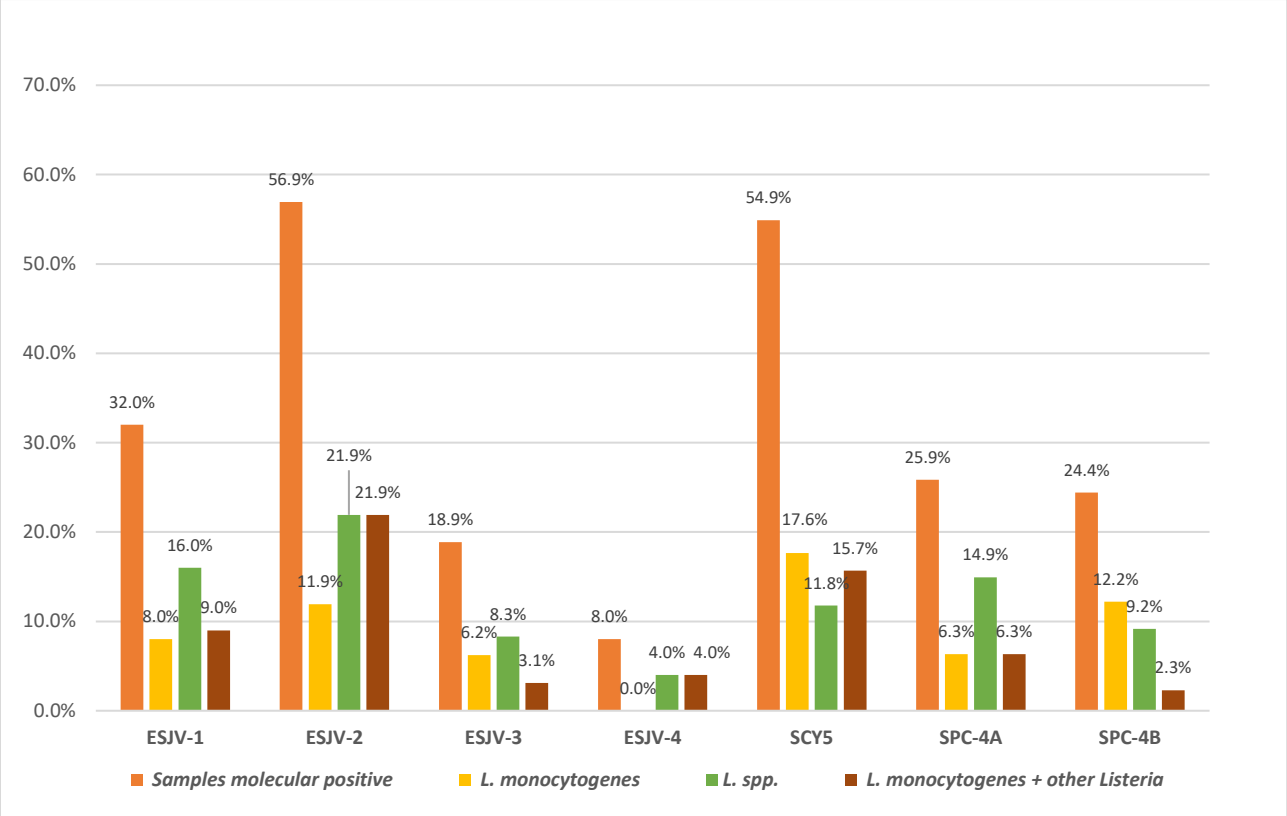


Figure 3. Percentage of samples molecular and culture positive from seven citrus packinghouses in different samplings

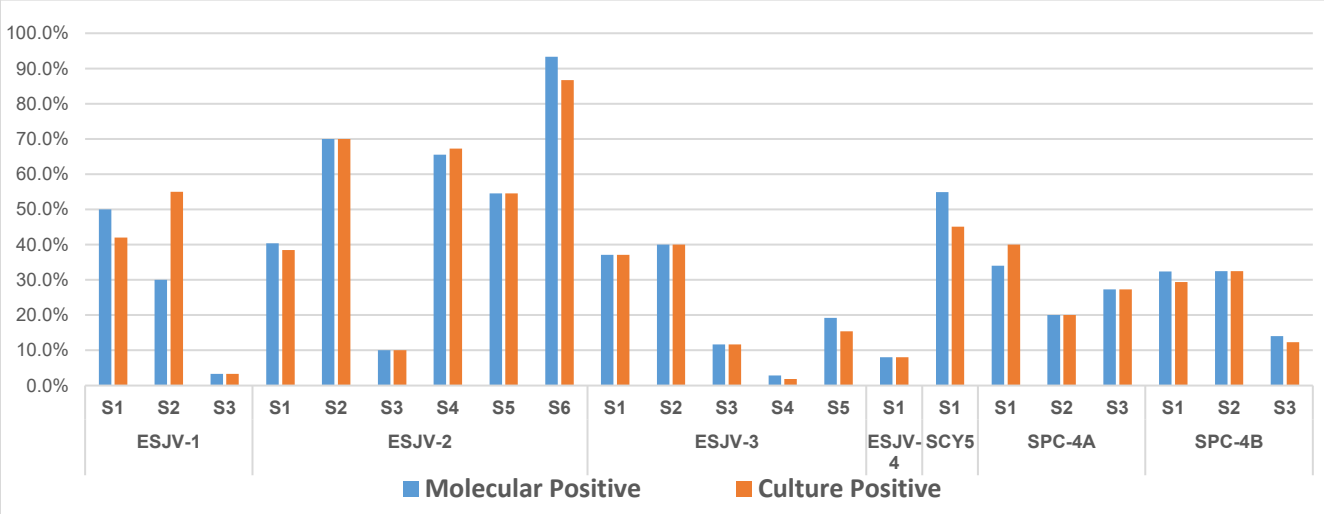


Figure 4. *Listeria* outcomes and impact of weather conditions before/during the sampling events

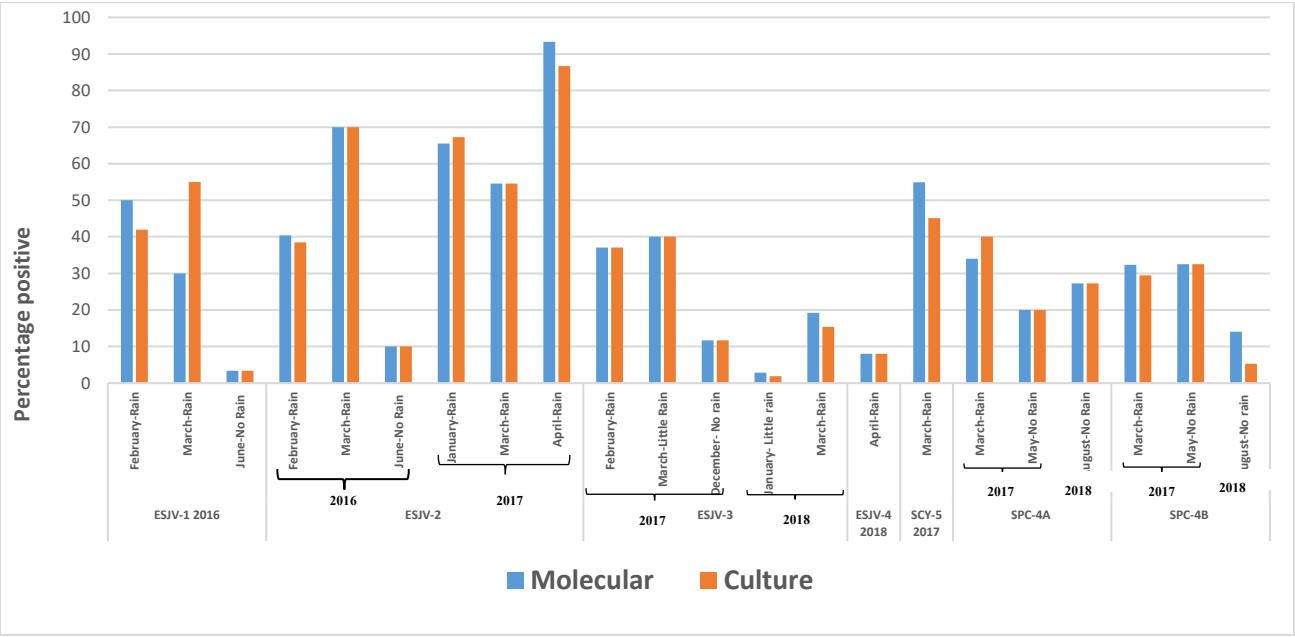


Figure 5. Molecular *Listeria* outcomes from non-food contact and food contact surfaces in citrus packinghouses

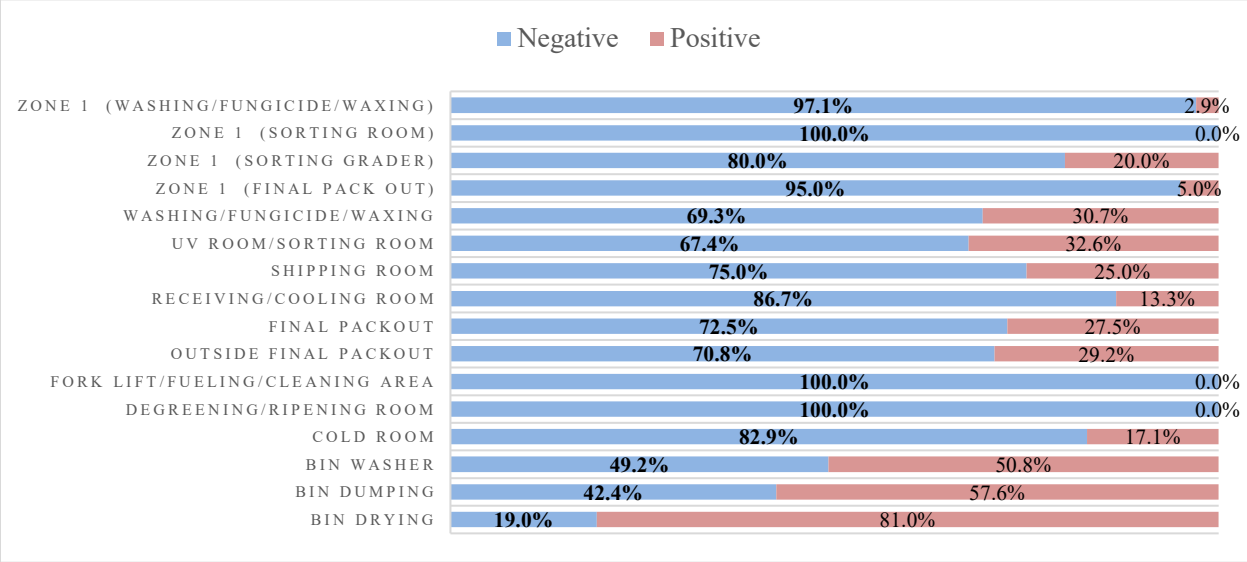


Figure 6. Percentage of *Listeria* positive samples by operational location in citrus packinghouses

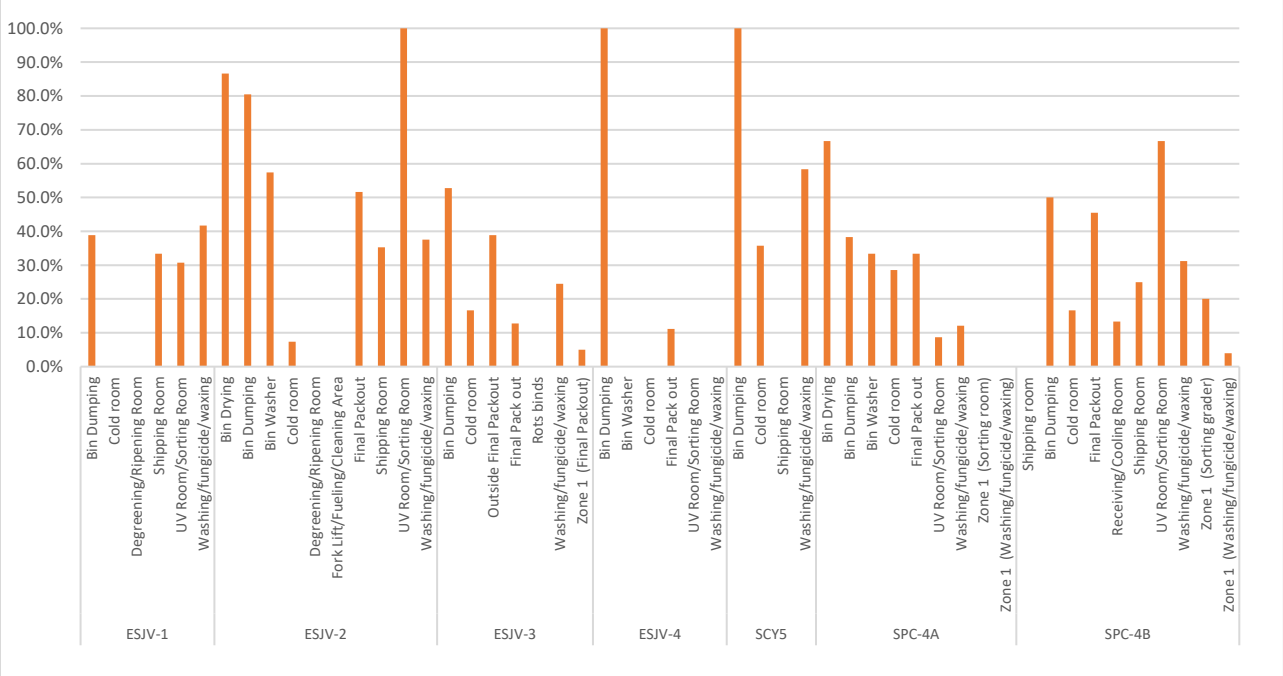


Figure 7. *Listeria monocytogenes* PCR serogroup diversity in citrus packinghouses

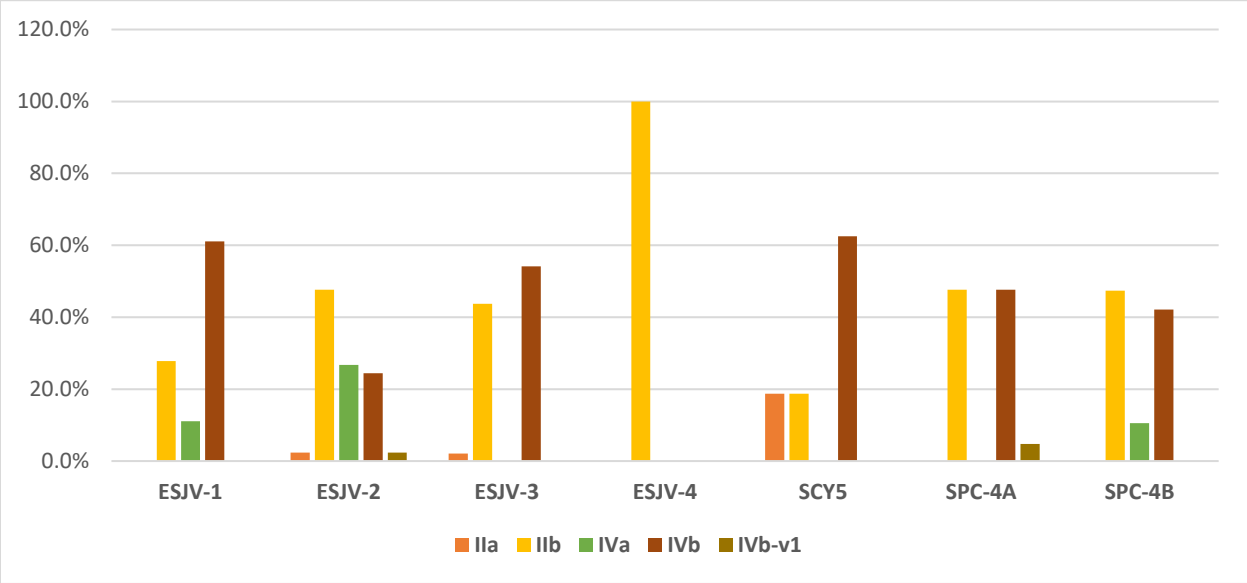


Figure 8. *Listeria monocytogenes* PCR-based serogroup diversity in citrus packinghouses over sampling times

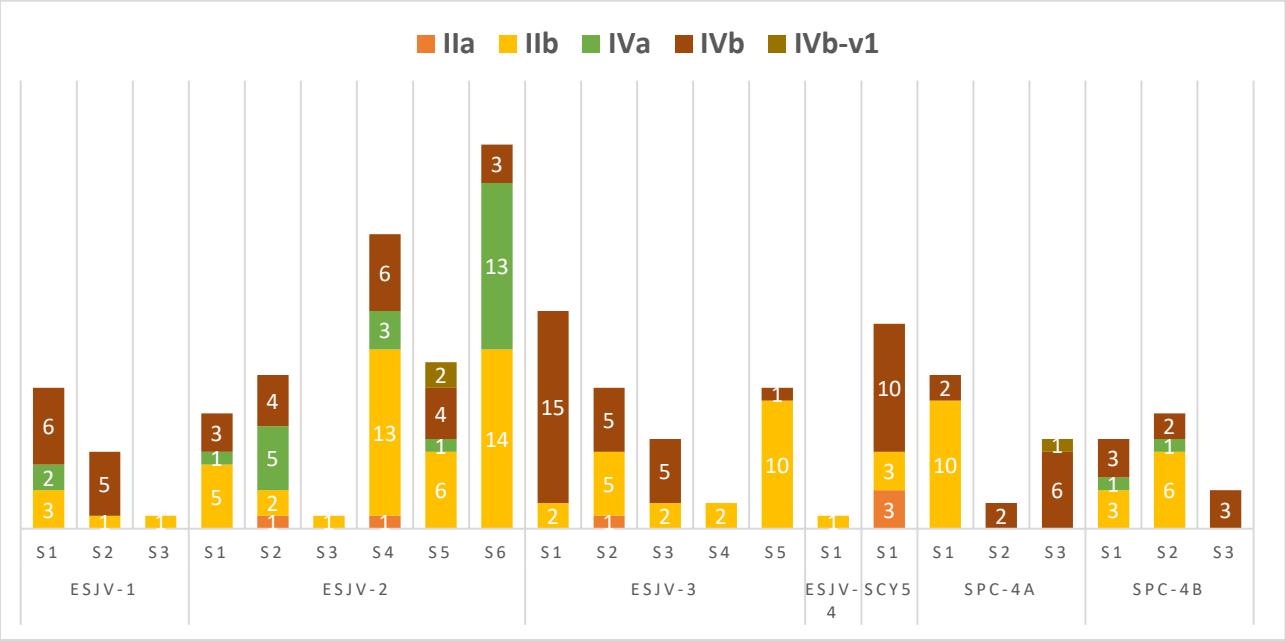


Figure 9. Cluster of *Listeria monocytogenes* isolated from ESJV-1 packinghouse by operational location in the first three samplings

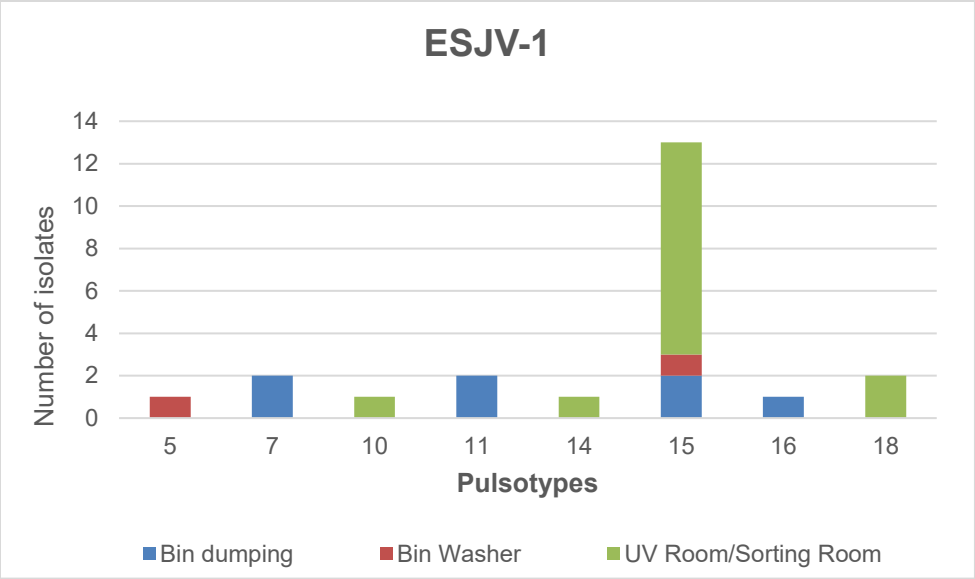


Figure 10. Cluster of *Listeria monocytogenes* isolated from ESJV-2 packinghouse by operational location in the first three samplings

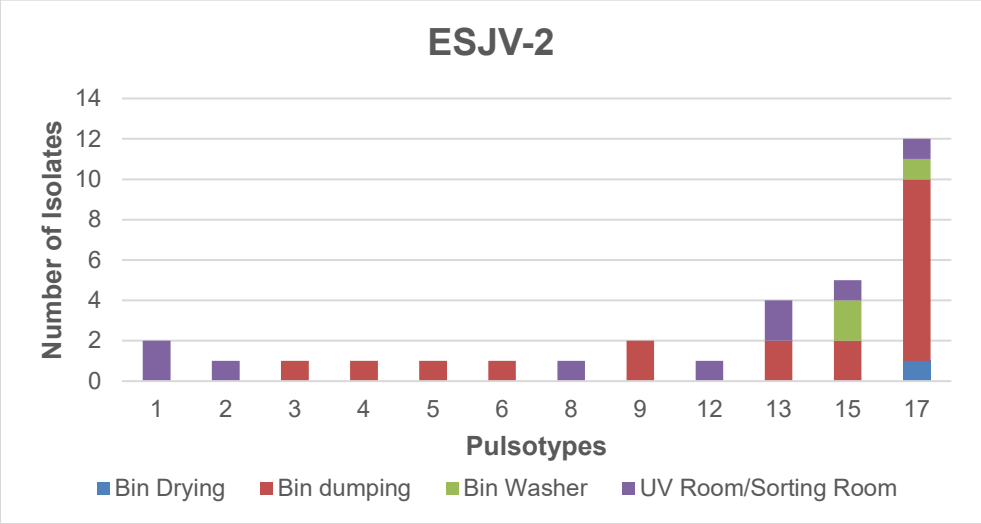


Figure 11. Diversity of *Listeria monocytogenes* isolated from two citrus packinghouses by operational location in three samplings

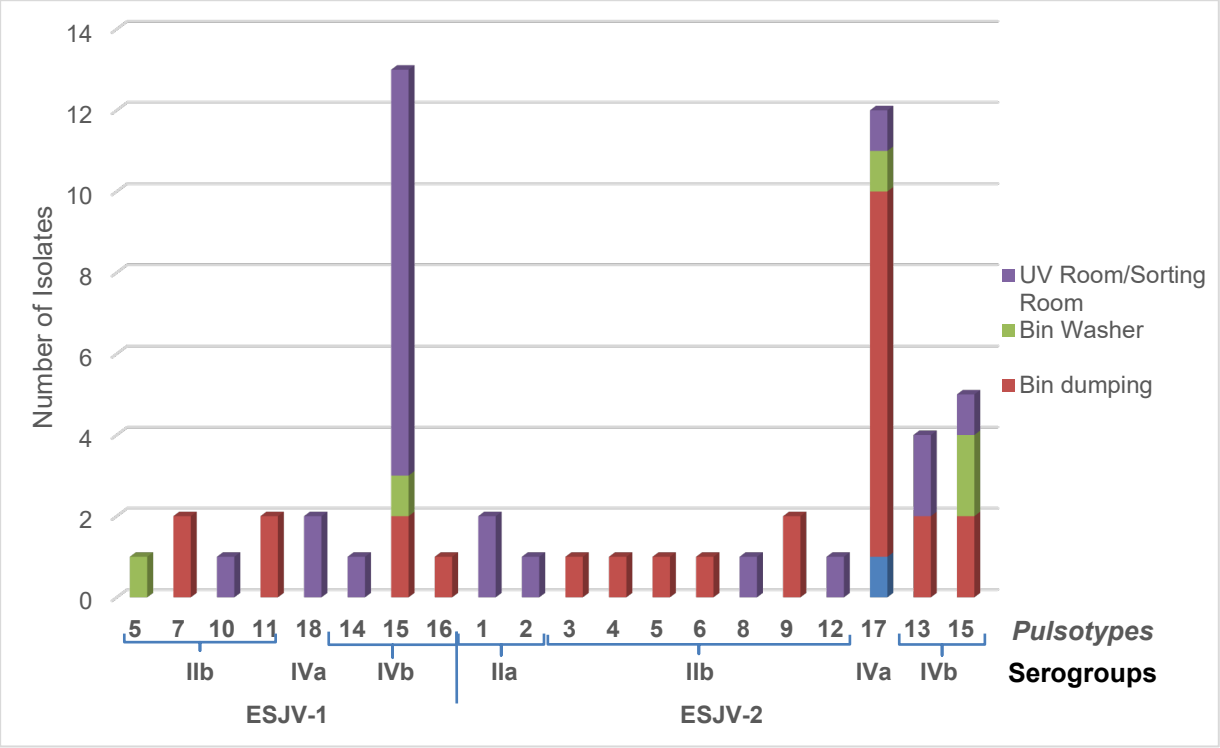
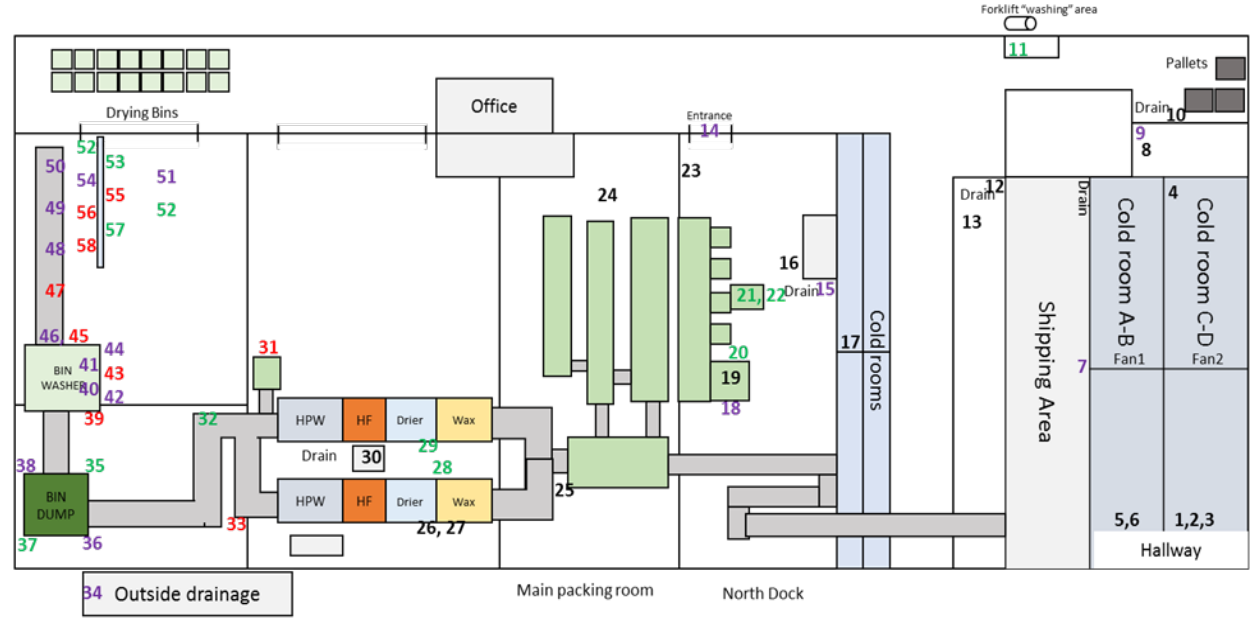
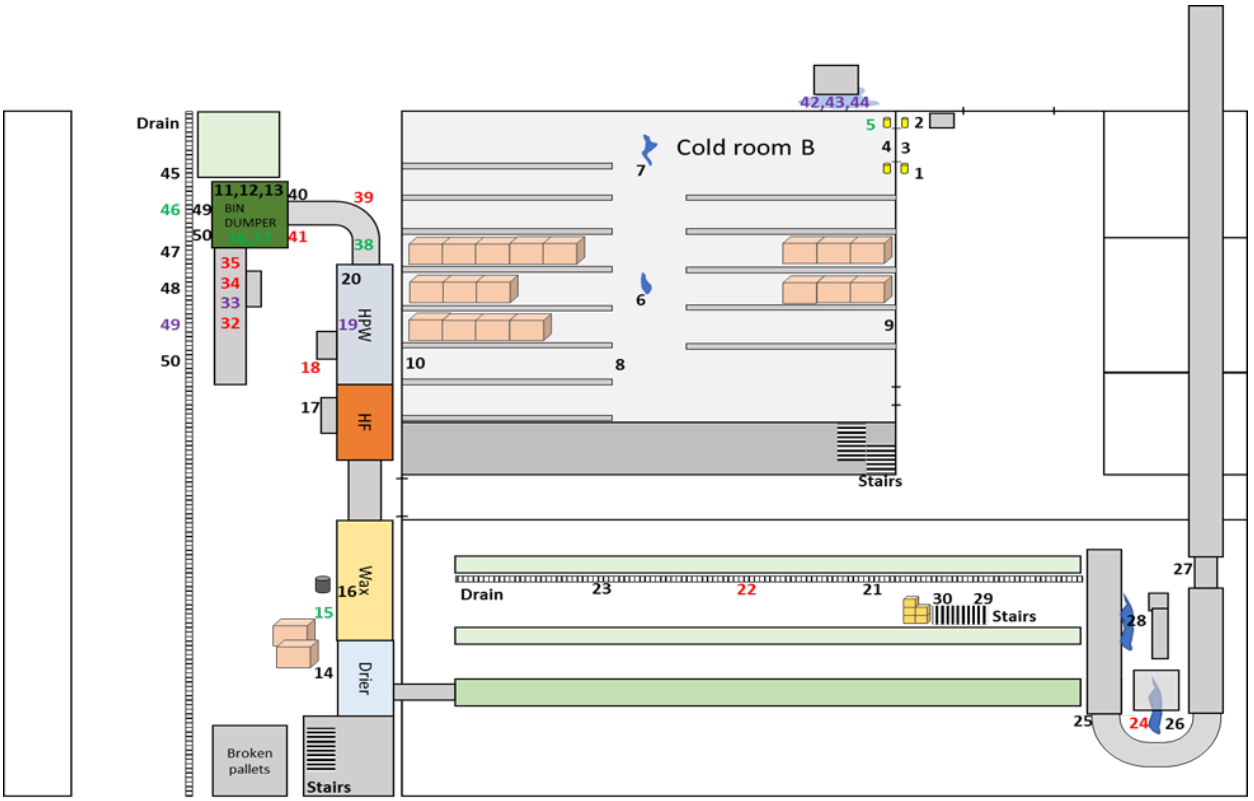


Figure 12. Spatial maps of operational locations of samples positive for *Listeria* in citrus packinghouse



Listeria spp.
Listeria monocytogenes
Both together



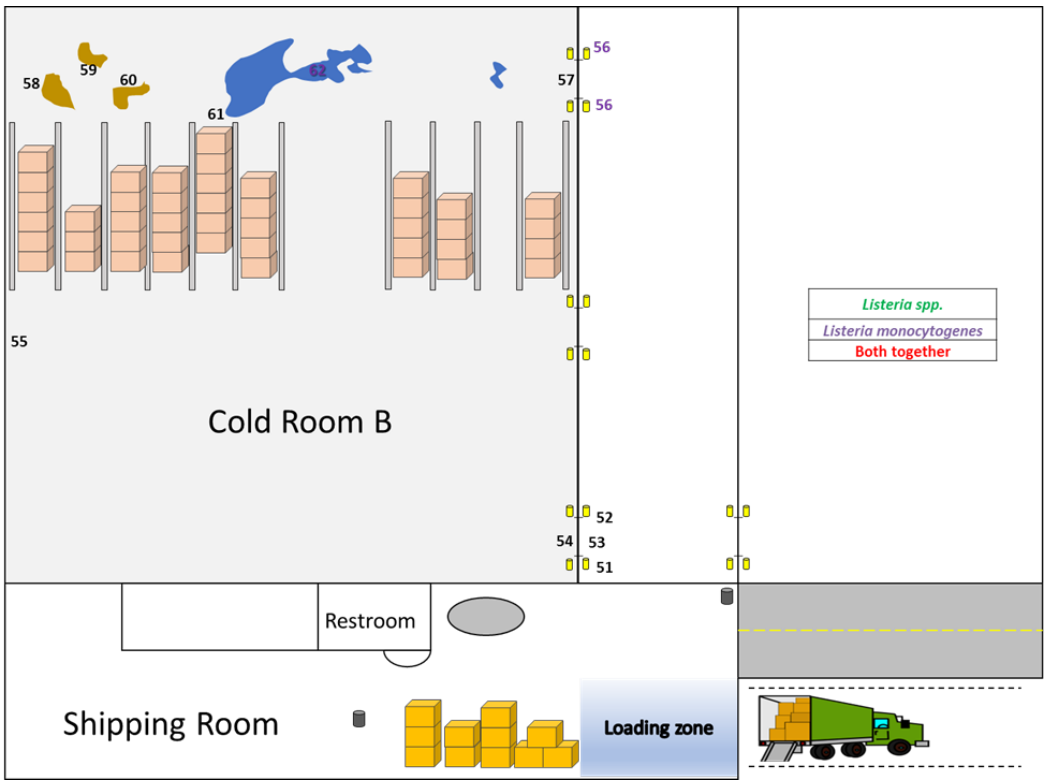
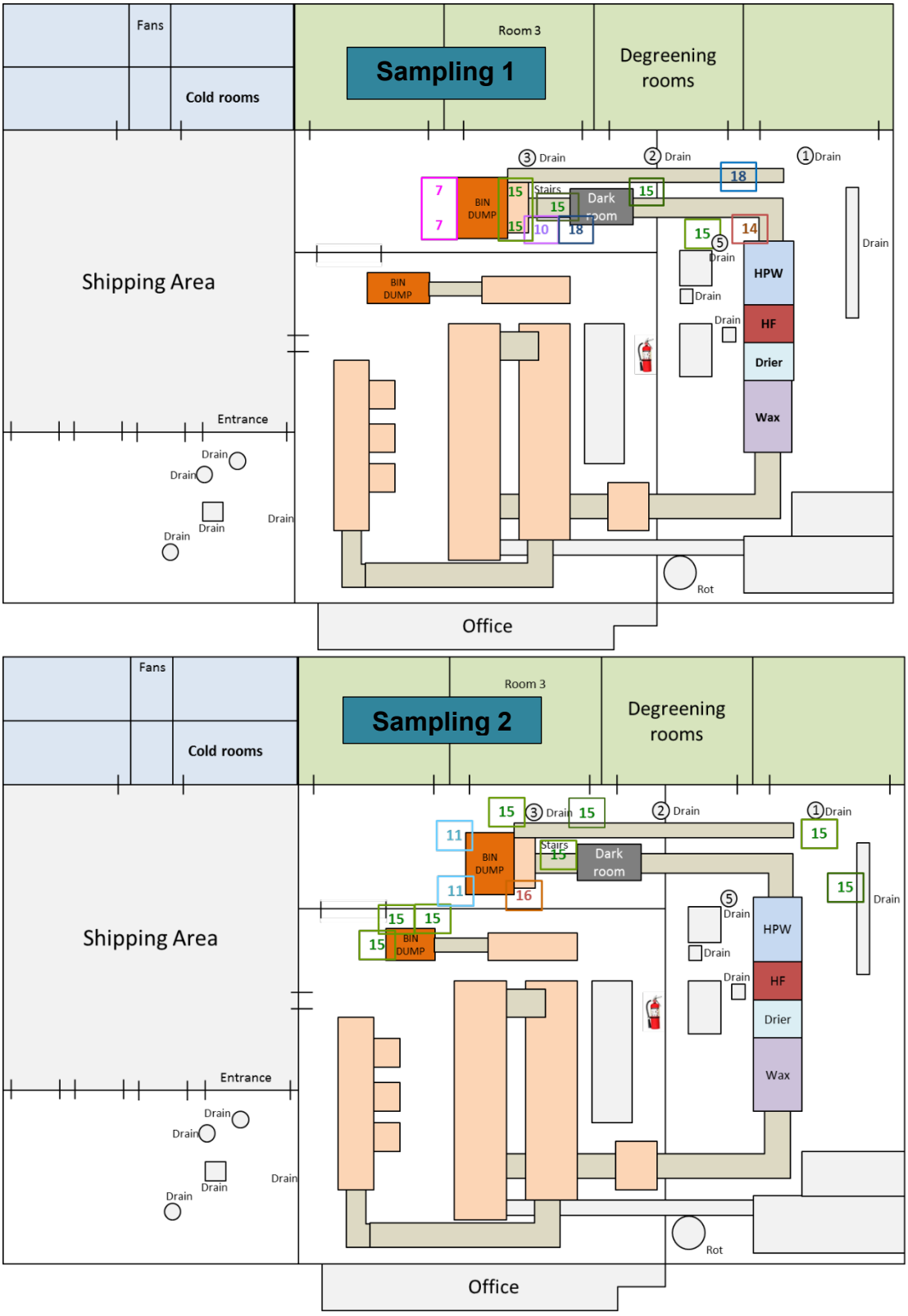

58
59
60
61
62
56
57
56
55
Cold Room B
52
54
53
51
Listeria spp.
Listeria monocytogenes
Both together
Restroom
Shipping Room
Loading zone

Figure 13. Spatial and temporal maps by operational location for *L. monocytogenes* in ESJV-1 packinghouse (for samplings 1–3)



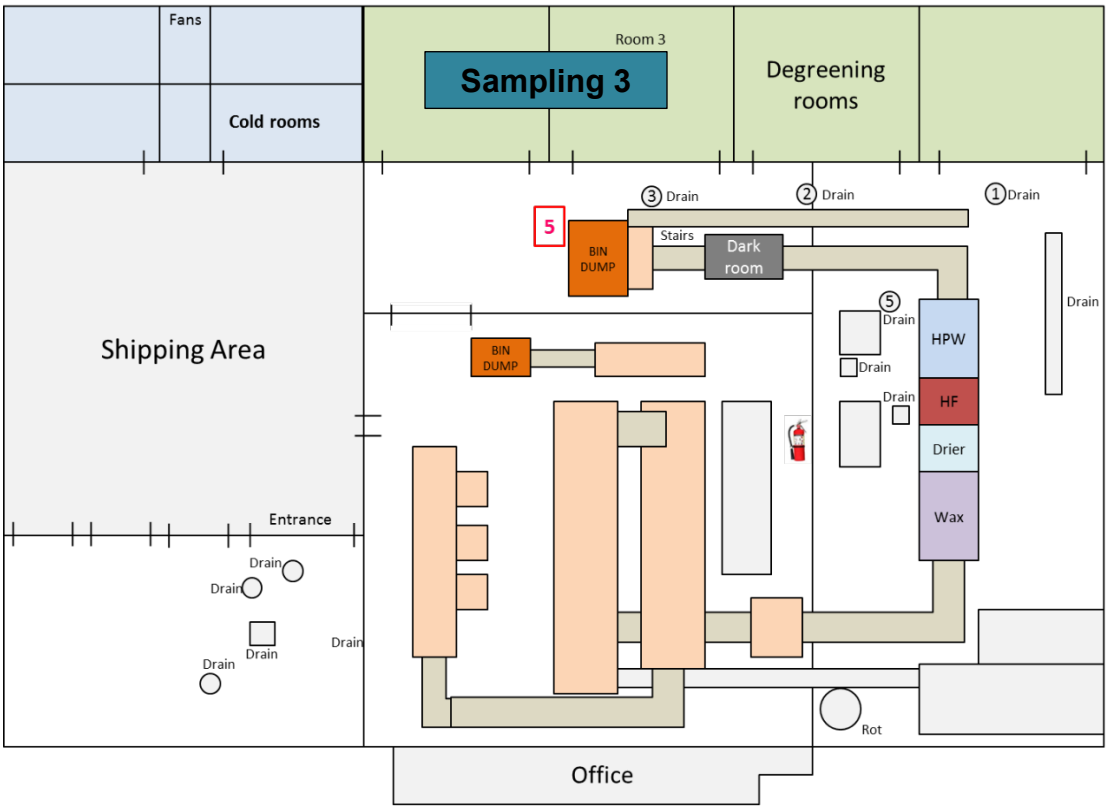
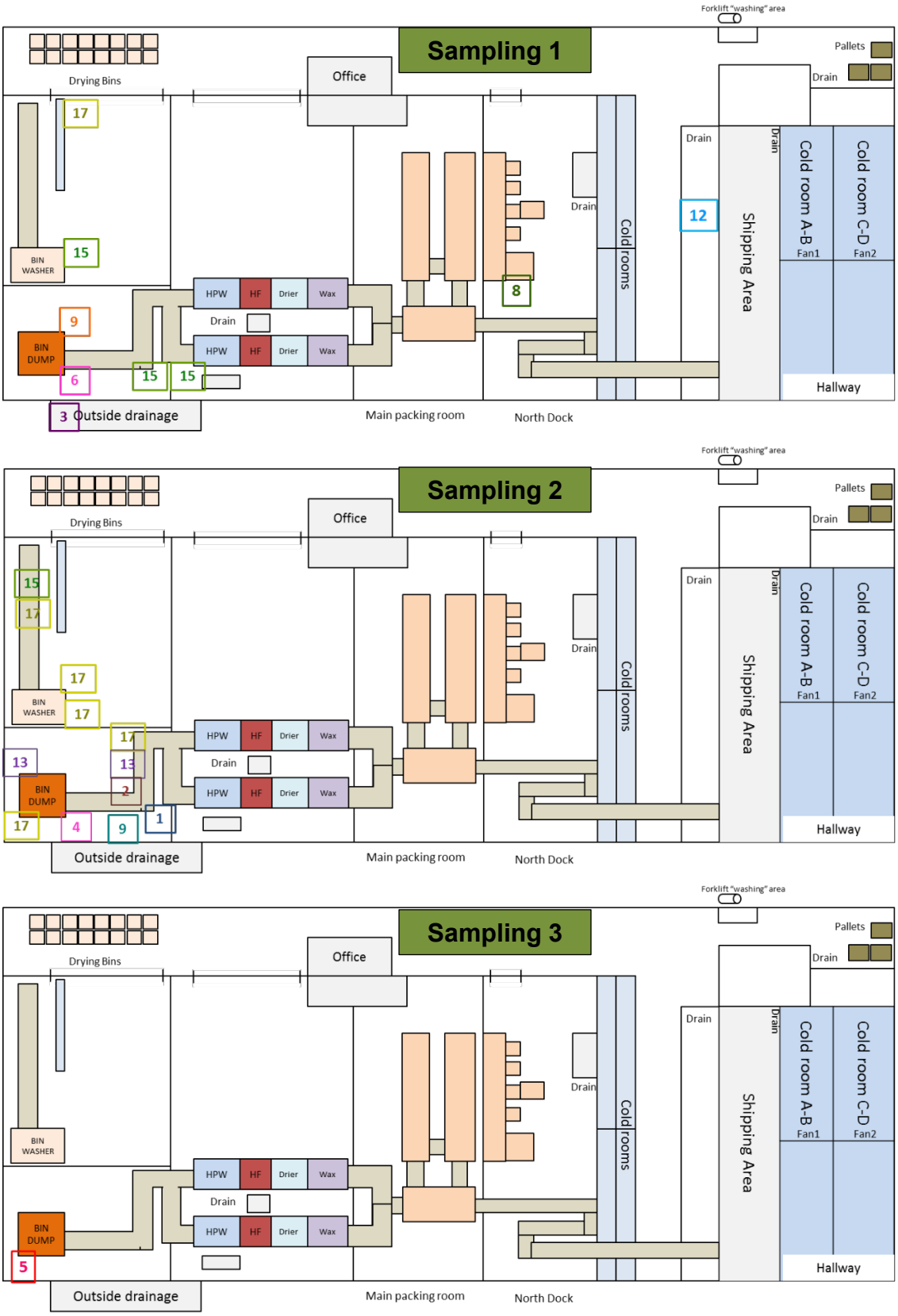

Fans
Cold rooms
Room 3
Sampling 3
Degreening rooms
Shipping Area
Entrance
Office
3 Drain
2 Drain
1 Drain
5
BIN DUMP
Stairs
Dark room
5 Drain
Drain
Drain
Drain
HPW
HF
Drier
Wax
BIN DUMP
Drain
Drain
Drain
Drain
Drain
Rot

Figure 14. Spatial and temporal maps by operational location for *L. monocytogenes* in ESJV-2 packinghouse (for samplings 1–3)



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