CENTER for PRODUCE SAFETY

CPS 2020 RFP
FINAL PROJECT REPORT

Project Title

Identification of quantitative and qualitative patterns of environmental contamination by *Listeria* spp. and *L. monocytogenes* in fresh produce processing facilities, and evaluation of practical control measures able to eliminate transient and persistent contamination

Project Period

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Objectives

1. *Assessment of the environmental contamination by Listeria spp. in fresh produce processing plants.*
2. *Establishment of the genetic correlations of the Listeria spp./L. monocytogenes isolates to understand the distribution patterns across different compartments within the same processing plant and among different processing facilities. Identification of persistent and transient Listeria spp. strains.*
3. *Evaluation of the efficacy of control measures currently implemented in commercial fresh produce processing plants against transient and persistent Listeria spp./L. monocytogenes contamination.*

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FINAL REPORT

Abstract

Well-established routine Environmental Monitoring (EM) programs should be designed on a risk-based approach, considering the nature and size of the food operation and reflecting aspects related to the raw materials, the production processes, and the final product but they also need to be regularly revised based on trend analysis. The aim of this project was the **generation of practical knowledge and solutions for the implementation of EM programs in fresh produce processing facilities as different industrial practices and processing environments may account for different contamination patterns**. Based on project outputs, it has been possible to identify and control potential sources of contamination that may affect the products during processing and storage. The project was divided in three main objectives. The **first objective** focused on the identification of hotspots of contamination, with an emphasis on understanding how different factors interact and affect the probability of contamination in various fresh produce processing facilities. This objective consisted of systematic sampling through an EM plan including zoning, sanitary design, location connectivity, and ranking with respect to the length and level of contamination of *Listeria* spp. In the **second objective**, the application of whole genome sequencing (WGS) in food processing environments (FPEs) was used to enhance the understanding of the origin, cross-contamination, reservoir, and possible persistence of specific *Listeria* spp./*L. monocytogenes* isolates. Establishment of the genetic correlations of the *Listeria* spp./*L. monocytogenes* isolates helped to understand the distribution patterns across different processing plants. The **third objective** focused on the evaluation of biocides used during cleaning and disinfection activities in the produce processing facilities of the monitored processing plants. The aim was the identification of potential strain adaptation to common biocides and its impact on the tolerance to different environmental stresses.

The results obtained within this project provided the following main outputs:

i) The use of a modified ISO protocol enhanced the detection of *L. monocytogenes* in the environmental monitoring samples.
ii) Sampling Zone 1 sites provides relevant information on hotspots of contamination that cannot be found in Zone 2 sites.
iii) Only two serotypes were found among the 100 isolates subjected to WGS from the cut lettuce and cut fruit facilities.
iv) The two serotypes (ST155 and ST6) found have been commonly associated with human listeriosis outbreaks, ST155 being the most abundant. Most of the ST6 isolates corresponded to Zone 3 sites and were obtained from the cut lettuce facility.
v) The Zone 3 sites of the two facilities seem to be potential *Listeria* niches mostly due to inadequate cleaning and disinfection procedures.
vi) Biocide resistance was not observed in the *L. monocytogenes* isolates, and the presence of *L. monocytogenes* in the cleaned and disinfected facilities could be mostly linked to incorrect performance of the activities rather than a lack of efficacy of the used biocides.

Background

Listeria monocytogenes is a human pathogen widespread in the environment (soil, water, and organic material) that unlike most bacteria can grow and multiply at low temperatures, making the bacteria a potential problem in ready-to-eat fresh fruit and vegetables (RTE-fF&V). Raw materials entering the process are considered potential sources of *L. monocytogenes* and the cause for the presence of the pathogen in the food processing environments (FPEs) (EFSA, 2018). *L.*

monocytogenes can survive over a decade in hiding places, and temporal breakdowns in hygiene barrier efficiency, poor hygiene practices and unhygienic design of equipment may trigger *L. monocytogenes* food plant contamination (EFSA, 2018). *L. monocytogenes* contamination from environmental sources has shown to play an important role in the finished product contamination (Norton et al., 2001; Simmons and Wiedmann, 2018), particularly in RTE-fF&V as there are no full mitigation strategies in the production process for the complete inactivation. Environmental contamination sources have been widely studied but most of the research has not been focused on fresh produce. Zoellner et al. (2018) conducted an exhaustive search to identify all available research, industry and regulatory documents on *Listeria* environmental monitoring (EM) in food processing facilities. Only 5.5% of the relevant references were focused on fresh produce, highlighting a research gap (Zoellner et al., 2018). The main objective of this project was to contribute generating practical knowledge and solutions for the implementation of EM programs in fresh produce processing facilities as different industrial practices and processing environments may account for different contamination patterns. Valuable data will be acquired to complement the current knowledge on *Listeria* spp. environmental monitoring (EM) in fresh produce processing facilities to prevent contamination risks.

L. monocytogenes can be spread throughout the facility due to contaminated contact materials, inappropriate personnel movement and food workflows (Ferreira et al., 2014). Such contamination can be an intermediate step in **transmission** from their original habitat in the environment to reservoirs such as from biofilms, process water and organic plant residues to food-contact surfaces and food under processing. Although *L. monocytogenes* is capable of a rapid attachment to various food processing surfaces, such as stainless steel, the debate continues about whether *L. monocytogenes* can form true biofilms. **Persistence of *L. monocytogenes* strains** in food facilities can often be traced back to the unhygienic design of equipment, infrastructure problems or inefficient cleaning and sanitation procedures. However, the high prevalence of **transient *L. monocytogenes* strains** may indicate that *L. monocytogenes* is being introduced repeatedly from outside sources (Jagadeesan et al., 2019).

Well-established routine EM programs should be designed on a risk-based approach, considering the nature and size of the food operation and reflecting aspects related to the raw materials, the production processes and the final product but they also need to be regularly revised based on trend analysis. As described by Zoellner et al. (2019), the current approach to designing environmental monitoring relies the most on zoning (standard division of surfaces in a facility with respect to the proximity and contact with foods) and sanitary design (whether a surface is cleanable or not, or how well it could be cleaned).

To accurately identify the origin of contamination, it is crucial to establish the relatedness of organisms involved in a single contamination event. The high-throughput capability and the increased speed of next-generation sequencing (NGS) followed by the steep drop in costs and the development of new bioinformatics tools to process the data has allowed the application of **whole genome sequencing** (**WGS**) in *L. monocytogenes* outbreak investigation in many countries including the USA and Europe (Schjørring et al., 2017; Allard et al., 2018; Toledo et al., 2018). Using WGS, base-by-base comparisons of entire genomes are possible as well as retrieval of additional information such as serotypes and virulence or antimicrobial resistance markers (Moura et al., 2016). The use of WGS in FPEs represents a powerful tool to enhance the understanding of the origin, cross-contamination, reservoir, and possible persistence of specific subpopulations along the food chain. Hurley et al. (2019) demonstrated the application of a WGS-based approach as a proactive tool to support food safety control of *L. monocytogenes* in combination with a bioinformatic analysis targeting known biomarkers associated with persistence, antimicrobial resistance, as well as predicted hypovirulent and hypervirulent phenotypes.

Research has been driven to establish strategies to control *L. monocytogenes* in processing plants. Simmons and Wiedmann (2018) indicated that key control strategies include sanitary equipment and facility design as well as the implementation of Sanitation Standard Operating Procedures (SSOPs). Cleaning and sanitizing are the most important aspects of a SSOP. The specific steps used to clean and sanitize equipment and environmental areas are unique to each processor but in most of the cases, the same sanitizers are applied for different processing practices. The food industry uses quaternary ammonium compounds (QACs), such as benzalkonium chloride (BC), and also sodium hypochlorite to sanitize equipment and environmental areas. However, inappropriate use of these chemicals, such as insufficient rinsing after disinfection and inadequate dosage, may lead to niches with sub-inhibitory concentrations of these compounds (Yu et al., 2018). *L. monocytogenes* has been shown to adapt to QACs and chlorine resulting in an increased survival of this microorganism in food environments (Bansal et al., 2018; Rodríguez-Melcón et al., 2019). Rotating sanitizers have been reported to provide greater long-term effectiveness and prevention of *L. monocytogenes* becoming established in niches in the environment (FDA, 2017).

Research Methods and Results

Objective 1

Three processing facilities (cut vegetables, cut fruits and prepared salads) were sampled in this study. After several meetings with the industry managers and visits to the processing operations, specific sites were selected for the three EM samplings (EM1, EM2 and EM3). The sampling sites were divided into three Zones following the FDA draft guidance on Lm control in ready-to-eat foods (FDA, 2017; Zoellner et al., 2019). Zone 1: corresponded to food-contact surfaces (FCS); Zone 2: proximity to food-contact surfaces (close to FCS), and Zone 3: remote from food-contact surfaces within the processing area (remote from FCS).

In all processing facilities, sites from areas of concern were visited first with one representative from the operation for mapping and identifying the processing operations. Sampling sites in the facilities were risk rated to determine the number of test points to sample considering the physical size of the processing operation and complexity and number of steps involved in the processes. A list of the selected sampling sites is included in **Tables 1, 2 and 3**. Each site was sampled three times. There were two samplings, one performed after processing, just before cleaning, and the other after cleaning and disinfection activities.

Once the sites were selected, the type of device used for the sampling (sponges, contact plates and swabs) was defined based on the size and accessibility of the area sampled. Hydrasponge sterile swabs (3M), pre-moistened with sterile water, were used for swabbing. In large surface areas (e.g., floors and walls), approx. >900 cm^2 was swabbed. In small areas, approx. <81 cm^2 was swabbed. For other surface areas, individual units (e.g., one drain or one wheel) were swabbed. Sponges were maintained in sterile bags with 100 mL of half Fraser broth until the analysis. Additionally, one water sample from the centrifuge drainage (2 L) was also collected.

The levels of *Listeria* spp. were quantified by filtration of 50 mL and 10 mL of the half Fraser broth (Scharlab), in which the sponges were placed and gently massaged. Filtration was performed using sterile filters (0.45 µm cellulose nitrate) coupled with a vacuum system (Sartorius, Spain). Then, the filters were placed in the selective media ALOA/OCLA agar (Scharlab) incubated for 18-24 h at 37°C. After incubation, *Listeria* spp. was enumerated as the blue-green colonies formed with or without a halo and the blue colonies with an inhibition halo as presumptive *L. monocytogenes*. For each plate, up to five presumptive isolates (i.e., colonies with consistent color and morphology) were sub-streaked onto Brain Heart Infusion (BHI) agar plates followed by

incubation at 37°C for 24 h. All Listeria isolates from the BHI agar plates were re-suspended and kept at -80ºC in 15% glycerol until confirmation by PCR.

Environmental samples were analyzed for the presence of *L. monocytogenes* by the ISO 11290-1 method using one selective media. Presumptive colonies were analyzed by conventional PCR (Bio-Rad® thermal cycler, CA) with specific primers to confirm the presence of *hly* and *iap* genes. For each PCR, positive *L. monocytogenes* CECT 5672 from the Spanish Culture Collection (CECT) and negative (sterile distilled water) controls were included. Template DNA for PCR was prepared by the boiling method. The PCR products were analyzed by agarose gel electrophoresis at 80 V70 min and Red-dye staining (Biotium Inc., CA). UV fluorescence emission was recorded and quantified by using ImageQuant™ LAS 500 (GE Healthcare Bio-Sciences AB, Björkgatan, Sweden). To determine whether there were statistically significant differences between Zones, EM and fresh-cut facilities data were analyzed and interpreted using R studio (version 1.2.1335).

Objective 2

L. monocytogenes isolates obtained from the environmental sampling of the cut vegetables and cut fruit processing plants after processing, just before cleaning (*Objective 1*), were sequenced using WGS. Sample IDs of the two processing plants are reported in **Table 4**. All isolates were streaked on BHI agar plates and incubated at 37°C for 24 h. Genomic DNA from all isolates was purified using the Gentra Puregene Yeast/Bacterial Kit (Qiagen). DNA quantification was performed by Qubit 3.0 (Life Technologies). A total 106 *L. monocytogenes* DNA extracts were sent to AllGenetics experts in sequencing. To prevent potential problems in the library preparation due to extract impurities, DNA was purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), following the instructions provided by the manufacturer. The DNA was quantified using the Qubit dsDNA HS assay (Thermo Fisher Scientific). A whole-genome sequencing library was prepared following Carøe and Bohmann (2020) with minor modifications. Briefly, DNA was randomly sheared using an enzyme cocktail (dsDNA Fragmentase, New England Biolabs). Then, DNA was end-repaired and A-tailed. The library was dual-indexed to allow for post-sequencing demultiplexing and enriched by PCR amplification using the Q5 High-Fidelity 2X Master Mix (New England Biolabs). Libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), following the instructions provided by the manufacturer. Then, libraries were pooled in equimolar amounts according to the quantification results of a Qubit dsDNA HS Assay. The pool was sequenced in a fraction of a NovaSeq PE150 run (Illumina) aiming for a total output of 30 gigabases. The quality of reads in the FASTQ files was assessed using the software FastQC. Adapter trimming of reads was performed using Trimmomatic v 0.39 and their quality were evaluated using FastQC (v 0.11). The previous quality-filtered reads were used to perform a spectrum analysis of the spectrum of k-mers per sample. The KMC k-mer counter tool was used to count the occurrences of all the different k-mers in the quality-filtered reads setting the kmer size parameter to k=21. Illumina reads were assembled by hybrid de novo assembly using Unicycler v0.4.8 with default settings. The quality control reports were summarized using MultiQC. All the genomes were annotated using the PATRIC genome annotation service which employs the algorithm RASTtk to provide annotation of genomic features. Contamination was assessed using CheckM, BWA and Qualimap which provides robust estimates of genome completeness and contamination by using collocated sets of genes that are ubiquitous and single-copy within a phylogenetic lineage included in the chekM tool.

For each assembled sequence, the software LisSerov.0.4.9 to assign each of the isolate to one of the *L. monocytogenes* serotype groups was used. A second serotyping strategy was applied using an in silico serovar prediction approach from multi-locus sequence types (MLST). The MLST allele sequence and profile data was obtained from the PubMLST database using the web

server MLST v2.0. Core-genome MLST (cgMLST) data were analysed to determine allele differences with the BIGSdb PasteurMLST Genome Comparator with the cgMLST schemes using 1,748 loci. Additionally, the assembled genomes were compared with the software ChewBBACA software using the core genome MLST scheme cgMLST1748 obtained from the GIBSdb *Listeria monocytogenes* database. This software is a comprehensive pipeline that includes a set of functions for the creation and validation of cgMLST schemes, providing an allele calling algorithm based on Blast Score Ratio and a set of functions to visualize and validate allele variation at the loci. Different minimum spanning trees based on the loci obtained with the cgMLST1748 scheme were generated using the option MSTree V2 implemented in the GrapeTree software.

Isolates obtained from the environmental sampling of the cut vegetables and cut fruit processing plants after cleaning and disinfection activities, were characterized using the Multiple-Locus Variable Number of Tandem Repeats analysis (MLVA). To perform the analysis, eight primer pairs were used in two 4-plex PCR reactions. The protocol consisted in 2 different multiplex-PCR reactions using the Type-it Microsatellite PCR Kit (Qiagen) and labelled primers. PCR reaction one (PCR1) contained primers for amplification of locus Lm-2, Lm-8 Lm-10 and Lm-11 and the second PCR (PCR2) contained primers for locus Lm-3, Lm-15, Lm-23 and Lm-32 (Sperry et al., 2008). Amplification reactions were performed in a T100™ thermal cycler (Bio-Rad, CA, USA). After amplification, fluorescent PCR products were diluted in nuclease-free water and analyses for MLVA profile were performed by automated capillary electrophoresis on a Spectrum Compact CE System (Promega, WI, USA) with Internal Lane Standard 600 (Promega). Estimated fragment size, dye, peak height, and area data for each isolate were exported into GeneMarker® Software (SoftGenetics®, PA, USA).

Objective 3

L. monocytogenes isolates obtained from the environmental sampling of the three processing plants after the cleaning and disinfection activities and representative of the main MLVA profiles were selected to determine their sensitivity to commercial biocides use by the industry. Briefly, isolates were grown on BHI agar plates at 37°C for 48 hours prior to harvesting. Overnight cultures were tested against each specific biocide at following concentrations: 33%, 5% and 2% from stock solution. Negative samples were included in all the tests. Plates with the overnight cultures and the treatments were transferred to the Infinite® M Plex (TECAN) microplate reader to monitor potential growth of the strains under the tested conditions.

Outcomes and Accomplishments

Objective 1

<u>EM of fresh-cut operations after processing, just before cleaning</u>

A total of 591 samples were collected from the three fresh-cut facilities and EM samplings. Among these samples, 204 corresponded to the cut vegetable facility, 177 to the cut fruit facility, and 210 to the prepared salads. Among them, 195 (33%) were from Zone 1, 132 (22%) from Zone 2 and 264 (45%) from Zone 3. The prevalence of *L. monocytogenes* and *Listeria* spp. was 30% and 40%, respectively, among all the tested samples. Per Zones, the number of *L. monocytogenes* detected samples was 25/195 (13%) in Zone 1, 18/132 (14%) in Zone 2 and 135/264 (51%) in Zone 3 (**Figure 1A**). The prevalence of *Listeria* spp. was higher than that of the pathogenic *L. monocytogenes* as it was 48/195 (25%) in Zone 1, 27/132 (21%) in Zone 2 and 160/264 (61%) in Zone 3 (**Figure 1B**).

Among fresh-cut facilities, *L. monocytogenes* prevalence ranged from 28% (56/204) in the cut vegetables, 25% (45/177) in the cut fruits and 37% (77/210) in the prepared salads (**Table 5**). Among the 56 positive swaps collected from the cut vegetable facility, 14% corresponded to Zone 1, 19% to Zone 2 and 41% to Zone 3 (**Figure 2A**). As could be expected, samples with the highest prevalence of *L. monocytogenes* corresponded to Zone 3 sites, such as drains, water on the floors, and stairs to access the weight filler. The results indicate that the detection of *L. monocytogenes* was in samples from the boot washer, floor cracks, forklift wheels and drains. By comparison, the prevalence of *Listeria* spp. in the cut vegetable facility was higher than that of the pathogenic *L. monocytogenes* as 22 (43%) samples corresponded to Zone 1, 18 (29%) to Zone 2 and 48 (53%) to Zone 3 (**Figure 2B**). In the cut fruit facility, among the 177 sites examined, *L. monocytogenes* average prevalence ranged from 7 (12%) in Zone 1, 3 (8%) in Zone 2 and 35 (45%) in Zone 3 (**Figure 2C**). Sample sites detected positive for *L. monocytogenes* corresponded to cover lid edges, floor covers, and the ramp used to go to the down area. Listeria spp. average prevalence in the cut fruit facility ranged from 15 (25%) in Zone 1, 6 (15%) in Zone 2, and 53 (68%) in Zone 3 (**Figure 2D**). In the prepared salad facility, among the 210 samples collected, *L. monocytogenes* prevalence ranged from 11 (13%) in Zone 1, to 3 (10%) in Zone 2 and 63 (66%) in Zone 3 (**Figure 2E**) and *Listeria* spp. prevalence accounted for 11 (13%) positive samples in Zone 1, 3 (10%) in Zone 2 and 59 (62%) in Zone 3 (**Figure 2F**).

Interestingly, sampling points with higher *Listeria* spp. counts corresponded to swaps collected in Zones 3 (mean value of 1.24 log cfu/unit), and lower counts were enumerated for samples of Zone 1 and Zone 2 (mean value of 0.35 log cfu/unit) (**Figure 3A**). Among the facilities, similar levels of *Listeria* spp. were observed with mean values of 0.79, 0.79 and 0.66 log cfu/unit for cut vegetables, cut fruits and prepared salads (**Figure 3B**). This study also focused on the identification of sites showing the persistence of *L. monocytogenes* and *Listeria* spp. over the EM samplings. Among the EM samplings, fewer detected sites were expected at the end of the sampling with repeat findings. However, *L. monocytogenes* and *Listeria* spp. were detected in the three EM samplings and their prevalence through the EM samplings differed without a clear trend in the three fresh-cut facilities. Thus, in the cut vegetable facility, the prevalence of *L. monocytogenes* among 68 samples was 19%, 40% and 15% in EM1, EM2 and EM3, respectively (**Figure 4A**). In the cut fruit facility, among 59 samples detected positive, *L. monocytogenes* decreased with the EM sampling, ranging from 38% to 15% and 12% in EM1, EM2 and EM3, respectively (**Figure 4C**). This decrease was due to meetings with the manager of this facility and the corrective actions taken. In the prepared salad facility, the prevalence of *L. monocytogenes* among 70 samples was 31%, 21% and 36% for EM1, EM2 and EM3, respectively (**Figure 4E**). On the other hand, the prevalence of *Listeria* spp. among EM samplings varied as well but the levels in the cut vegetables and prepared salads facilities were similar among EM samplings (39-44% for cut vegetables and 28-29%, for cut fruits). The greatest differences in the prevalence of *Listeria* spp. were observed in the cut fruit facility among EM samplings (40%, 20% and 48% for EM1, EM2 and EM, respectively) (**Figure 4B, D and F**).

In this study of almost 10 months of EM performed in three different fresh-cut operations, *L. monocytogenes* and *Listeria* spp. prevalence and contamination patterns were characterized. *L. monocytogenes* was found in the three facilities sampled in this study, with an average prevalence of 30% and 40% for *Listeria* spp. among 591 environmental samples. In this set of experiments, sampling was conducted after production, just before cleaning, which explains the high prevalence compared with previous studies. In this study, particular attention was paid to the identification of potential niches of contamination and entrenched *Listeria* that are exposed by equipment movement, becoming sources of cross-contamination. The data reported here helped the identification of specific points where *Listeria* was recovered across different EM samplings. These sampling sites could be identified as hotspots of contamination such as rollers and drains. This information is very useful to improve the management of these hotspots during the cleaning

and disinfection activities and improve *Listeria* control strategies in these fresh-cut facilities. An intensified EM sampling was conducted for the sites identified as potential sources of *L. monocytogenes* (264 sites in Zone 3, 132 sites in Zone 2 and 195 sites in Zone 1). The upstream from the positive FCS helped the identification of potential sources of contamination placed within the processing area, showing a higher risk. There is a consensus supporting that there is a considerable likelihood that *Listeria* spp.-positive samples are positive for *L. monocytogenes* (Sullivan and Wiedmann, 2020). FDA guidelines indicate that there is minimal value in determining whether *Listeria* spp. detected on a non-FCS is *L. monocytogenes* because processors should eliminate the *Listeria* spp. regardless of whether it is *L. monocytogenes* (FDA, 2017). In the three fresh-cut facilities studied here, 178 (30%) samples tested positive for *L. monocytogenes* while 235 (40%) samples were positive for *Listeria* spp. According with our results, Cornell and CALS (2019), have shown that 67% of *Listeria*-positive samples contain *L. monocytogenes*, which reinforces the application of this approach. Regarding Zones, another main difference between the present study and the previous ones is that environmental sampling was conducted in sites located in Zone 1, Zone 2 and Zone 3 versus the majority of the studies that avoided sampling in Zone 1 sites (food-contact surfaces) (Sullivan and Wiedmann, 2020). In our study, the highest prevalence of *L. monocytogenes*/*Listeria* spp. was in Zone 3 in the three facilities, but surprisingly higher *L. monocytogenes* prevalence was observed in Zone 1 than in Zone 2 in two of the facilities, the cut fruit and the prepared salads facilities. In the case of *Listeria* spp., Zone 1 in the three facilities always showed a higher prevalence than Zone 2. The sampling conducted in this study allowed the detection of *L. monocytogenes* on several FCS surfaces, highlighting the interest in including FCS in the EM. The samples taken after production reflected the risk of activities likely to contribute to equipment and product cross-contamination. Although *L. monocytogenes* detections after production may only be transients, repeat detections in the same area indicate areas that require extensive investigation. Results obtained in the present study identified two Zone 1 sites (bins and output rollers) from the prepared salad facility to be positive in two EM samplings, indicating the relevance of these sites to contribute to product cross-contamination. In all the facilities, drains were recurrently found positive for *L. monocytogenes* in different EM samplings, confirming these sites as harborage places for this pathogen. After each EM, the results were presented and interpreted by the companies. Some intensive corrective actions for *Listeria* control, including intensive and modified cleaning and sanitation protocols, and maintenance procedures and repaired actions (e.g., floor cracks), were planned and executed.

EM of fresh-cut operations after cleaning and disinfection

In the first EM (EM1), a total of 231 points from the three fresh-cut processing facilities (cut lettuce, cut fruit, and prepared salads) were sampled. The results showed 22/231 positive samples for *L. monocytogenes*. The positive sampling sites are described in **Table 6**. As could be expected, most of the positive samples for *L. monocytogenes* corresponded to Zone 3 sites (e.g., boot washer, drains, floors, etc.). However, one Zone 1 site from the cut fruit processing line (point 33: waste hole to conveyor belt) resulted positive for *L. monocytogenes*, indicating failures in the cleaning and disinfection activities performed in this processing line. Based on this result, corrective actions should be put in place to eliminate the source of contamination and prevent its spread. This sampling site was also *L. monocytogenes*-positive when the EM was performed after processing, just before cleaning. **Table 7** shows the specific sampling zones where the positive *L. monocytogenes* samples were found in the three EM plans. In the EM2 and 3 only samples that tested positive in the first EM sampling were monitored, aiming to find those sites that showed persistent presence of *L. monocytogenes*. The sampling site found positive in EM1, did not test positive in the rest of the EM sampling. However, 13 and 9 sampling sites from EM2 and EM3 where positive for *L. monocytogenes* in all the EM samplings. The genetic characterization of these isolates will help us to understand the nature of this contamination (transient or persistent).

Objective 2

EM of fresh-cut operations after processing, just before cleaning

A total of 100 isolates from the cut lettuce and cut fruit processing plants were sequenced as described in the materials and methods section. The whole-genome sequencing (WGS) yielded between 471,264 and 1,983.932 reads (R1 plus R2 reads) per sample. The high-quality reads obtained for each isolate (average coverage above 10X) were used for assembling to draft the genome sequences. The De novo assembly resulted in genome assemblies from 11 to 301 contigs. The average size of the assemblies was 2,982,015.5. The average N50 value was 253,121.5 bp, indicating a satisfied completeness quality of the assemblies. The results of assemblies for each isolate are summarized in **Table 4.** The genome completeness ranged between 98 to 100%, except for isolate FI1E11-1, which showed contamination and its genome completeness was only 20.9% completed (**Table 8**). The average nucleotide identity (ANI) of the isolates was higher than 99% in most pairs of genomes with the same serotype. However, ANI dropped drastically to 94% between different serotypes. In general, it is accepted that the average whole genome ANI in organisms belonging to the same species is ≥95% (Jain et al., 2018). The 100 isolates were then subjected to molecular serotyping by WGS using different strategies and software. The results obtained indicated that 80% of the isolates (80/100) belonged to serotype 1/2a-3a, 19% (19/100) to serotype 4b-4d-4e, and 1% (1/100) unknown. Secondly, based on the seven-gene MLST in silico serovar prediction approach from PubMLST database using the web server MLST v2.0, *L. monocytogenes* isolates were classified in linage I and linage II, 2 Sequence type (ST) and 2 Clonal Complexes (CCs). The most abundant ST/CC was ST155/CC155 (85%), followed by ST6/CC6 (15%). Lastly, assemblies sequence data of *L. monocytogenes* isolates were genotyped with core genome MLST (cgMLST) based on 1748 genes scheme using the web server BIGSdb-Pasteur v1.36.7 (Moura et al., 2016). The results showed that each of the strains from ST155 and ST6 belonged to a different core-genome type (CT). In general, most of assembled sequence were identified with two CTs 29464 and 17837 (**Table 9**), except for few isolates that were identified with more than one profile. Minimum spanning trees were built based on the loci obtained with the cgMLST1748 scheme, and show the similarity among the isolates, grouping all of them in two clusters. The first cluster ST155 (CC155) was 1018 alleles distant from the second cluster ST6 (CC6), as can be seen in **Figure 5**. The first cluster consists of 85 isolates, which belong to the ST155. All isolates of this cluster, irrespective of origin, share the same similar allelic profiles with distance of one, two, and three alleles, except for the isolate FI4E-54-5, which showed an allelic distance of 9. A similar trend was observed among the *L. monocytogenes* isolates classified in the ST6 cluster, which differed between 0 and 3 allelic variants, indicating the low diversity among the isolates. Minimum spanning trees were also generated based on the type of commodity, sampling zones and sampling points, for each processing line. In the case of commodity type, the tree shows that most of the ST6 was found in the cut lettuce processing line, indicating a different origin for this specific ST (**Figure 6**). Only four ST6 isolates were obtained from the cut fruit processing line, while 15 isolates were obtained from six different points of the cut lettuce processing line (**Figure 6**). To differentiate among the different sampling zones, **Figure 7** shows that all the ST6 correspond to samples obtained from Zone 3. However, 80 isolates belonging to the ST155 were obtained from all the Zones (Zone 1, 2 and 3). It has been reported that *L. monocytogenes* serogroup 1/2a strains might have a competitive growth advantage over serotype 4b during refrigerated storage, which could explain the predominance of this serotype in both processing lines. Taking the origin of the sample (sampling point) into account, the obtained MST indicates that most of the ST6 isolates have been obtained from wheels (cart and box holder wheels) as well as floor cracks, showing a relationship among the samples (**Figure 8**).

EM of fresh-cut operations after cleaning and disinfection

The *L. monocytogenes* isolates obtained in the processing facilities after the cleaning and disinfection activities were characterized using the MLVA typing method. In the first EM sampling (EM1) of the cut lettuce processing line, a total of 11 isolates were obtained from 6 positive sampling points, while 24 isolates were obtained from the cut fruit processing lines (7 positive sampling points), and 53 isolates from the prepared salads processing lines (9 positive sampling points). In the second and third EM samplings 13 and 9 sampling points were positive, respectively. A total of 102 (EM2) and 63 (EM3) isolates were obtained. Among the isolates obtained after EM2, 33 isolates were collected from cut lettuce processing lines, 9 isolates from cut fruit and 60 isolates from prepared salads processing line. In the case of EM3, a total of 63 isolates were collected: 5 isolates from the cut lettuce processing line and 58 isolates from the prepared salads processing line. In the EM3, none of the samples taken in the cut fruit processing line was positive for Lm. All isolates were classified based on their MLVA detection profile. Among all the isolates only two different MLVA profiles were obtained. The MLVA profiles detected were 1) 3-13-1-3-15-1-3-15 (96.4% of the isolates) and 2) 5-16-1-3-20-3-3-11 (3.2 % of the isolates). **Table 10** shows a summary of all the MLVA profiles detected for each processing plant and their prevalence. Based on the WGS of a pool of the isolates, the MLVA profile 3-13-1-3-15-1-3-15 corresponds to CC155 and MLVA profile 5-16-1-3-20-3-3-11 corresponds to CC6. CC155 1 is characterized for being a more persistent isolate detected through EM. In addition, a decrease in the prevalence of CC6 was observed throughout the samplings. In most of the cases, when analyzing the isolates obtained from the same sampling point, only one serotype was observed, with three exceptions: isolate 31.3 from EM2 of the cut fruit processing plant; isolate 14.1 from EM1 of the prepared salads line; and isolate 64.2 from EM2 of the prepared salads line. A different profile (MLVA profile 3: 3-13-1-3-19-3-3-14) was detected in EM1 in the prepared salads processing line (point 39), but this profile was not found in any other sample. Regarding the abundance of the two clonal complex, 155 and 6, these results suggested that these serotypes were more resistant to the cleaning and disinfection activities performed by the company.

Objective 3

The evaluation of the efficacy of control measures currently implemented in the monitored processing plants was performed against isolates obtained in the EM performed after cleaning and disinfection activities. The processing lines included in this study subcontracted the cleaning and disinfection activities within the plant. This external company uses a protocol that includes three different biocides and three rinsings, one between each biocide. The biocides included in this study are summarized in **Table 11**. Isolates representative of the two main MLVA profiles (9 strains from each processing line; 66.6% MLVA1 and 33.3% MLVA2) were selected for the resistance tests, using the biocides applied by the industry. When the concentration recommended by the manufacturer was applied, none of the tested isolates survived. The same was observed when the biocide concentration was reduced by 50% and also 2%, indicating that all the isolates obtained after the cleaning and disinfection activities were sensitive to the applied biocides (**Figure 9**). Therefore, the reason for finding positive *L. monocytogenes* isolates, even after the cleaning and disinfection activities, was not due to the presence of persister cells but mostly because of an incomplete cleaning and disinfection. Therefore, it is very important to verify the cleaning and disinfection activities to avoid the creation of persister niches, which could be the source of contamination.

Summary of Findings and Recommendations

Objective 1

- A modified protocol of the ISO method was performed to enhance the detection of *L. monocytogenes* in the environmental monitoring samples. The improvements in the protocol included the transport of the swaps from the plant to the lab in the pre-enrichment broth (half Fraser broth) instead of buffered peptone water (BPW) and the use of filtration of 100 mL of the pre-enrichment broth instead of 1 mL of the pre-enrichment to the enrichment step to decrease the detection limit (detected in 100 mL).
- Two types of environmental samplings were performed: 1) after processing, just before cleaning; and 2) after cleaning and disinfection. The information obtained from the sampling of the processing environment after processing was very valuable to obtain information about the routes for the entrance of contamination in the processing environment.
- In all the EM samplings, samples were also taken from FCS (Zone 1). It was found that the prevalence of *L. monocytogenes* in these samples was, in many cases, higher than in Zone 2 samples. Therefore, additional information can be obtained including Zone 1 samples, mostly for the identification of hotspots of contamination.
- After processing, just before cleaning, the highest *L. monocytogenes* prevalence was observed in Zone 3 (61%, 160/264), followed by Zone 1 (25%, 48/195) and Zone 2 (21%, 27/132).
- Relationship between *Listeria* spp. counts and *L. monocytogenes* detection: Detection of *L. monocytogenes* was associated with variable *Listeria* spp. counts. In samples taken after processing, *L. monocytogenes* was detected in about 80% of the sampling points that showed counts of *Listeria* spp. Therefore, analyses of *Listeria* spp. in all the sampling zones represent a valuable strategy and an appropriate approach for an EM sampling program.
- The EM sampling performed after the cleaning and disinfection operations demonstrated that *L. monocytogenes* was still detected mostly in sites of Zone 3, but also in one site of Zone 1.
- The high prevalence of *L. monocytogenes* found in Zone 3 indicates the difficulties of the cleaning and disinfection of these areas and the need for improving the sampling frequency and the number of test points to ensure that the corrective actions have been undertaken.

Objective 2

- The collection of multiple isolates (up to five confirmed positive colonies from filtration and enrichment) per sample point helped to capture the diversity of *L. monocytogenes* in the processing facilities. In the two sampling approaches, only two different serotypes were observed.
- WGS was performed on isolates obtained from the cut lettuce and cut fruit facilities. The genetic characterization of the *L. monocytogenes* isolates (lineage groups and serotypes) evidenced the low diversity within the different zones of the processing environment as well as different processing facilities.
- Two *L. monocytogenes* serotypes, ST155 and ST6, were identified in samples taken after processing. Each strain belonged to a different core-genome type (CT), with CT155 and CT6 for ST155 and ST6, respectively. ST155 belongs to lineage groups 1/2a, 3a while ST6 belongs to lineage 4b, 4d, 4e.
- Among the 100 isolates included in the WG, 19 corresponded to the ST6 and 78 to the ST155. Most of the isolates belonging to the ST6 (n=15) were found in the cut lettuce facility, while only four ST6 isolates were in the cut fruit facility. These results could indicate potential reservoir sites in the cut lettuce facility and the potential risks of recontamination.

- All the ST6 isolates corresponded to samples obtained from Zone 3 (e.g., wheels and floors), indicating the potential presence of *Listeria* niches in these areas mostly due to inadequate cleaning and disinfection procedures.
- The same serotypes were found in the cut facilities after the cleaning and disinfection activities.
- Our results indicate that the diversity of *L. monocytogenes* serotypes was very low. This reduced diversity could be due to the fact that all the isolates were obtained after conventional culture methods. The use of a metagenomic approach could open the possibility that less adapted serotypes could have been identified.
- Interventions after the sampling events changed contamination scenarios substantially.

Objective 3

- Isolates obtained from the EM performed after the cleaning and disinfection activities were tested against the biocides used by the industry for the cleaning and disinfection activities.
- All the isolates were sensitive to concentrations of the biocides much lower than the doses recommended by the manufacturer.
- The results obtained highlight the need for validating the cleaning activities because even if there are no persister cells in the processing environment if the cleaning activities are not well performed, contamination niches might occur.
- Intensive cleaning and sanitation protocols, and maintenance procedures and repair strategies for drains, floor cracks and boot washers were some of the corrective actions that the company implemented.

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APPENDICES

Publications

Gil, M.I., Truchado, P., Tudela, J.A., Allende, A. 2023. Environmental monitoring of three fresh-cut processing facilities reveals harborage sites for *Listeria monocytogenes*. Food Microbiology, to be submitted.

Truchado, P., Férez, J.A., Gil, M.I., Allende, A. 2023. Longitudinal Study of *Listeria monocytogenes* Isolates from Fresh Produce Processing Plants: Establishment of Genetic Diversity using Whole-Genome Sequencing. Food Control, to be submitted.

Gomez-Galindo, M., Truchado, P., Gil, M.I., Allende, A. 2023. Identification of Multilocus Variable-Number Tandem Repeat Analysis (MLVA) Typing of *Listeria monocytogenes* Isolates from Fresh Produce Processing Plants. Food Microbiology, to be submitted.

Presentations

Title: "*Listeria monocytogenes* in the processing environment of fruits and vegetables: A challenge for the production industry in Europe". Ana Allende, In: Barcelona Biofilm Summit (BBS) 2022, Organized by IRTA, UAB and CHRISTEYNS together with Alimentaria FoodTech, Barcelona, 10th November 2022.

Budget Summary

A total of $256,468 was awarded to this project, and all funds were spent.

The budget allocated to this project was spent as described in the following table:

Institution:	**CEBAS-CSIC**			**PI: Ana Allende**			
CPS No.:	**2021CPS01**			Truchado, Co-PI	Maria I, Co-PI		
Total Research Funded: $256,468				PI	aallende@cebas.csic.es		
CPS Research Term	1/1/2021-12/31/2022 **NCE 3.31.23, Report due 3.31.23**			Accounting	rminguez@cebas.csic.es		
CPS Project Term:	1/1/2021-6/30/2023						
Title:	Identification of quantitative and qualitative patterns of environmental contamination by Listeria spp. and L. monocytogenes in fresh produce processing facilities and evaluation of practical control measures able to eliminate transient and persistent contamination.						
Award Budget		Salaries	Travel	Supplies	Other	Indirect Costs	Total Research Funded
Year 1		$28,740.00	$17,116.00	$51,920.00	$3,900.00	$2,299.00	$103,975.00
Year 2		$73,114.00	$12,058.00	$49,172.00	$3,900.00	$5,849.00	$144,093.00
Year 3			$6,500.00	$0.00	$1,900.00		$8,400.00
TOTALS		**$101,854.00**	**$35,674.00**	**$101,092.00**	**$9,700.00**	**$8,148.00**	**$256,468.00**

Invoice Date	Invoice Number	Salaries	Travel	Supplies	Other	Indirect Costs	Total Research Funded		**Actual exchange rate**	**Billed exchange rate**
05/29/21	09020221050SB-01	$0.00	$0.00	$7,639.07	$33.59	$0.00	$7,672.66	1-3/21	1.2517010	1.20700
07/19/21	09020221091SB-02	$6,478.04	$0.00	$16,239.57	$12.24	$518.25	$23,248.09	4-6/21	1.2235798	1.18019
10/21/21	09020221134SB-03	$7,668.27	$0.00	$9,283.92	$12.02	$613.47	$17,577.68	7-9/21	1.2020000	1.16550
01/14/22	09020222001SB-04	$8,646.43	$0.00	$7,051.87	$0.00	$691.71	$16,390.01	10-12/21	1.1405000	1.14630
05/12/22	116008622050010-05	$6,636.26	$0.00	$5,482.95	$1,660.14	$530.91	$14,310.26	1-3/22	1.0858990	1.0583000
07/25/22	11608622070033-06	$18,788.83	$1,274.13	$33,917.34	$10.66	$1,503.11	$55,494.07	4-6/22	1.0664000	1.01310
10/24/22	OPE02039_3T/2022-07	$18,519.52	$0.00	$17,191.52	$10.57	$1,481.56	$37,203.17	7-9/22	1.0572999	0.97300
01/23/23	OPE02039_4T/2022	$24,746.19	$332.21	$57,864.94	$128.41	$1,979.71	$85,051.46	10-12/22	1.1122000	1.08260
Cumulative Balance		$91,483.53	$1,606.34	$154,671.18	$1,867.63	$7,318.73	$256,947.40	Sum	9.1395798	8.8259897
Remaining Balance		$10,370.47	$34,067.66	-$53,579.18	$7,832.37	$829.27	-$479.40	Average	1.1424475	1.10324871

Tables 1–11 and Figures 1–9 (see below)

Table 1. List of sampling sites per zone tested three times in the cut vegetable facility.

Cut vegetables		
Sites Zone 1	Sites Zone 2	Sites Zone 3
Feedstock boxes	Prepared product conveyor belt roller	Boot washer
Unloading table	Waste product conveyor belt roller (inside)	White room entrance boot washer
Cutting board	Waste product conveyor belt roller (outside)	Floor crack
Cutting blades	Conveyor belt button	Drain near to elevator
Corer	Waste shovel	Rusty corner near forklift
Slicer's blades	Frame under slicer	Chromed wall
First washing station	Roller of the outlet conveyor belt of the first washing station	Water retained on floor
First washing station filter	Water outlet pipes from first washing station	Crack in the floor under the slicer
First washing station outlet conveyor belt	Channel around washing station	Forklift's wheel
Second washing station	Waste conveyor belt roller	Forklift's fork
Conveyor belt to centrifuge	Blue conveyor belt roller	Floor near elevator
Start of vibratory belt	Metallic conveyor belt roller	Rusty pipe
Weigher cone	Centrifuge cage	Portable ladder wheel
Vibratory channels from weight filler	Centrifuge b	Instrument box handle
Weigher plates	Conveyor belt by-pass roller	Floor around centrifuge
Packaging machine outlet	Conveyor belt to vibratory belt roller	Floor around centrifuge outlet
Spinning table	Under vision system	Floor's holes near centrifuge
	Floor under weigher belt	Drain before vibratory belt
	Electrical panel's rubber junction	Drain under vision system
	Weight filler's rubber seals	Stairs to weight filler
	Blue conveyor belt button	Floor metal cover edges
		White room exit door
		Cart wheels
		Cart wheels
		Cart wheels
		Floor's holes near pillar
		Pillar junction
		Floor in the packaging area
		Drain
		Box holder wheels

Table 2. List of sampling sites per zone tested three times in the cut fruit facility.

Cut fruits		
Sites Zone 1	Sites Zone 2	Sites Zone 3
Washing station	Waste conveyor belt	Down ramp
Wash filter	Waste conveyor belt roller	Drain near waste area
Carrot cutting board	Apple cores conveyor belt	Floor near apple wash station
Carrot strips conveyor belt	Apple dipping tray	Drain near washing station
Kiwi peeler	Dipping conveyor belt roller	Forklift fork
Pineapple radial blades	Edge of roller conveyor belt for snack	Forklift wheel
Pineapple cutter	Weigher panel rubber junction forming tube area ceiling	Knife box handle
Knife	Packer output roller	Cart weight
Grey box for product	Conveyor belt roller for packaging bags	Tray rack wheels
White box for product	Packer infeed roller	Grey trolley wheels
Cutting board	Tray holder	Pillar corner
Waste hole to conveyor belt	Under conveyor belt equipment	Pillar junction near strip area
Apple cutting support		Waste shovel
Apple blade		Cleaning tool box
Vibratory belt plate		Rubbish bin
Final carrot strips hopper		Floor cover
Forming tube		Floor crack
Cup packaging machine		Pillar junction near waste area
Conveyor belt of sachet packing machine		Stairs to the skewer area
Weighing output conveyor belt		Drain waste line
		Drain dipping
		Floor cover apple area
		Strip drain
		Boot washer
		Forklift fork
		Forklift wheel

Table 3. List of sampling sites per zone tested three times in the prepared salad facility.

Prepared salads		
Sites Zone 1	Sites Zone 2	Sites Zone 3
Inner washing basket equipment	Operating panel	Boot washer
Bell pepper cutting board	L2 conveyor belt roller	Preparation room drain
Scale	L2 box holder	Trash pallet
Vegetable inlet	L3 weight filler cage	Stairs in pasta cooking room
First conveyor belt	L3 topping hopper equipment	Red rolling base
Rolling bin	L3 transport chain	Freezer chamber floor
Trays	Plastic roll	Faucet foot pedal
Tomato slicer blades	Sealing machine exit	Floor hole
Slicer entry	Lid sealing machine	Blue rolling base wheels
Washing basket equipment	Under vibratory channel	Tray rack wheels
Cutting table		Blue rolling base
Washing basket equipment in cooking room		Basket drain
Cooking table		Cooking room ceiling
Cooling room trays		Pasta drain
Line2 initial conveyor belt		Cooking table wheels
Line 3 (L3) initial conveyor belt		Floor hole
L3 inlet topping hopper		Cooling room tray rack wheels
L3 salad hopper		Cooling room air filter
L3 green bin		Cooling room floor hole
L3 check weigh belt		Cooling room wall
L3 waste output roller		Door handle
Topping outlet tray		Cart wheels 1
Output roller		Cart wheels 2
First vibratory channel		Line 2 drain
Vibratory channels to weight filler		L2 waste shovel
Weight filler cone		L2 floor
L3 rotary table		L3 topping weigher filler waste
L2 rotary table		L3 drain

		L3 floor hole Steps Platform floor Platform waste shovel

Table 4. Sample ID, processing plant (cut lettuce and cut fruit), sampling point, zone, assembly quality statistics and annotation data associated with *L. monocytogenes* whole genomes from Illumina paired-end sequencing data.

Sample ID	Processing plant	Sampling point	Zone	Nº of raw reads	Quality-filtered reads	N.º contigs	Genomes Sizes	GC (%)	N50
FF2E 19.1	Fruit	Cart weight	3	2871912	1442852	11	2915370	37.86	406497
FF2E 26.7	Fruit	White box for product	1	1859472	927326	20	3000208	37.86	393166
FF2E 30.9	Fruit	Floor cover	3	2116448	1067258	19	2879504	37.89	314087
FF2E 32.2	Fruit	Cutting board	1	1944156	961956	15	2926350	37.87	299063
FF2E 34.3	Fruit	Waste conveyor belt	2	2108974	1009272	16	3015946	37.83	314175
FF2E 3.4	Fruit	Waste conveyor belt roller	2	2930004	1395244	25	2928661	37.88	345519
FF2E 35.5	Fruit	Waste conveyor belt roller	2	2122968	1008706	24	2928478	37.88	228132
FF2E 37.6	Fruit	Stairs to the skewer area	3	1995862	929590	25	2949569	37.86	228132
FF2E 44.1	Fruit	Drain dipping	3	2471864	1280044	19	3015917	37.83	393100
FF2E 45.4	Fruit	Floor cover apple area	3	2198862	1060330	14	2984421	37.84	481957
FF2E 4.6	Fruit	Drain near waste area	3	2604666	1293872	21	2920136	37.86	301745
FF2E 47.3	Fruit	Weigher panel rubber junction	2	2149790	1038070	24	2992153	37.85	243380
FF2E 49.4	Fruit	Final carrot strips hopper	1	2041330	922042	23	2928626	37.87	229095
FF2E 53.5	Fruit	Cup packaging machine	1	2755790	1290140	17	2937105	37.86	304483
FF2E 54.8	Fruit	Conveyor belt of sachet packing machine	1	2313778	1103644	17	2985166	37.84	393082
FF2E 58.1	Fruit	Strip drain	3	3099874	1405346	15	2985495	37.84	393083
FF2R 18.1	Fruit	Knife	1	2101850	1048458	27	2922737	37.89	240900
FF2R 48.1	Fruit	Vibratory belt plate	1	1514778	726806	30	2922860	37.89	172941
FF2R 61.1	Fruit	Spinning table/Rotary table	1	3342630	1626548	19	3015006	37.82	559909
FF5E 19.5	Fruit	Cart weight	3	1595786	749584	26	2896933	37.93	219808
FF5E 28.7	Fruit	Cleaning tool box	3	2026694	999262	17	2909536	37.92	257202
FF5E 30.1	Fruit	Floor cover	3	2574748	1400562	14	2840922	37.91	359852
FF5E 33.4	Fruit	Waste hole to conveyor belt	1	1584496	793764	33	2921947	37.9	187434

FF5E 37.3	Fruit	Stairs to the skewer area	3	2572254	1206922	12	2886527	37.91	385354
FF5E 44.5	Fruit	Drain dipping	3	1916120	991684	30	2914883	37.91	221648
FF5E 45.10	Fruit	Floor cover apple area	3	1564572	778852	28	2912166	37.9	250670
FF5E 58.7	Fruit	Strip drain	3	1884668	1011366	20	2834707	37.93	244069
FF8E 28.3	Fruit	Cleaning tool box	3	2368226	1265656	14	2979219	37.86	522439
FF8E 37.5	Fruit	Stairs to the skewer area	3	1825416	932532	33	2886979	37.92	119328
FF8E 38.6	Fruit	Drain waste cover	3	1544044	779726	18	2825179	37.94	304456
FF8E 44.2	Fruit	Drain dipping	3	3141978	1585302	18	2937333	37.86	512588
FF8E 48.10	Fruit	Vibratory belt plate	1	1969902	989554	22	2921592	37.88	262453
FF8E 58.6	Fruit	Strip drain	3	1888934	827914	24	2948591	37.89	225865
FF8E 5.8	Fruit	Floor near apple wash station	3	2167136	1090854	18	2936114	37.85	348944
FI1E11.1	Iceberg	Floor crack	3	3801110	1983932	168	7429081	48.80	75362
FI1E12.1	Iceberg	Drain near to elevator	3	2706280	1429068	29	2917097	37.87	191580
FI1E15.1	Iceberg	Waste product conveyor belt	2	2262328	1193586	28	2966826	37.88	229095
FI1E18.1	Iceberg	Water retained on floor	3	2845628	1373142	35	2884214	37.94	178632
FI1E2.1	Iceberg	White room boot washer	3	3211934	1639636	19	2912362	37.9	248315
FI1E29.1	Iceberg	Forklift's wheel	3	1839576	832918	52	2892490	37.96	85488
FI1E31.1	Iceberg	Floor near elevator	3	2502964	1327038	18	2935936	37.86	476005
FI1E64.1	Iceberg	Stairs to weight filler	3	2245114	1073342	40	2948129	37.91	132821
FI1E67.1	Iceberg	Cart wheels	3	2296210	1143138	23	3000752	37.86	228132
FI1E70.1	Iceberg	Floor's holes near pillar	3	2237472	1146502	39	2979582	37.89	139691
FI1E76.1	Iceberg	Pillar junction	3	2311182	1142786	34	2917526	37.9	200004
FI1E80.1	Iceberg	Drain	3	1798114	893186	33	2896030	37.95	207896
FI1E82.1	Iceberg	Box holder wheels	3	2816400	1355148	20	2910574	37.92	294358
FI4E 11.3	Iceberg	Floor crack	3	1703986	636292	70	2901109	37.94	71449
FI4E 16.9	Iceberg	Conveyor belt button	2	1889026	766950	49	2895491	37.88	112074
FI4E 17.1	Iceberg	Waste shovel	2	2545194	1006118	29	2953116	37.86	175289
FI4E 20.10	Iceberg	Slicer's blades	1	2157238	1060444	26	2952805	37.86	193089
FI4E 20.1	Iceberg	Slicer	1	1368900	683940	49	2986126	37.89	75018
FI4E 20.3	Iceberg	Slicer	1	2131148	1071900	23	2966072	37.83	299063
FI4E 20.5	Iceberg	Slicer	1	1832192	945866	24	3000801	37.86	223575

FI4E 20.7	Iceberg	Slicer	1	1819226	884132	21	3016679	37.83	362990
FI4E 20.9	Iceberg	Slicer	1	1900044	979208	27	3008909	37.85	195762
FI4E 21.1	Iceberg	Slicer	1	1932474	928510	22	2920734	37.9	228311
FI4E 22.6	Iceberg	Frame under slicer	2	1735384	812988	31	2917370	37.9	273410
FI4E 23.4	Iceberg	Crack in the floor under the slicer	3	1831246	930846	21	3018319	37.83	262446
FI4E 24.6	Iceberg	First washing station	1	2582750	1244688	29	3007099	37.85	206105
FI4E 2.4	Iceberg	White room entrance boot washer	3	2477438	1102432	31	3000218	37.85	222708
FI4E 25.4	Iceberg	First washing station filter	1	2356666	1164740	28	2818448	37.96	228132
FI4E 26.3	Iceberg	First washing station outlet conveyor belt	1	1534286	801622	46	2985814	37.9	139534
FI4E 27.2	Iceberg	Roller of the outlet conveyor belt of the first washing station	2	2157916	1080538	40	2989700	37.89	204383
FI4E 28.1	Iceberg	Water outlet pipes from first washing station	2	2307970	1117120	17	3004298	37.84	387137
FI4E 30.7	Iceberg	Forklift's fork	3	2313764	1070114	24	2999515	37.85	228216
FI4E 31.5	Iceberg	Floor near elevator	3	2444610	1091436	17	3012509	37.83	227128
FI4E 34.2	Iceberg	Instrument box handle	3	2370718	1177046	17	2963006	37.83	405027
FI4E 41.1	Iceberg	Metallic conveyor belt roller	2	2333432	953208	21	2996017	37.86	307495
FI4E 54.5	Iceberg	Drain before vibratory belt	3	1031010	471264	301	2821361	38.24	15745
FI4E 59.6	Iceberg	Floor under weigher belt	2	2225376	958388	25	2926066	37.86	247798
FI4E 62.3	Iceberg	Weigher plates	1	1949658	833632	46	2958510	37.89	117958
FI4E 69.10	Iceberg	Cart wheels	3	2259692	964198	38	2895897	37.95	108757
FI4E 69.1	Iceberg	Cart wheels	3	2275126	949452	25	2895656	37.94	156602
FI4E 69.2	Iceberg	Cart wheels	3	1533558	534606	72	2871523	38.02	61080
FI4E 69.4	Iceberg	Cart wheels	3	1808032	718724	38	2881520	37.98	102655
FI4E 69.6	Iceberg	Cart wheels	3	2117904	837934	42	2881258	37.98	108793
FI4E 69.8	Iceberg	Cart wheels	3	1612624	642652	79	2875248	38	50749
FI4E 70.4	Iceberg	Floor's holes near pillar	3	2735184	1324504	16	2867132	37.86	345585
FI4E 79.7	Iceberg	Spinning table	1	1876246	875226	36	2939500	37.92	117261

FI4E 8.5	Iceberg	Waste conveyor belt roller	2	1842304	910492	45	2972119	37.92	125486
FI4E I1.5	Iceberg	Final product	P	1463204	726098	50	2955675	37.9	113556
FI4R 1.17	Iceberg	Boot washer	3	1982362	974408	26	2997845	37.83	191294
FI4R 20.1	Iceberg	Slicer's blades	1	2027642	957316	21	3014781	37.83	228090
FI4R 20.2	Iceberg	Slicer's blades	1	2111776	997916	21	3014126	37.83	345528
FI4R 69.13	Iceberg	Cart wheels	3	2708626	1162026	17	2890398	37.92	254778
FI4R 69.17	Iceberg	Cart wheels	3	1853986	720948	47	2882177	37.99	113232
FI4R 69.4	Iceberg	Cart wheels	3	2430908	991538	35	2887810	37.97	109719
FI4R 69.9	Iceberg	Cart wheels	3	1946368	837386	60	2888283	37.98	82627
FI7E 11.5	Iceberg	Floor crack	3	2930638	1525464	13	2853390	37.95	294295
FI7E 12.3	Iceberg	Drain near to elevator	3	2050048	1006316	20	2903212	37.92	294295
FI7E 2.10	Iceberg	White room boot washer	3	2103330	906576	24	2973430	37.86	228217
FI7E 23.1	Iceberg	Crack in the floor under the slicer	3	2885438	1325506	17	3018679	37.82	411029
FI7E 29.4	Iceberg	Forklift's wheel	3	2393542	1148064	19	2935269	37.84	420054
FI7E 30.8	Iceberg	Forklift's fork	3	2022144	886824	27	2965715	37.85	196755
FI7E 31.3	Iceberg	Floor near elevator	3	1746976	799144	25	2849565	37.93	256699
FI7E 6.4	Iceberg	Final product conveyor belt roller	2	2816754	1452440	14	2969382	37.85	511583
FI7E 70.4	Iceberg	Floor's holes near pillar	3	2074246	936580	12	2912045	37.88	750750
FI7E 77.3	Iceberg	Floor in the packaging area	3	2622872	1238294	15	2981930	37.85	313736
FI7E 80.9	Iceberg	Drain	3	1809320	847904	22	2910957	37.92	257143

Table 5. Positive *Listeria monocytogenes* (Lm) and *Listeria* spp. samples and prevalence (%) per zone (Zones 1, 2 and 3) through three environmental monitoring (EM) samplings (1, 2 and 3) in three fresh-cut processing facilities.

Fresh-cut facility	Zones	EM Samplings	Number of samples	Positive Lm samples	Prevalence Lm	Positive *Listeria* spp. samples	Prevalence *Listeria* spp.
Cut vegetables	Zone 1	1	17	0	0.0	7	41.2
		2	17	7	41.2	8	47.1
		3	17	0	0.0	7	41.2
		Total	**51**	**7**	**13.7**	**22**	**43.1**
	Zone 2	1	21	2	9.5	6	28.6
		2	21	9	42.9	6	28.6
		3	21	1	4.8	6	28.6
		Total	**63**	**12**	**19.0**	**18**	**28.6**
	Zone 3	1	30	14	46.7	14	46.7
		2	30	11	36.7	15	50.0
		3	30	12	40.0	19	63.3
		Total	**90**	**37**	**41.1**	**48**	**53.3**
Total in the operation			**204**	**56**	**27.5**	**88**	**43.1**
Cut fruits	Zone 1	1	20	6	30.0	4	20.0
		2	20	1	5.0	3	15.0
		3	20	0	0.0	8	40.0
		Total	**60**	**7**	**11.7**	**15**	**25.0**
	Zone 2	1	13	3	23.1	3	23.1
		2	13	0	0.0	0	0.0
		3	13	0	0.0	3	23.1
		Total	**39**	**3**	**7.7**	**6**	**15.4**
	Zone 3	1	26	16	61.5	20	76.9
		2	26	10	38.5	12	46.2
		3	26	9	34.6	21	80.8
		Total	**78**	**35**	**44.9**	**53**	**67.9**
Total in the operation			**177**	**45**	**25.4**	**74**	**41.8**

Prepared salads	Zone 1	1	28	4	14.3	3	10.7
		2	28	3	10.7	5	17.9
		3	28	4	14.3	3	10.7
		Total	**84**	**11**	**13.1**	**11**	**13.1**
	Zone 2	1	10	1	10.0	1	10.0
		2	10	0	0.0	1	10.0
		3	10	2	20.0	1	10.0
		Total	**30**	**3**	**10.0**	**3**	**10.0**
	Zone 3	1	32	22	68.8	21	65.6
		2	32	17	53.1	18	56.3
		3	32	24	75.0	20	62.5
		Total	**96**	**63**	**65.6**	**59**	**61.5**
Total in the operation			**210**	**77**	**36.7**	**73**	**34.8**

Prevalence: percentage of confirmed positive among the tested samples in each zone.
Zone 1: food-contact surfaces (FCS); Zone 2: proximity to food-contact surfaces (close to FCS); Zone 3: remote from food-contact surfaces in processing areas (remote from FCS).

Table 6. Positive sampling points to Lm in EM1, EM2 and EM3

Line	Zone	Nº Point	Point	EM1 (C) (C)	EM2 (C)	EM3 (C)
I	3	1	Boot washer	+ (1)	+ (10)	+ (1)
I	3	2	White room boot washer	+ (1)	+ (22)	+ (3)
I	3	11	Floor crack	+ (6)	-	-
I	3	12	Drain near to elevator	+ (2)	+ (1)	-
I	3	54	Drain before vibratory belt	+ (1)	-	-
I	3	80	Drain	+ (1)	-	+ (1)
F	3	1	Ramp down area	+ (1)	+ (1)	-
F	3	22	Trolley wheels	+ (2)	-	-
F	3	30	Floor cover	+ (12)	-	-
F	3	31	Floor crack	+ (5)	+ (7)	-
F	1	33	Waste hole to conveyor belt	+ (1)	-	-
F	3	45	Floor cover apple area	+ (2)	+ (1)	-
F	3	58	Strip drain	+ (1)	-	-
CS	3	1	Boot washer	+ (1)	+ (9)	-
CS	3	11	Faucet foot pedal	+ (1)	+ (21)	+ (34)
CS	3	14	Blue rolling base wheels	+ (13)	+ (20)	+ (9)
CS	3	39	Drain 1	+ (7)	+ (2)	-
CS	3	41	Trolley wheels 1	+ (1)	-	+ (1)
CS	3	43	Trolley wheels 3	+ (1)	-	-
CS	3	44	L2 drain	+ (13)	+ (1)	+ (2)
CS	3	58	L3 floor hole	+ (1)	+ (3)	+ (1)
CS	3	64	Drain 2	+ (14)	+ (4)	+ (11)
TOTAL				88	102	63

****The number in parentheses refers to the number of colonies collected on OCLA plate with inhibitory halo.*

Table 7. Positive samples for *Listeria monocytogenes* (Lm) and prevalence in the environmental monitoring EM1, EM2 and EM3 as well as sampling Zones of different processing plants.

Zones	EM Samplings	Positive Lm samples	*Prevalence (%) Lm*
Zone 1	1	1/70	0.0
	2	0/1	0.0
	3	0/1	0.0
	Total	**1/72**	**1.4**
Zone 2	1	0/47	0.0
Zone 3	1	21/91	23.1
	2	13/21	61.9
	3	9/21	42.9
	Total	**43/133**	**32.3**

****Prevalence: percentage of confirmed positive combining all the tested samples for each EM and Zone.*

Table 8. Summary of the results obtained with CheckM, BWA and Qualimap: completeness, potential contamination, average coverage and mapping quality of each genome assembly.

Sample ID	Processing plant	Sampling point	Zone	Completeness (%)	Potential contamination (%)	Percentage of Mapped Reads (%)	Mean Mapping Quality	Mean depth of coverage (X)	Standard deviation of depth of coverage (X)
FF2E 19.1	Fruit	Cart weight	3	98.9	0.6	97.9	59.9	63.0	41.4
FF2E 26.7	Fruit	White box for product	1	99.5	0.6	99.7	60.0	39.7	27.7
FF2E 30.9	Fruit	Floor cover	3	99.5	0.6	99.9	59.9	61.5	43.2
FF2E 32.2	Fruit	Cutting board	1	98.9	0.6	98.0	59.9	47.0	33.1
FF2E 34.3	Fruit	Waste conveyor belt	2	98.9	0.6	99.7	60.0	42.4	32.8
FF2E 3.4	Fruit	Waste conveyor belt roller	2	99.5	0.6	100.0	59.9	43.2	30.7
FF2E 35.5	Fruit	Waste conveyor belt roller	2	99.5	0.6	99.9	60.0	44.6	30.2
FF2E 37.6	Fruit	Stairs to the skewer area	3	98.9	0.6	99.4	59.9	40.1	28.7
FF2E 44.1	Fruit	Drain dipping	3	99.5	0.6	98.2	59.9	56.2	42.3
FF2E 45.4	Fruit	Floor cover apple area	3	99.5	0.6	100.0	59.8	55.3	34.1
FF2E 4.6	Fruit	Drain near waste area	3	99.5	0.6	100.0	60.0	45.7	32.9
FF2E 47.3	Fruit	Weigher panel rubber junction	2	99.5	0.6	99.4	59.9	44.3	33.1
FF2E 49.4	Fruit	Final carrot strips hopper	1	98.9	0.6	99.8	59.9	40.1	27.9
FF2E 53.5	Fruit	Cup packaging machine	1	99.5	0.6	100.0	59.9	57.1	40.9
FF2E 54.8	Fruit	Conveyor belt of sachet packing machine	1	99.5	0.6	100.0	59.9	47.6	34.1
FF2E 58.1	Fruit	Strip drain	3	99.5	0.6	100.0	59.9	60.7	42.9
FF2R 18.1	Fruit	Knife	1	99.5	0.6	99.8	60.0	46.5	33.9
FF2R 48.1	Fruit	Vibratory belt plate	1	99.5	0.6	99.8	59.7	32.0	23.0
FF2R 61.1	Fruit	Spinning table/Rotary table	1	99.5	0.6	100.0	59.9	69.8	51.3
FF5E 19.5	Fruit	Cart weight	3	98.4	0.0	99.6	59.9	33.2	22.5
FF5E 28.7	Fruit	Cleaning tool box	3	99.5	0.0	99.9	59.9	44.3	29.2
FF5E 30.1	Fruit	Floor cover	3	98.9	0.6	97.7	59.9	62.6	40.5
FF5E 33.4	Fruit	Waste hole to conveyor belt	1	99.5	0.6	99.8	59.9	35.0	25.8
FF5E 37.3	Fruit	Stairs to the skewer area	3	99.5	0.6	100.0	60.0	53.7	38.4
FF5E 44.5	Fruit	Drain dipping	3	99.5	0.0	100.0	59.9	44.0	30.3
FF5E 45.10	Fruit	Floor cover apple area	3	98.4	0.6	99.6	60.0	34.4	22.8
FF5E 58.7	Fruit	Strip drain	3	99.5	0.0	99.4	59.9	46.1	29.9
FF8E 28.3	Fruit	Cleaning tool box	3	99.5	0.6	97.8	59.9	53.8	38.0
FF8E 37.5	Fruit	Stairs to the skewer area	3	99.5	0.6	99.9	59.9	41.8	31.1
FF8E 38.6	Fruit	Drain waste cover	3	99.5	0.0	99.4	59.9	35.5	26.2
FF8E 44.2	Fruit	Drain dipping	3	99.5	0.6	100.0	59.8	69.9	44.3
FF8E 48.10	Fruit	Vibratory belt plate	1	99.5	0.6	99.6	59.9	43.7	28.7
FF8E 58.6	Fruit	Strip drain	3	99.5	0.6	99.9	60.0	48.1	31.9
FF8E 5.8	Fruit	Floor near apple wash station	3	98.9	0.0	99.9	59.9	35.9	25.8
FI1E11.1	Iceberg	Floor crack	3	100.0	96.5	20.9	59.9	18.7	9.3

FI1E12.1	Iceberg	Drain near to elevator	3	98.4	0.6	98.1	59.9	62.4	46.9
FI1E15.1	Iceberg	Waste product conveyor belt	2	98.4	0.6	99.8	59.9	51.9	44.2
FI1E18.1	Iceberg	Water retained on floor	3	98.9	0.6	99.5	60.0	61.8	61.3
FI1E2.1	Iceberg	White room boot washer	3	98.4	0.6	99.6	59.9	72.6	60.9
FI1E29.1	Iceberg	Forklift's wheel	3	99.5	0.6	99.7	59.9	37.2	29.9
FI1E31.1	Iceberg	Floor near elevator	3	99.5	0.6	100.0	59.8	58.4	31.2
FI1E64.1	Iceberg	Stairs to weight filler	3	98.4	0.6	99.6	59.9	46.6	39.2
FI1E67.1	Iceberg	Cart wheels	3	99.5	0.6	99.9	59.9	49.0	34.0
FI1E70.1	Iceberg	Floor's holes near pillar	3	98.9	0.6	99.7	59.9	49.6	40.7
FI1E76.1	Iceberg	Pillar junction	3	99.5	0.6	99.5	59.9	50.4	44.6
FI1E80.1	Iceberg	Drain	3	98.9	0.6	99.7	60.0	39.7	35.3
FI1E82.1	Iceberg	Box holder wheels	3	99.5	0.0	99.9	59.9	59.9	44.0
FI4E 11.3	Iceberg	Floor crack	3	98.9	0.6	99.6	59.9	27.8	23.3
FI4E 16.9	Iceberg	Conveyor belt button	2	98.4	0.6	95.5	59.9	32.5	21.7
FI4E 17.1	Iceberg	Waste shovel	2	98.9	0.6	97.8	59.9	43.2	30.4
FI4E 20.10	Iceberg	Slicer's blades	1	98.9	0.6	99.7	60.0	47.0	36.2
FI4E 20.1	Iceberg	Slicer	1	99.5	0.6	99.6	59.9	29.3	20.7
FI4E 20.3	Iceberg	Slicer	1	98.9	0.6	97.6	59.9	45.3	32.2
FI4E 20.5	Iceberg	Slicer	1	98.9	0.6	97.9	59.9	45.9	35.1
FI4E 20.7	Iceberg	Slicer	1	99.5	0.6	99.7	59.9	40.8	27.8
FI4E 20.9	Iceberg	Slicer	1	99.5	0.6	100.0	59.8	37.7	26.7
FI4E 21.1	Iceberg	Slicer	1	99.5	0.6	99.9	60.0	42.2	28.0
FI4E 22.6	Iceberg	Frame under slicer	2	98.9	0.6	99.8	59.9	41.0	32.5
FI4E 23.4	Iceberg	Crack in the floor under the slicer	3	99.5	0.6	99.7	60.0	35.6	26.8
FI4E 24.6	Iceberg	First washing station	1	99.5	0.6	100.0	59.9	39.6	22.9
FI4E 2.4	Iceberg	White room entrance bootwasher	3	99.5	0.6	99.8	60.0	53.9	35.6
FI4E 25.4	Iceberg	First washing station filter	1	98.9	0.6	97.5	59.8	52.4	38.7
FI4E 26.3	Iceberg	First washing station outlet conveyor belt	1	98.9	0.6	99.5	59.9	34.9	25.2
FI4E 27.2	Iceberg	Roller of the outlet conveyor belt of the first washing station	2	99.5	0.6	99.7	60.0	46.9	33.9
FI4E 28.1	Iceberg	Water outlet pipes from first washing station	2	99.5	0.6	99.7	59.8	47.9	33.1
FI4E 30.7	Iceberg	Forklift's fork	3	99.5	0.6	99.7	59.9	45.8	34.0
FI4E 31.5	Iceberg	Floor near elevator	3	99.5	0.6	99.9	59.9	46.6	29.7
FI4E 34.2	Iceberg	Instrument box handle	3	98.9	0.6	97.9	59.9	50.2	34.4
FI4E 41.1	Iceberg	Metallic conveyor belt roller	2	99.5	0.6	99.6	59.9	41.0	29.9
FI4E 54.5	Iceberg	Drain before vibratory belt	3	99.5	0.6	99.0	60.0	21.1	18.5
FI4E 59.6	Iceberg	Floor under weigher belt	2	99.5	0.6	98.2	59.9	41.5	31.5
FI4E 62.3	Iceberg	Weigher plates	1	99.5	0.6	99.6	59.9	36.2	27.7
FI4E 69.10	Iceberg	Cart wheels	3	98.9	0.0	99.5	59.9	42.3	32.6
FI4E 69.1	Iceberg	Cart wheels	3	99.5	0.0	99.7	60.0	42.7	35.8
FI4E 69.2	Iceberg	Cart wheels	3	99.5	0.0	99.6	60.0	23.4	18.1

FI4E 69.4	Iceberg	Cart wheels	3	98.4	0.0	99.5	59.9	31.8	25.7
FI4E 69.6	Iceberg	Cart wheels	3	98.9	0.0	99.7	60.0	37.1	30.1
FI4E 69.8	Iceberg	Cart wheels	3	99.5	0.0	99.5	60.0	28.3	23.0
FI4E 70.4	Iceberg	Floor's holes near pillar	3	95.1	0.6	97.2	60.0	57.9	39.9
FI4E 79.7	Iceberg	Spinning table	1	99.5	0.6	99.5	59.9	38.3	31.6
FI4E 8.5	Iceberg	Waste conveyor belt roller	2	99.5	0.6	99.6	59.9	39.7	30.8
FI4E I1.5	Iceberg	Final product	P	98.9	0.6	99.7	60.0	31.7	25.4
FI4R 1.17	Iceberg	Boot washer	3	99.5	0.6	99.5	59.8	41.7	29.4
FI4R 20.1	Iceberg	Slicer's blades	1	99.5	0.6	99.9	59.9	40.8	27.9
FI4R 20.2	Iceberg	Slicer's blades	1	99.5	0.6	99.9	60.0	42.7	29.6
FI4R 69.13	Iceberg	Cart wheels	3	99.5	0.0	99.4	60.0	51.6	41.3
FI4R 69.17	Iceberg	Cart wheels	3	99.5	0.0	99.5	60.0	31.9	25.2
FI4R 69.4	Iceberg	Cart wheels	3	98.9	0.0	99.6	59.9	44.1	35.1
FI4R 69.9	Iceberg	Cart wheels	3	98.9	0.0	99.6	60.0	37.2	31.1
FI7E 11.5	Iceberg	Floor crack	3	99.5	0.0	99.5	59.9	69.0	47.6
FI7E 12.3	Iceberg	Drain near to elevator	3	98.9	0.0	99.8	59.8	44.7	29.0
FI7E 2.10	Iceberg	White room boot washer	3	99.5	0.6	99.9	60.0	39.4	30.5
FI7E 23.1	Iceberg	Crack in the floor under the slicer	3	99.5	0.6	100.0	59.7	56.6	36.6
FI7E 29.4	Iceberg	Forklift's wheel	3	98.9	0.6	98.0	59.9	49.3	36.7
FI7E 30.8	Iceberg	Forklift's fork	3	98.6	0.6	99.6	59.9	38.4	28.2
FI7E 31.3	Iceberg	Floor near elevator	3	98.9	0.0	99.4	60.0	36.0	26.3
FI7E 6.4	Iceberg	Final product conveyor belt roller	2	99.5	0.6	100.0	60.0	63.6	40.2
FI7E 70.4	Iceberg	Floor's holes near pillar	3	98.9	0.6	99.4	59.9	41.1	29.8
FI7E 77.3	Iceberg	Floor in the packaging area	3	99.5	0.6	99.9	59.9	53.8	38.5
FI7E 80.9	Iceberg	Drain	3	99.5	0.0	99.9	59.9	37.5	26.1

Table 9. Sample ID, processing plant (cut lettuce and cut fruit), sampling point, zone and serovar prediction results.

Sample ID	Processing plant	Sampling point	Zone	In silico Serotype	Lineage	MLST Sequence Type (ST)	Clonal complex (CC)	cgMLST1748 profile (CT)	Loci matched
FF2E 19.1	Fruit	Cart weight	3	1/2a; 3a	II	155	CC155	29464	1715/1748 (98.1%)
FF2E 26.7	Fruit	White box for product	1	1/2a; 3a	II	155	CC155	29464	1736/1748 (99.3%)
FF2E 30.9	Fruit	Floor cover	3	1/2a; 3a	II	155	CC155	29464	1707/1748 (97.7%)
FF2E 32.2	Fruit	Cutting board	1	1/2a; 3a	II	155	CC155	29464	1736/1748 (99.3%)
FF2E 34.3	Fruit	Waste conveyor belt	2	1/2a; 3a	II	155	CC155	29464	1742/1748 (99.7%)
FF2E 3.4	Fruit	Waste conveyor belt roller	2	1/2a; 3a	II	155	CC155	29464	1739/1748 (99.5%)
FF2E 35.5	Fruit	Waste conveyor belt roller	2	1/2a; 3a	II	155	CC155	29464	1737/1748 (99.4%)
FF2E 37.6	Fruit	Stairs to the skewer area	3	1/2a; 3a	II	155	CC155	29464	1727/1748 (98.8%)
FF2E 44.1	Fruit	Drain dipping	3	1/2a; 3a	II	155	CC155	29464	1740/1748 (99.5%)
FF2E 45.4	Fruit	Floor cover apple area	3	1/2a; 3a	II	155	CC155	29464	1742/1748 (99.7%)
FF2E 4.6	Fruit	Drain near waste area	3	1/2a; 3a	II	155	CC155	29464	1716/1748 (98.2%)
FF2E 47.3	Fruit	Weigher panel rubber junction	2	1/2a; 3a	II	155	CC155	29464	1732/1748 (99.1%)
FF2E 49.4	Fruit	Final carrot strips hopper	1	1/2a; 3a	II	155	CC155	29464	1731/1748 (99.0%)
FF2E 53.5	Fruit	Cup packaging machine	1	1/2a; 3a	II	155	CC155	29464	1740/1748 (99.5%)
FF2E 54.8	Fruit	Conveyor belt of sachet packing machine	1	1/2a; 3a	II	155	CC155	29464	1738/1748 (99.4%)
FF2E 58.1	Fruit	Strip drain	3	1/2a; 3a	II	155	CC155	29464	1736/1748 (99.3%)
FF2R 18.1	Fruit	Knife	1	1/2a; 3a	II	155	CC155	29464	1737/1748 (99.4%)
FF2R 48.1	Fruit	Vibratory belt plate	1	1/2a; 3a	II	155	CC155	29464	1736/1748 (99.3%)
FF2R 61.1	Fruit	Spinning table/Rotary table	1	1/2a; 3a	II	155	CC155	29464	1740/1748 (99.5%)
FF5E 19.5	Fruit	Cart weight	3	4b; 4d; 4e	I	6	CC6	17837/33032	1724/1748 (98.6%)
FF5E 28.7	Fruit	Cleaning tool box	3	4b; 4d; 4e	I	6	CC6	17837/33032	1732/1748 (99.1%)
FF5E 30.1	Fruit	Floor cover	3	1/2a; 3a	II	155	CC155	29464	1716/1748 (98.2%)
FF5E 33.4	Fruit	Waste hole to conveyor belt	1	1/2a; 3a	II	155	CC155	29464	1734/1748 (99.2%)
FF5E 37.3	Fruit	Stairs to the skewer area	3	1/2a; 3a	II	155	CC155	29464	1742/1748 (99.7%)
FF5E 44.5	Fruit	Drain dipping	3	4b; 4d; 4e	I	6	CC6	17837	1730/1748 (99.0%)
FF5E 45.10	Fruit	Floor cover apple area	3	1/2a; 3a	II	155	CC155	29464	1726/1748 (98.7%)
FF5E 58.7	Fruit	Strip drain	3	1/2a; 3a	II	155	CC155	29464	1727/1748 (98.8%)
FF8E 28.3	Fruit	Cleaning tool box	3	1/2a; 3a	II	155	CC155	29464	1743/1748 (99.7%)
FF8E 37.5	Fruit	Stairs to the skewer area	3	1/2a; 3a	II	155	CC155	29464	1730/1748 (99.0%)

FF8E 38.6	Fruit	Drain waste cover	3	1/2a; 3a	II	155	CC155	29464	1725/1748 (98.7%)
FF8E 44.2	Fruit	Drain dipping	3	1/2a; 3a	II	155	CC155	29464	1745/1748 (99.7%)
FF8E 48.10	Fruit	Vibratory belt plate	1	1/2a; 3a	II	155	CC155	29464	1732/1748 (99.1%)
FF8E 58.6	Fruit	Strip drain	3	4b; 4d; 4e	I	6	CC6	17837/33032	1731/1748 (99.0%)
FF8E 5.8	Fruit	Floor near apple wash station	3	1/2a; 3a	II	155	CC155	29464	1738/1748 (99.4%)
FI1E11.1	Iceberg	Floor crack	3	1/2a; 3a	II	155	CC155	29464	1653/1748 (94.6%)
FI1E12.1	Iceberg	Drain near to elevator	3	1/2a; 3a	II	155	CC155	29464	1706/1748 (97.6%)
FI1E15.1	Iceberg	Waste product conveyor belt	2	1/2a; 3a	II	155	CC155	29464	1731/1748 (99.0%)
FI1E18.1	Iceberg	Water retained on floor	3	1/2a; 3a	II	155	CC155	29464	1718/1748 (99.3%)
FI1E2.1	Iceberg	White room boot washer	3	1/2a; 3a	II	155	CC155	29464	1732/1748 (99.1%)
FI1E29.1	Iceberg	Forklift's wheel	3	1/2a; 3a	II	155	CC155	29464	1723/1748 (98.6%)
FI1E31.1	Iceberg	Floor near elevator	3	1/2a; 3a	II	155	CC155	29464	1742/1748 (99.7%)
FI1E64.1	Iceberg	Stairs to weight filler	3	1/2a; 3a	II	155	CC155	29464	1731/1748 (99.0%)
FI1E67.1	Iceberg	Cart wheels	3	1/2a; 3a	II	155	CC155	29464	1738/1748 (99.4%)
FI1E70.1	Iceberg	Floor's holes near pillar	3	1/2a; 3a	II	155	CC155	29464	1730/1748 (99.0%)
FI1E76.1	Iceberg	Pillar junction	3	1/2a; 3a	II	155	CC155	29464	1731/1748 (99.0%)
FI1E80.1	Iceberg	Drain	3	1/2a; 3a	II	155	CC155	29464	1729/1748 (98.9%)
FI1E82.1	Iceberg	Box holder wheels	3	4b; 4d; 4e	I	6	CC6	17837/33032	1734/1748 (99.2%)
FI4E 11.3	Iceberg	Floor crack	3	1/2a; 3a	II	155	CC155	29464	1712/1748 (97.9%)
FI4E 16.9	Iceberg	Conveyor belt button	2	1/2a; 3a	II	155	CC155	29464	1668/1748 (95.4%)
FI4E 17.1	Iceberg	Waste shovel	2	1/2a; 3a	II	155	CC155	29464	1708/1748 (97.7%)
FI4E 20.10	Iceberg	Slicer's blades	1	1/2a; 3a	II	155	CC155	29464	1710/1748 (97.8%)
FI4E 20.1	Iceberg	Slicer	1	1/2a; 3a	II	155	CC155	29464	1720/1748 (98.4%)
FI4E 20.3	Iceberg	Slicer	1	1/2a; 3a	II	155	CC155	29464	1710/1748 (97.8%)
FI4E 20.5	Iceberg	Slicer	1	1/2a; 3a	II	155	CC155	29464	1736/1748 (99.3%)
FI4E 20.7	Iceberg	Slicer	1	1/2a; 3a	II	155	CC155	29464	1740/1748 (99.5%)
FI4E 20.9	Iceberg	Slicer	1	1/2a; 3a	II	155	CC155	29464	1736/1748 (99.3%)
FI4E 21.1	Iceberg	Slicer	1	1/2a; 3a	II	155	CC155	29464	1735/1748 (99.3%)
FI4E 22.6	Iceberg	Frame under slicer	2	1/2a; 3a	II	155	CC155	29464	1728/1748 (98.9%)
FI4E 23.4	Iceberg	Crack in the floor under the slicer	3	1/2a; 3a	II	155	CC155	29464	1740/1748 (99.5%)
FI4E 24.6	Iceberg	First washing station	1	1/2a; 3a	II	155	CC155	29464	1735/1748 (99.3%)
FI4E 2.4	Iceberg	White room entrance bootwasher	3	1/2a; 3a	II	155	CC155	29464	1731/1748 (99.0%)
FI4E 25.4	Iceberg	First washing station filter	1	1/2a; 3a	II	155	CC155	29464	1711/1748 (97.9%)

FI4E 26.3	Iceberg	First washing station outlet conveyor belt	1	1/2a; 3a	II	155	CC155	29464	1725/1748 (98.7%)
FI4E 27.2	Iceberg	Roller of the outlet conveyor belt of the first washing station	2	Non typeable	II	155	CC155	29464	1734/1748 (99.2%)
FI4E 28.1	Iceberg	Water outlet pipes from first washing station	2	1/2a; 3a	II	155	CC155	29464	1736/1748 (99.3%)
FI4E 30.7	Iceberg	Forklift's fork	3	1/2a; 3a	II	155	CC155	29464	1726/1748 (98.7%)
FI4E 31.5	Iceberg	Floor near elevator	3	1/2a; 3a	II	155	CC155	29464	1737/1748 (99.4%)
FI4E 34.2	Iceberg	Instrument box handle	3	1/2a; 3a	II	155	CC155	29464	1710/1748 (97.8%)
FI4E 41.1	Iceberg	Metallic conveyor belt roller	2	1/2a; 3a	II	155	CC155	29464	1729/1748 (98.9%)
FI4E 54.5	Iceberg	Drain before vibratory belt	3	1/2a; 3a	II	155	CC155	29464	1582/1748 (90.5%)
FI4E 59.6	Iceberg	Floor under weigher belt	2	1/2a; 3a	II	155	CC155	29464	1714/1748 (98.1%)
FI4E 62.3	Iceberg	Weigher plates	1	1/2a; 3a	II	155	CC155	29464	1730/1748 (99.0%)
FI4E 69.10	Iceberg	Cart wheels	3	4b; 4d; 4e	I	6	CC6	17837/33032	1719/1748 (98.3%)
FI4E 69.1	Iceberg	Cart wheels	3	4b; 4d; 4e	I	6	CC6	17837	1723/1748 (98.6%)
FI4E 69.2	Iceberg	Cart wheels	3	4b; 4d; 4e	I	6	CC6	17837/33032	1704/1748 (97.5%)
FI4E 69.4	Iceberg	Cart wheels	3	4b; 4d; 4e	I	6	CC6	17837/33032	1723/1748 (98.6%)
FI4E 69.6	Iceberg	Cart wheels	3	4b; 4d; 4e	I	6	CC6	17837/33032	1722/1748 (98.5%)
FI4E 69.8	Iceberg	Cart wheels	3	Non typeable	I	6	CC6	17837/33032	1701/1748 (97.3%)
FI4E 70.4	Iceberg	Floor's holes near pillar	3	Non typeable	Unknown	Unknown	Unknown	29464	1709/1748 (97.8%)
FI4E 79.7	Iceberg	Spinning table	1	1/2a; 3a	II	155	CC155	29464	1726/1748 (98.7%)
FI4E 8.5	Iceberg	Waste conveyor belt roller	2	1/2a; 3a	II	155	CC155	29464	1731/1748 (99.0%)
FI4E I1.5	Iceberg	Final product	P	1/2a; 3a	II	155	CC155	29464	1715/1748 (98.1%)
FI4R 1.17	Iceberg	Boot washer	3	1/2a; 3a	II	155	CC155	29464	1725/1748 (98.7%)
FI4R 20.1	Iceberg	Slicer's blades	1	1/2a; 3a	II	155	CC155	29464	1740/1748 (99.5%)
FI4R 20.2	Iceberg	Slicer's blades	1	1/2a; 3a	II	155	CC155	29464	1740/1748 (99.5%)
FI4R 69.13	Iceberg	Cart wheels	3	4b; 4d; 4e	I	6	CC6	17837/33032/28409	1722/1748 (98.5%)
FI4R 69.17	Iceberg	Cart wheels	3	4b; 4d; 4e	I	6	CC6	17837/33032	1719/1748 (98.3%)
FI4R 69.4	Iceberg	Cart wheels	3	4b; 4d; 4e	I	6	CC6	17837/33032	1729/1748 (98.9%)
FI4R 69.9	Iceberg	Cart wheels	3	4b; 4d; 4e	I	6	CC6	17837	1715/1748 (98.1%)
FI7E 11.5	Iceberg	Floor crack	3	4b; 4d; 4e	I	6	CC6	17837/33032	1735/1748 (99.3%)
FI7E 12.3	Iceberg	Drain near to elevator	3	4b; 4d; 4e	I	6	CC6	17837/33032	1732/1748 (99.1%)
FI7E 2.10	Iceberg	White room boot washer	3	1/2a; 3a	Unknown	155*	Unknown	29464	1740/1748 (99.5%)

FI7E 23.1	Iceberg	Crack in the floor under the slicer	3	1/2a; 3a	II	155	CC155	29464	1737/1748 (99.4%)
FI7E 29.4	Iceberg	Forklift's wheel	3	1/2a; 3a	II	155	CC155	29464	1715/1748 (98.1%)
FI7E 30.8	Iceberg	Forklift's fork	3	1/2a; 3a	II	155	CC155	29464	1717/1748 (98.2%)
FI7E 31.3	Iceberg	Floor near elevator	3	4b; 4d; 4e	I	6	CC6	17837	1724/1748 (98.6%)
FI7E 6.4	Iceberg	Final product conveyor belt roller	2	1/2a; 3a	II	155	CC155	29464	1743/1748 (99.7%)
FI7E 70.4	Iceberg	Floor's holes near pillar	3	1/2a; 3a	II	155	CC155	29464	1729/1748 (98.9%)
FI7E 77.3	Iceberg	Floor in the packaging area	3	1/2a; 3a	II	155	CC155	29464	1738/1748 (99.4%)
FI7E 80.9	Iceberg	Drain	3	4b; 4d; 4e	I	6	CC6	17837/33032	1731/1748 (99.0%)

Table 10. MLVA profiles of EM1, EM2 and EM3 in each processing plant obtained from positive samples for *Listeria monocytogenes*.

Processing plants	**EM Samplings**		**Positive *Listeria monocytogenes* samples**	**Prevalence (%)**
Cut lettuce	1	MLVA 1	9/12	75.0
		MLVA2	3/12	25.0
	2	MLVA1	33/33	100.0
		MLVA 2	0/33	0.0
	3	MLVA 1	5/5	100.0
		MLVA 2	0/5	0.0
	TOTAL	**MLVA 1**	**47/50**	**94.0**
		MLVA 2	**3/50**	**6.0**
Cut fruits	1	MLVA 1	23/24	95.8
		MLVA2	1/24	4.2
	2	MLVA1	8/9	88.8
		MLVA 2	1/9	11.2
	3	MLVA 1	-	-
		MLVA 2	-	-
	TOTAL	**MLVA 1**	**31/33**	**94.0**
		MLVA 2	**2/33**	**6.0**
Prepared salads	1	MLVA 1	49/52	94.2
		MLVA2	2/52	3.8
	2	MLVA1	59/60	98.3
		MLVA 2	1/60	1.7
	3	MLVA 1	58/58	100.0
		MLVA 2	0/58	0.0
	TOTAL	**MLVA 1**	**166/170**	**97.6**
		MLVA 2	**3/170**	**1.8**
	TOTAL	**MLVA 1**	**244/253**	**96.4**
		MLVA 2	**8/253**	**3.2**

Table 11. Commercial biocides tested.

COMMERCIAL PRODUCT	MAIN COMPONENTS
P3-oxonia active	Hydrogen peroxide Acetic acid Peracetic acid
Topactive 314	Sodium hydroxide Sodium hypochlorite Soap Alkyl amine oxide
P3-topax 990	Alkyl amine oxide Acetic acid Alcohols, C13-15, branched and linear, ethoxylated N-(3-Aminopropyl)-Ndodecilpropane-1,3- diamine
P3-topax 66	Sodium hypochlorite Sodium hydroxide Alkyl amine oxide
Topaz AC3	Phosphoric acid Alkyl amine oxide Ester phosphates 2-(2- butoxyethoxy)etanol

Figure 1. Prevalence of *Listeria monocytogenes* and *Listeria* spp. associated with each zone. Zone 1: food-contact surfaces (FCS); Zone 2: proximity to food-contact surfaces (close to FCS); Zone 3: remote from food-contact surfaces in or near the processing areas (remote from FCS). Boxplots show the mean and median prevalence with the 25th and 75th percentile values. Points represent the values determined.

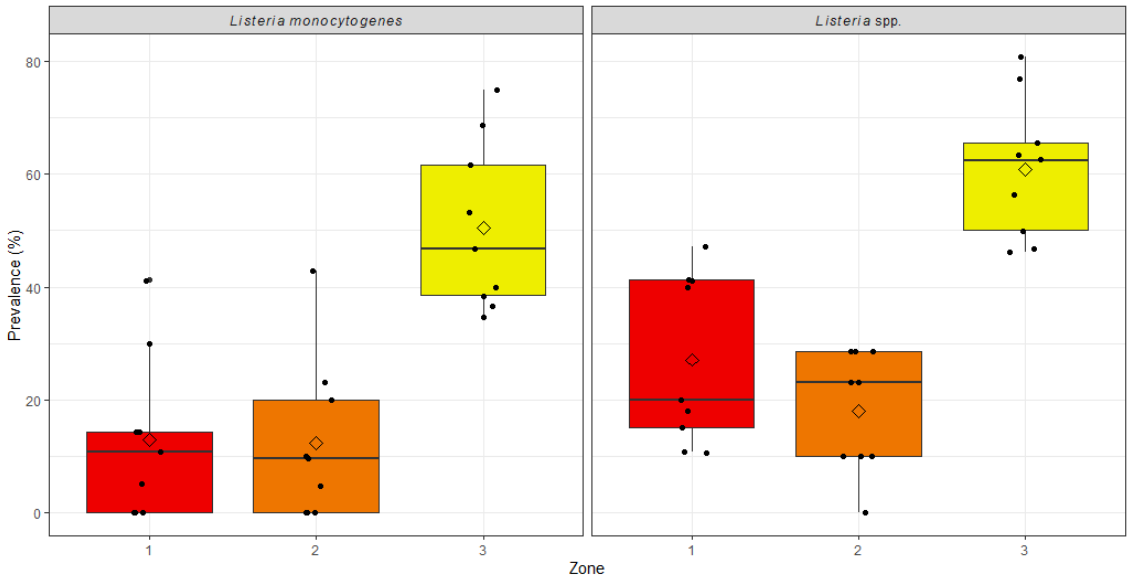

Figure 2. Prevalence of *Listeria monocytogenes* and *Listeria* spp. in the three fresh-cut facilities associated with each zone. Zone 1: food-contact surfaces (FCS); Zone 2: proximity to food-contact surfaces (close to FCS); Zone 3: remote from food-contact surfaces in or near the processing areas (remote from FCS). Boxplots show the mean and median prevalence with the 25th and 75th percentile values. Points represent the values determined.

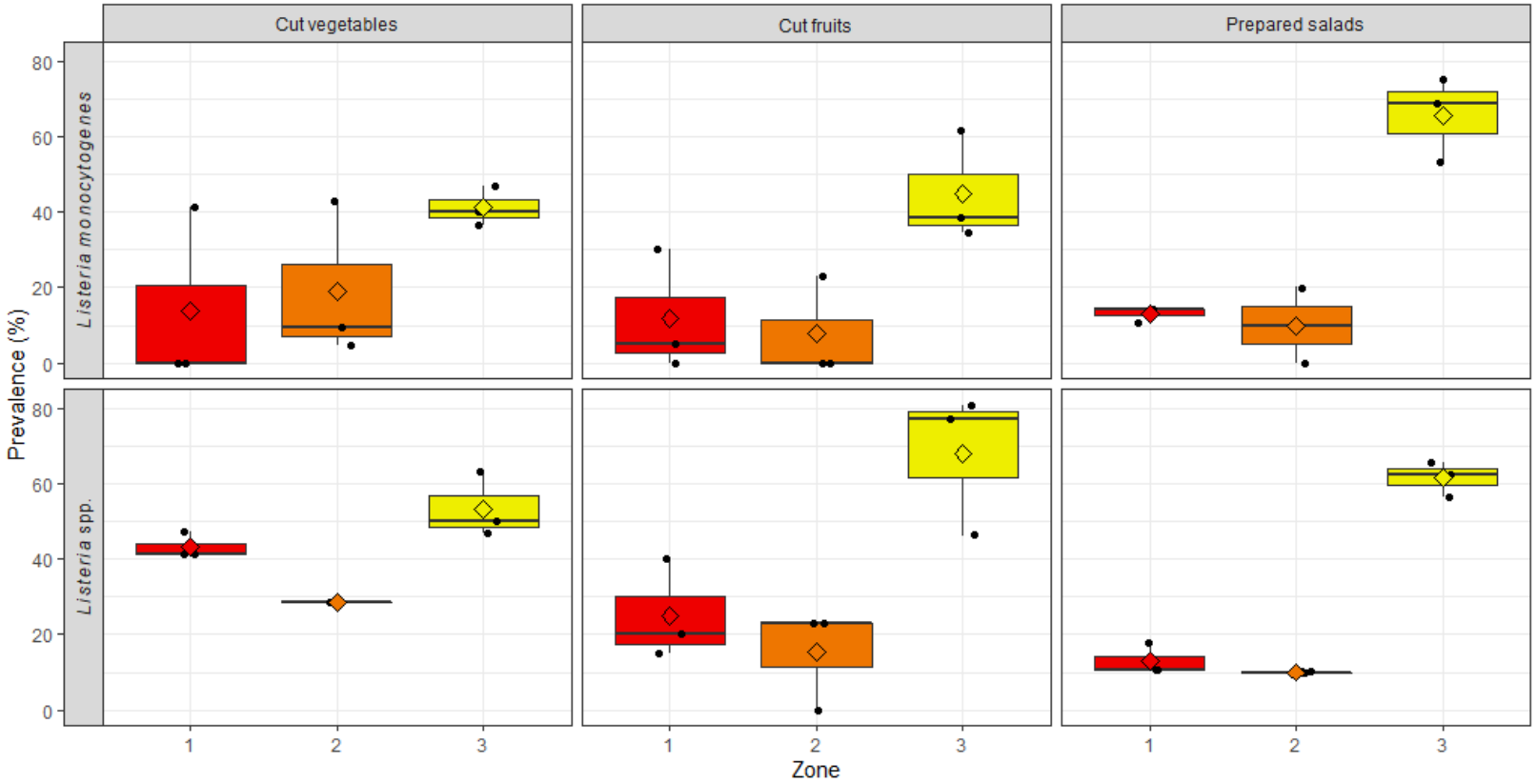

Figure 3. Levels of *Listeria* spp. (log cfu/unit) associated with each zone (A) and fresh-cut processing facility (B). Zone 1: food-contact surfaces (FCS); Zone 2: proximity to food-contact surfaces (close to FCS); Zone 3: remote from food-contact surfaces in or near the processing areas (remote from FCS). Boxplots show the mean and median prevalence with the 25th and 75th percentile values. Points represent the values determined.

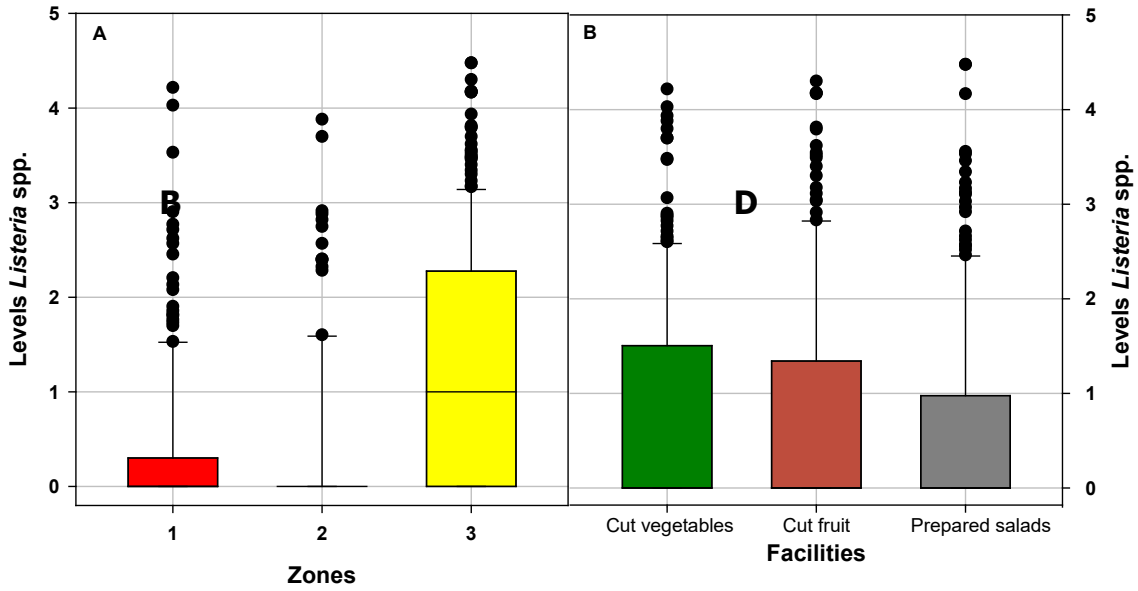

Figure 4. Prevalence of *Listeria monocytogenes* (A, C and E) and *Listeria* spp. (B, D and F) in the three fresh-cut facilities associated with the environmental monitoring (EM) samplings. Boxplots show the mean and median prevalence with the 25th and 75th percentile values. Points represent the values determined.

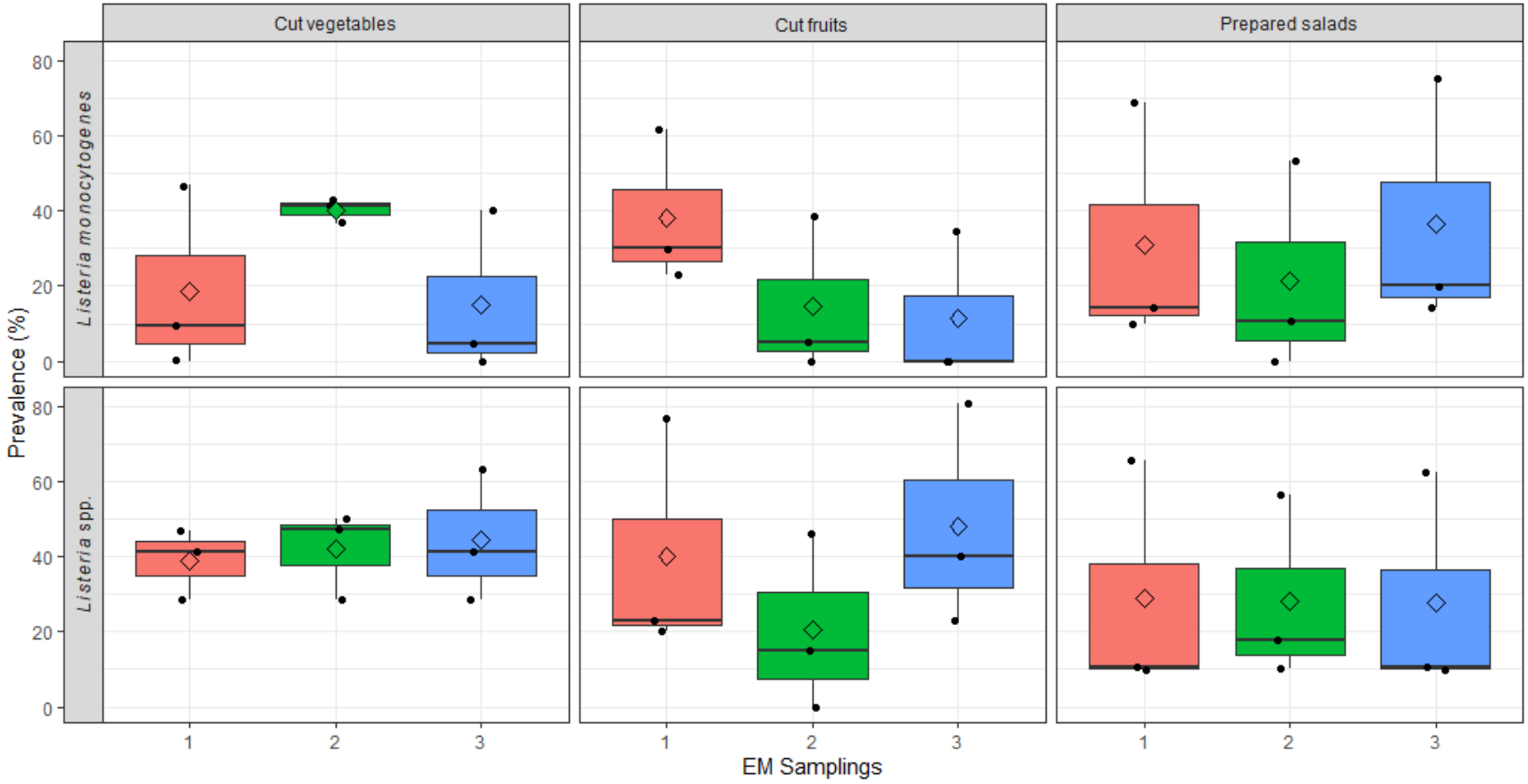

Figure 5. Minimum-spanning tree based on cgMLST allelic profles of 100 *Listeria monocytogenes* isolated from processing plants. Each circle represents an allelic profile based on sequence analysis of 1748 cgMLST scheme. Numbers correspond with allele distances between two nodes in the tree. Each circle contains the strain ID, and CCs are color coded. Lineages and serotypes are indicated.

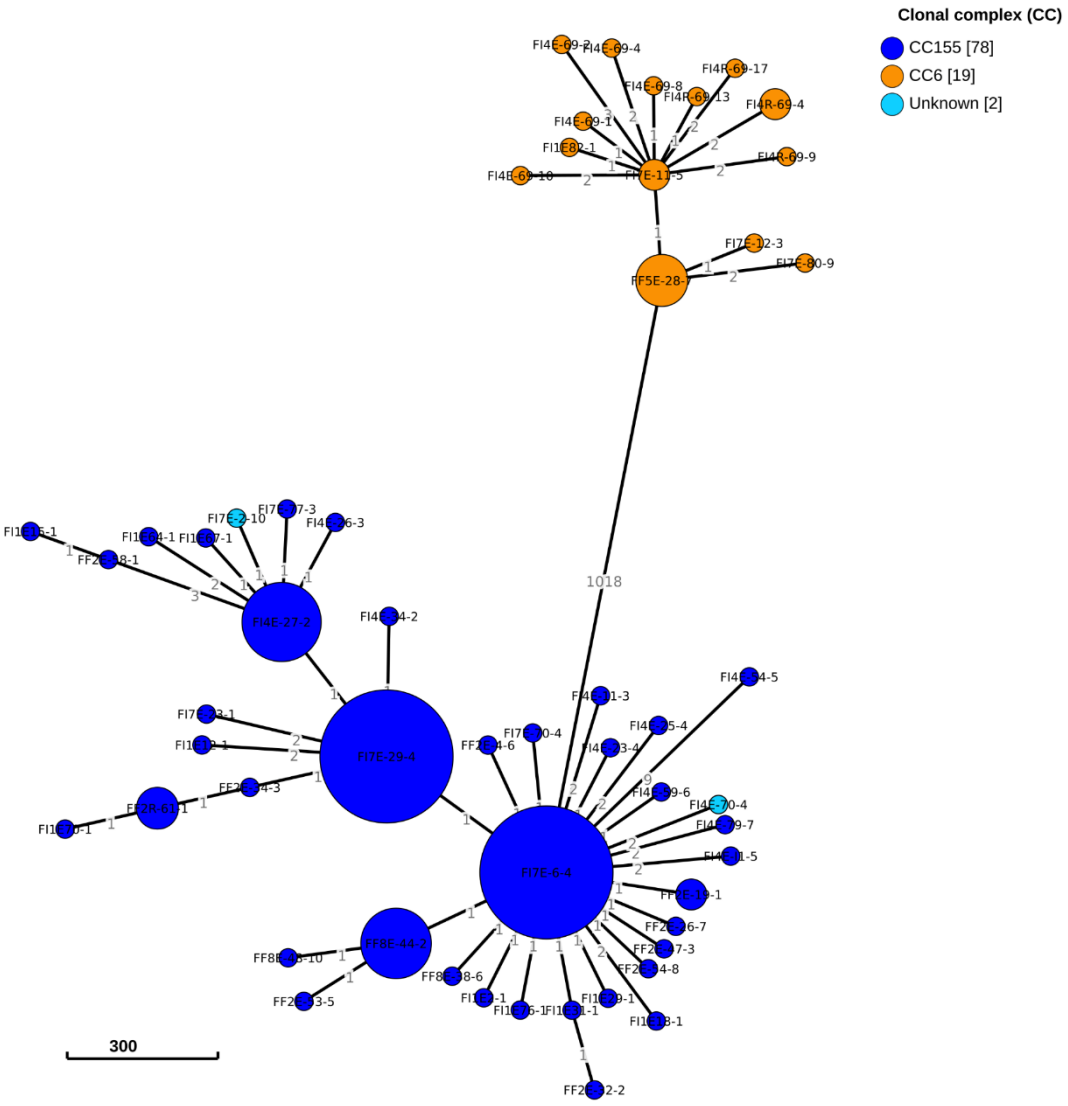

Figure 6. Minimum spanning tree generated with GrapeTree based on cgMLST allelic and visualized with iTOL including only iceberg line samples (A) and fruit line samples(B). Orange branches correspond to CC6, and blue branches correspond to CC155. Numbers correspond with allele distances between two nodes in the tree.

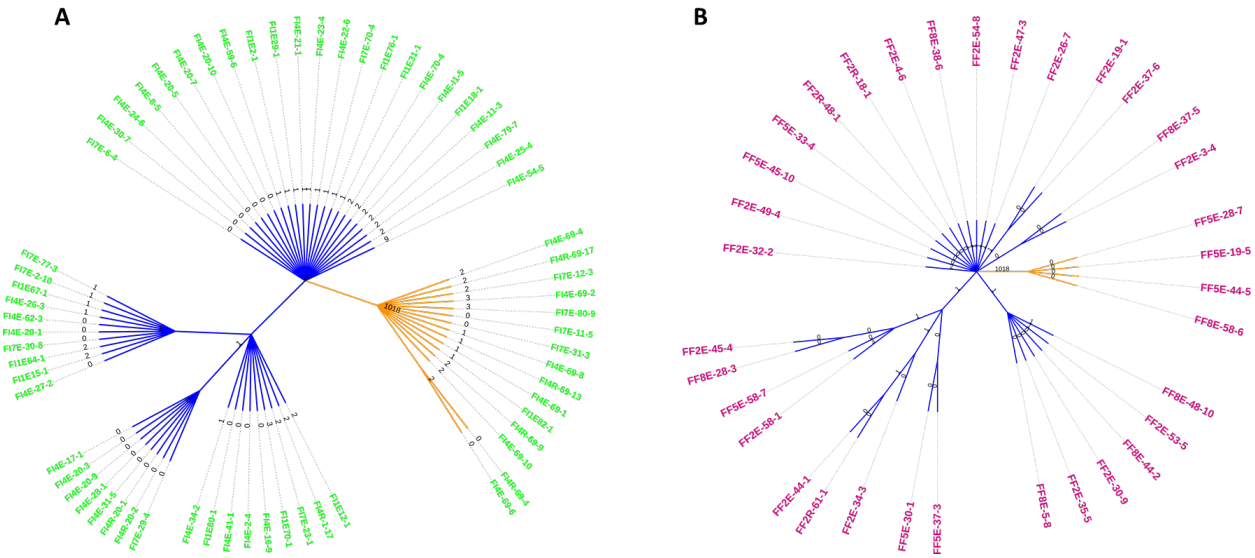

Figure 7. Minimum spanning tree generated with GrapeTree based on cgMLST allelic profiles including the 100 isolates obtained from the cut lettuce and cut fruit processing plants using the EM sampling zone as color identification.

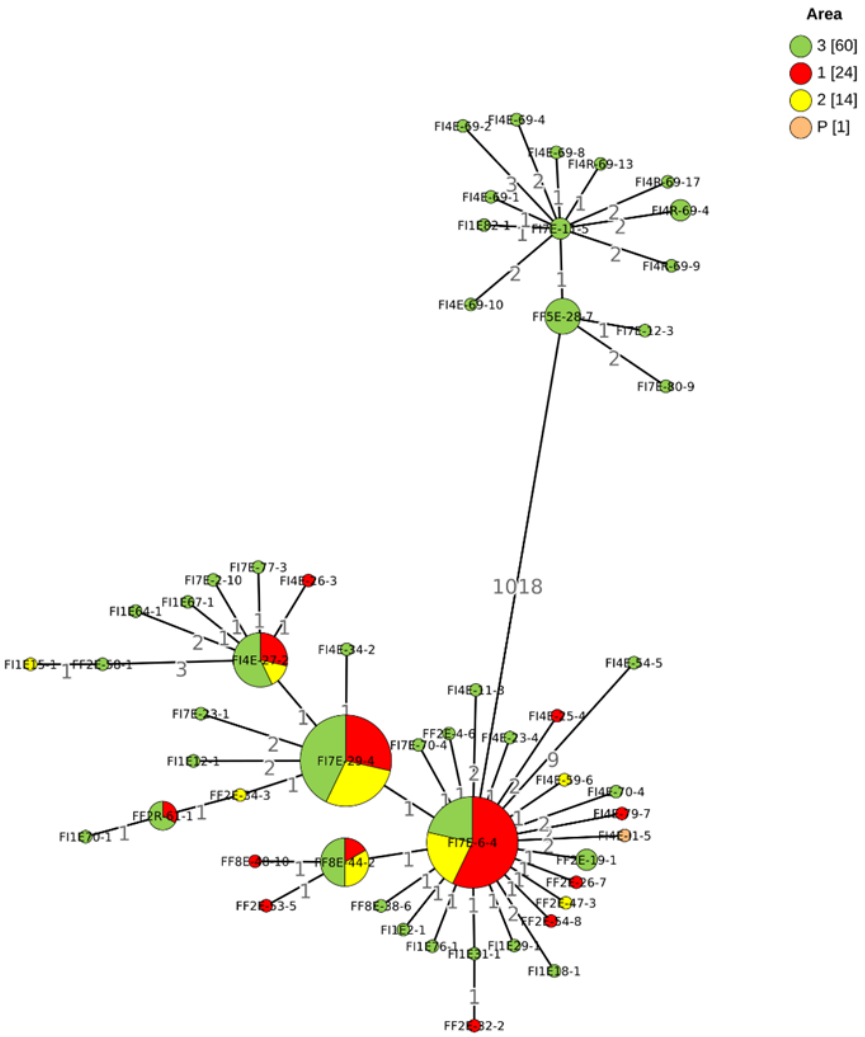

Figure 8. Minimum spanning tree generated with GrapeTree based on cgMLST allelic profiles including the 100 isolates obtained from the cut lettuce and cut fruit processing plants using the origin of the sample as color identification.

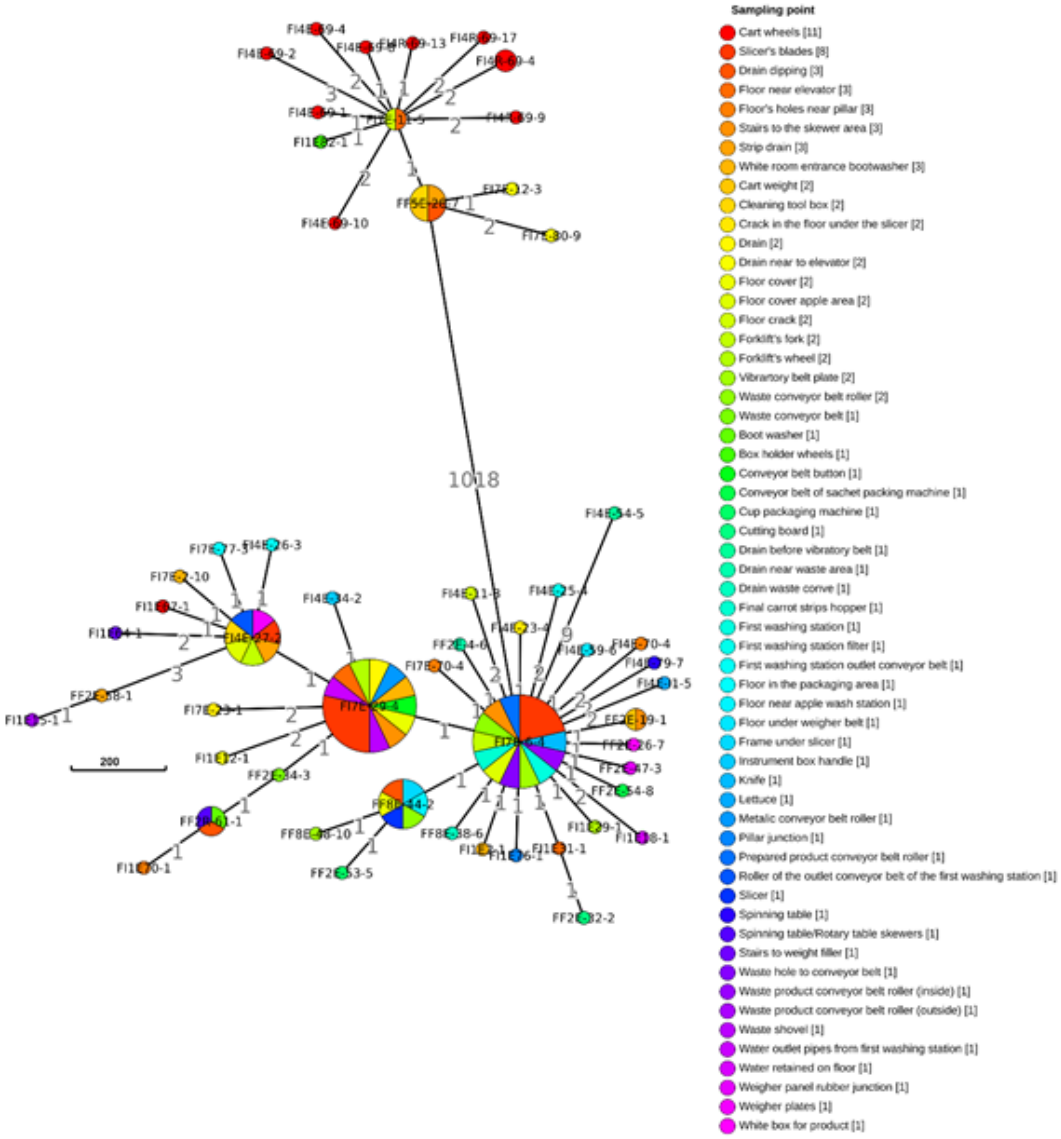

Figure 9. Growth kinetic test performed for strains 80.1 (cut lettuce), 58.1 (cut fruit) and 64.1 (prepared salads) using the biocides used by the industry at each EM sampling.