



Harnessing Whole Genome Sequence Data for Facility-Specific Signatures for *Listeria monocytogenes*: A Case Study With Turkey Processing Plants in the United States

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OPEN ACCESS

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Specialty section:

This article was submitted to
Agro-Food Safety,
a section of the journal
Frontiers in Sustainable Food Systems

Received: 16 July 2021

Accepted: 10 September 2021

Published: 13 October 2021

Citation:

Brown P, Chen Y, Siletzky R,
Parsons C, Jaykus L-A, Eifert J,
Ryser E, Logue CM, Stam C, Brown E
and Kathariou S (2021) Harnessing
Whole Genome Sequence Data for
Facility-Specific Signatures for *Listeria
monocytogenes*: A Case Study With
Turkey Processing Plants in the
United States.
Front. Sustain. Food Syst. 5:742353.
doi: 10.3389/fsufs.2021.742353

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Listeria monocytogenes is a Gram-positive foodborne pathogen responsible for the severe disease listeriosis and notorious for its ability to persist in food processing plants, leading to contamination of processed, ready-to-eat foods. *L. monocytogenes* persistence in various food processing environments (FPEs) has been extensively investigated by various subtyping tools, with increasing use of whole genome sequencing (WGS). However, major knowledge gaps remain. There is a need for facility-specific molecular signatures not only for adequate attribution of *L. monocytogenes* to a specific FPE but also for improved understanding of the ecology and evolution of *L. monocytogenes* in the food processing ecosystem. Furthermore, multiple strains can be recovered from a single FPE sample, but their diversity can be underestimated with common molecular subtyping tools. In this study we investigated a panel of 54 *L. monocytogenes* strains from four turkey processing plants in the United States. A combination of WGS and phenotypic assays was employed to assess strain persistence as well as identify facility-specific molecular signatures. Comparative analysis of allelic variation across the whole genome revealed that allelic profiles have the potential to be specific to individual processing plants. Certain allelic profiles remained associated with individual plants even when closely-related strains from other sources were included in the analysis. Furthermore, for certain sequence types (STs) based on the seven-locus multilocus sequence typing scheme, presence and location of premature stop codons in *inlA*, *inlB* length, prophage sequences, and the sequence content of a genomic hotspot could serve as plant-specific signatures. Interestingly, the analysis of different isolates from the same environmental sample revealed major differences not only in

serotype and ST, but even in the sequence content of strains of the same ST. This study highlights the potential for WGS data to be deployed for identification of facility-specific signatures, thus facilitating the tracking of strain movement through the food chain. Furthermore, deployment of WGS for intra-sample strain analysis allows for a more complete environmental surveillance of *L. monocytogenes* in food processing facilities, reducing the risk of failing to detect strains that may be clinically relevant and potentially novel.

Keywords: *Listeria monocytogenes*, *inlA*, whole genome sequencing, turkey processing plant, persistence, plant-specific signatures, cgMLST, wgMLST

INTRODUCTION

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen that is ubiquitous in the environment and responsible for the severe foodborne disease listeriosis in humans and other animals. Most cases of human listeriosis involve strains of two major lineages, i.e., lineage I and II, and three serotypes, specifically serotypes 4b and 1/2b (both of lineage I) and serotype 1/2a (lineage II) (Kathariou, 2002; Swaminathan and Gerner-Smidt, 2007; Orsi et al., 2011). Human populations at high risk include immunocompromised individuals, the elderly, pregnant women and their fetuses, and listeriosis can have severe outcomes including septicemia, meningitis, stillbirths, and death (Painter and Slutsker, 2007; Scallan et al., 2011; de Noordhout et al., 2014; Schlech, 2019). *L. monocytogenes* is responsible for about 1,600 illnesses and an estimated \$2.8 billion in adverse economic impacts each year in the United States alone (Scallan et al., 2011; de Noordhout et al., 2014; Hoffman et al., 2015). The severe outcomes of listeriosis and the associated high economic burden render *L. monocytogenes* a highly problematic agent.

L. monocytogenes can persist in food processing environments (FPEs) for impressively long periods of time, sometimes more than a decade (Kathariou, 2002; Gandhi and Chikindas, 2007; Carpentier and Cerf, 2011). Such persistence is mediated by several adaptations, including the ability to form persistent biofilms, tolerance to sanitizers such as quaternary ammonium compounds, growth at low temperatures, and phage resistance (Kathariou, 2002; Gandhi and Chikindas, 2007; Kim et al., 2008; Carpentier and Cerf, 2011; Kathariou et al., 2017). The increasing employment of whole genome sequencing (WGS) has significantly facilitated the assessment of *L. monocytogenes* persistence in individual food processing plants, greatly improving the resolution afforded by previous tools such as pulsed-field gel electrophoresis or the seven-locus multilocus sequence typing (MLST) scheme (Fagerlund et al., 2016, 2020; Knudsen et al., 2017; Hurley et al., 2019; Stoller et al., 2019; Harrant et al., 2020; Yang et al., 2020).

Analysis of WGS data from various FPEs has been utilized to identify genetic elements, especially those mediating biocide resistance, that can contribute to persistence (Cherifi et al., 2018; Muhterem-Uyar et al., 2018; Pasquali et al., 2018; Hurley et al., 2019; Cooper et al., 2021). Furthermore, WGS analysis has allowed rigorous, targeted inter-FPE comparisons of strains of the same genotype, e.g., the same MLST-based sequence

type (ST). Such strains can sometimes be clearly differentiated based on the facility of their origin (Knudsen et al., 2017; Hurley et al., 2019; Fagerlund et al., 2020; Kwon et al., 2020; Yang et al., 2020). However, they can also exhibit impressive similarity, as suggested by WGS-based detection of virtually identical strains of ST8 isolated 15 years apart from two different salmon processing plants, one in Norway and one in Denmark (Fagerlund et al., 2016). Additional data from such inter-FPE comparisons are critically needed to determine the potential for molecular signatures that may be unique to and specific for particular food processing plants, thus greatly facilitating the tracking of contamination and effectively directing mitigation strategies to the relevant food processing facilities. Furthermore, such comparisons may yield critically-needed insights on specific attributes and adaptations that can mediate persistence of *L. monocytogenes* in a particular FPE, and can contribute importantly to our understanding of *Listeria's* ecology and evolution in the food processing ecosystem. Integration of WGS-based data with phenotypic assessments of specific adaptive traits of high relevance to FPE persistence may be especially valuable in this regard.

In previous studies we isolated *L. monocytogenes* via selective enrichments of environmental samples from selected turkey processing plants in the United States and analyzed the strains for resistance to the quaternary ammonium disinfectant benzalkonium chloride (BC) and to heavy metals (Mullapudi et al., 2008). A high proportion of the serotype 1/2a and 1/2b strains were resistant to BC, with resistance typically accompanied by resistance to the heavy metal cadmium (Mullapudi et al., 2008). Furthermore, the serotype 1/2a and 1/2b strains were also frequently resistant to three wide-host-range lytic phages, including two phages isolated from environmental swabs from the same turkey processing plants (Kim et al., 2008). Recently, phage resistance of some of these turkey processing plant-derived strains of ST321 and ST391 was further characterized via WGS analysis and investigation of phage receptors on the teichoic acid of the cell wall (Brown et al., 2021).

In the current study, a panel of 54 *L. monocytogenes* strains and two *L. welshimeri* strains from these turkey processing plants were investigated via an approach that integrated WGS analysis with phenotypic assessments of resistance to BC, cadmium and a panel of four wide-host-range lytic phages. Our objectives were to obtain WGS-based assessments of possible persistence and dissemination among different turkey processing plants, as

well as to determine the potential for WGS-based analysis to identify molecular signatures specific to individual facilities. The panel of strains included several that were isolated from the same environmental sample, including different isolates of the same serotype and ST. Therefore, an additional objective was to characterize the effectiveness of WGS-based analysis to reveal genomic diversity among isolates from the same FPE sample.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The *L. monocytogenes* and *L. welshimeri* strains used in this study (**Supplementary Table 1**) were isolated from selective enrichments of environmental samples from four different turkey processing plants (A–D) in the United States from 2003 to 2006 as described previously (Eifert et al., 2005). Multiplex PCR was employed to determine serotype designations as previously described (Doumith et al., 2004), with designations confirmed via analysis of the whole genome sequence data. Unless otherwise indicated, bacterial strains were grown overnight in brain heart infusion broth (BHI; Becton, Dickinson and Co., Sparks, MD) or on BHI agar at 37°C, and were preserved at –80°C in BHI with 20% glycerol.

Whole Genome Sequencing and Analysis of WGS Data

Listeria strains were grown overnight in BHI at 37°C and genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Valencia, CA). Library preparations were completed using a Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) with 1 ng of genomic DNA. Genomic DNA was sequenced using a NextSeq 500 sequencer and the NextSeq 500/500 high-output kit v2.5 (300 cycles, 2 × 150 bp) (Illumina) or a MiSeq desktop sequencer with the MiSeq kit v2 (500 cycles, 2 × 250 bp) (Illumina), according to the manufacturer's instructions. Raw sequence reads were quality-trimmed and assembled *de novo* using SPAdes v 3.14.1 (Bankevich et al., 2012). The quality of the assemblies was assessed using QUAST v.4.6.4 (Gurevich et al., 2013). Default parameters were used with all software.

Sequence type (ST) designations using the seven-locus MLST scheme and heavy metal/antimicrobial resistance gene allele designations were determined using BIGSdb-*Lm* hosted by the Institut Pasteur (Moura et al., 2016). Whole-genome MLST (wgMLST) and core-genome MLST (cgMLST) data were analyzed to determine allele differences with the BIGSdb PasteurMLST Genome Comparator (Jolley et al., 2018), with the cgMLST and wgMLST schemes using 1,748 and 3,273 loci, respectively. Incomplete alleles and loci that had identical alleles among all strains of a given sequence type were excluded from analysis. For certain STs (i.e., 3, 5, 6, 9 and 321), strains from additional sources were included in genomic comparisons with the strains from the turkey processing plants. Strains from other sources were chosen based on publicly-available whole genome sequence data and origin from diverse sources, e.g., clinical origin and USDA-FSIS surveillance. For ST321, we also included in the comparisons a panel of six ST321

strains from a cold-smoked salmon facility in the United States (Harrand et al., 2020) while for ST5 we included 12 additional strains of meat origin from Ireland (Hurley et al., 2019). Allelic analysis of selected virulence determinants employed the BIGSdb Pasteur MLST Genome Comparator (Jolley et al., 2018). A phylogenetic tree was created by using 1,748 *L. monocytogenes* core genes from the PasteurMLST into Ridom SeqSphere+, as previously described (Chen et al., 2016). Minimum spanning trees were constructed with Bionumerics¹ version 8.0 (<https://www.applied-maths.com>) using the seven-locus MLST scheme. Prophage identification employed the proteome comparison function from PATHosystems Resource Integration Center (PATRIC) Bioinformatic Resource Center (PATRICBRC; Davis et al., 2020) and the Phage Search Tool Enhanced Release (PHASTER) (Zhou et al., 2011; Arndt et al., 2016). SNP cluster designations were identified using the NCBI Pathogen Detection Pipeline (<https://www.ncbi.nlm.nih.gov/pathogens/>) (The NCBI Pathogen Detection Project, 2016). The SNP calls are made in the context of all members of a specific cluster following SNP-calling algorithms developed by NCBI (https://www.ncbi.nlm.nih.gov/pathogens/pathogens_help/#data-processing-clustering).

Determination of Resistance to BC, Cadmium, and to Wide-Host-Range Lytic Phages

Phage susceptibility assays were done at 25°C as described previously using the wide-host-range lytic phages A511, P100, 20422-1, and 805405-1 (Brown et al., 2021). Resistance to BC and cadmium was determined as described previously (McLauchlin et al., 1997; Mullanpudi et al., 2008). Strains were designated resistant if confluent growth was observed on Iso-Sensitest agar (Oxoid, Hampshire, England) supplemented with 70 µg/mL anhydrous cadmium chloride (Sigma, St. Louis, MO, USA) or on Mueller-Hinton agar (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with 10 µg/mL BC (Acros, Morris Plains, NJ, USA). Plates were examined following incubation for 48 h at 37°C.

Genome Sequence Accession Numbers

Whole genome sequence data for the strains used in this project can be found in **Supplementary Table 1** and under BioProject Accession PRJNA215355.

RESULTS AND DISCUSSION

Sampling of four different turkey processing plants (A–D) from 2003 to 2006 resulted in 1,584 samples. The recovery of *L. monocytogenes* and other *Listeria* spp. from these samples will be described in a separate presentation. A panel of 54 *L. monocytogenes* isolates from the four turkey processing plants and two *L. welshimeri* from Plant A were chosen for whole genome sequencing based on their serotype as well as phenotypic assays including resistance to benzalkonium chloride (BC), cadmium and a panel of four wide-host-range lytic phages

¹BioNumerics version 8.0 created by Applied Maths NV. Available online at: <https://www.applied-maths.com>.

TABLE 1 | Composition of the panel of *L. monocytogenes* strains investigated in this study.

FPE	<i>L. monocytogenes</i> serotype				Timeframe	Sequence Types (STs)	
	1/2a	1/2b	1/2c	4b			Total
A	11	10	2	7	30	9/1/03–3/1/06 (30 months)	2, 5 , 6 , 9 , 87, 321 , 391, 506, 554, 1,084
B	2	4	0	7	13	8/1/03–7/1/05 (23 months)	1, 3 , 6 , 321 , 372, 550, 552
C	4	2	1	1	8	4/1/04–3/1/06 (23 months)	3, 5 , 7 , 9 , 29, 451, 570
D	3	0	0	0	3	10/1/04–6/1/05 (9 months)	7 , 581
Total	20	16	3	15	54	8/1/03–3/1/06 (31 months)	1, 2, 3 , 5 , 6 , 7 , 9 , 29, 87, 321 , 372, 391, 451, 506, 550, 552, 554, 570, 1,084

Plant A: Southeastern USA, Plant B: Northeastern USA, Plant C: Midwestern USA 1, Plant D: Midwestern USA 2. Sequence types (STs) in bold font indicate those isolated from two or more FPEs.

(Supplementary Table 1). The *L. monocytogenes* panel consisted predominantly of strains from Plants A ($n = 30$) and B ($n = 13$), with Plants C ($n = 8$) and D ($n = 3$) making smaller contributions, and included strains of all three serotypes most commonly encountered in human listeriosis: 1/2a ($n = 20$), 1/2b ($n = 16$), and 4b ($n = 15$) (Table 1). *In-silico* MLST revealed a total of 19 STs among the 54 *L. monocytogenes* strains, with several STs encountered in multiple plants (Table 1; Figure 1A). Several STs were also encountered at multiple time points over the three-year sampling period (Table 1; Figure 1B). The two *L. welshimeri* strains had the same ST, i.e., ST1084 (Supplementary Table 1).

BC resistance was found to be common (43%) among these *L. monocytogenes* strains, with all BC-resistant strains being also resistant to cadmium as previously described (Mullapudi et al., 2008), while several additional strains were resistant to cadmium but susceptible to BC, leading to 65% of the strains exhibiting cadmium resistance (Figure 2; Supplementary Table 1). Phage resistance was also common, with 29/54 (54%) of the strains exhibiting resistance against all four wide-host-range phages in the panel and 39 (69%) exhibiting resistance to at least one of the phages (Supplementary Table 1). Strains of the same ST and from the same plant tended to exhibit the same phage resistance profile, with the notable exceptions of ST5, ST391, and ST554 from Plant A (Supplementary Table 1). While strains of the same ST were found over extended periods of time in the same processing plant, no apparent increase in resistance to BC or phage could be noted, potentially because the selective pressures associated with BC or phage exposure may well have selected for specific resistance traits before sampling began.

Antibiotic resistance determinants identified via the NCBI Pathogen Detection Pipeline were only *fosX* and *lin*, both of which are widely conserved across *L. monocytogenes*, while an additional determinant, *abc-F*, was identified in ST391 and a small number of other strains (Supplementary Table 1). No other antibiotic resistance determinants were revealed in this panel of strains (Supplementary Table 1).

Evidence for Persistent Strains and Cross-Plant Dissemination

Strains of the same ST were isolated during multiple sampling months in both Plant A and B. In Plant A, strains of ST 2, 5, 9, 321, 391, and 554 were isolated at least 4

months apart, with six strains of ST391 isolated over a 21-month period (Table 1; Supplementary Table 1; Figure 1). ST391 strains were noteworthy, as they were isolated from Plant A over a 29-month period and belonged to the same SNP cluster (PDS000025393.5), with 19–47 wgMLST allele differences (Supplementary Table 2; Figure 2), in spite of the differences in their phage resistance profiles (Supplementary Table 1). A similar finding was obtained with ST321, which was isolated from Plants A and B and for which strains belonged to the same SNP cluster (PDS000000366.424) with 16–114 wgMLST allele differences (Supplementary Table 2; Figure 2). Even though the ST321 strains from plant A were resistant to all four phages, the strain from Plant B was susceptible (Supplementary Table 1). Several other STs from Plant A had high similarity between strains isolated on different dates, including ST9 (27 wgMLST allele differences, SNP cluster PDS000025433.45) and ST554 (7–12 wgMLST allele differences, SNP cluster PDS000024310.3) (Supplementary Tables 1, 2; Figure 2). In Plant B, strains of ST1 and ST6 were isolated at least 3 months apart, with two highly similar ST1 strains (15 wgMLST allele differences, SNP cluster PDS000083087.1) from different dates and five strains of ST6 in the same SNP cluster (PDS000025190.4) over a 30-month period (Table 1; Supplementary Table 1; Figure 2).

In comparison to these STs, noticeably more divergence was noted among ST5 strains from Plant A, with 27–282 wgMLST allele differences (Supplementary Table 2). The relatively large number of differences in ST5 strains may reflect either multiple introductions of these ST5 strains into Plant A or possibly earlier introductions enabling greater diversification over a longer period of time. However, the findings may also reflect the greater diversification potential of this ST. A previous study reported broad differences in strain diversity among ST5 strains from the same ready-to-eat meat processing plant in China and identified eight clusters of ST5 strains with one such cluster having >70 cgMLST allele differences from the others (Zhang et al., 2021).

While strains of the same ST isolated on different dates from Plant C or D were not identified in the panel, two ST551 strains were isolated on the same day from different samples from Plant D (Supplementary Table 1). These two strains (L1009a, L1014a) differed by 59 wgMLST alleles, indicating that, while they are related and likely diverged from the same introduction into Plant D, they had persisted in this plant long enough to diverge sufficiently to be differentiated by WGS (Supplementary Table 2;

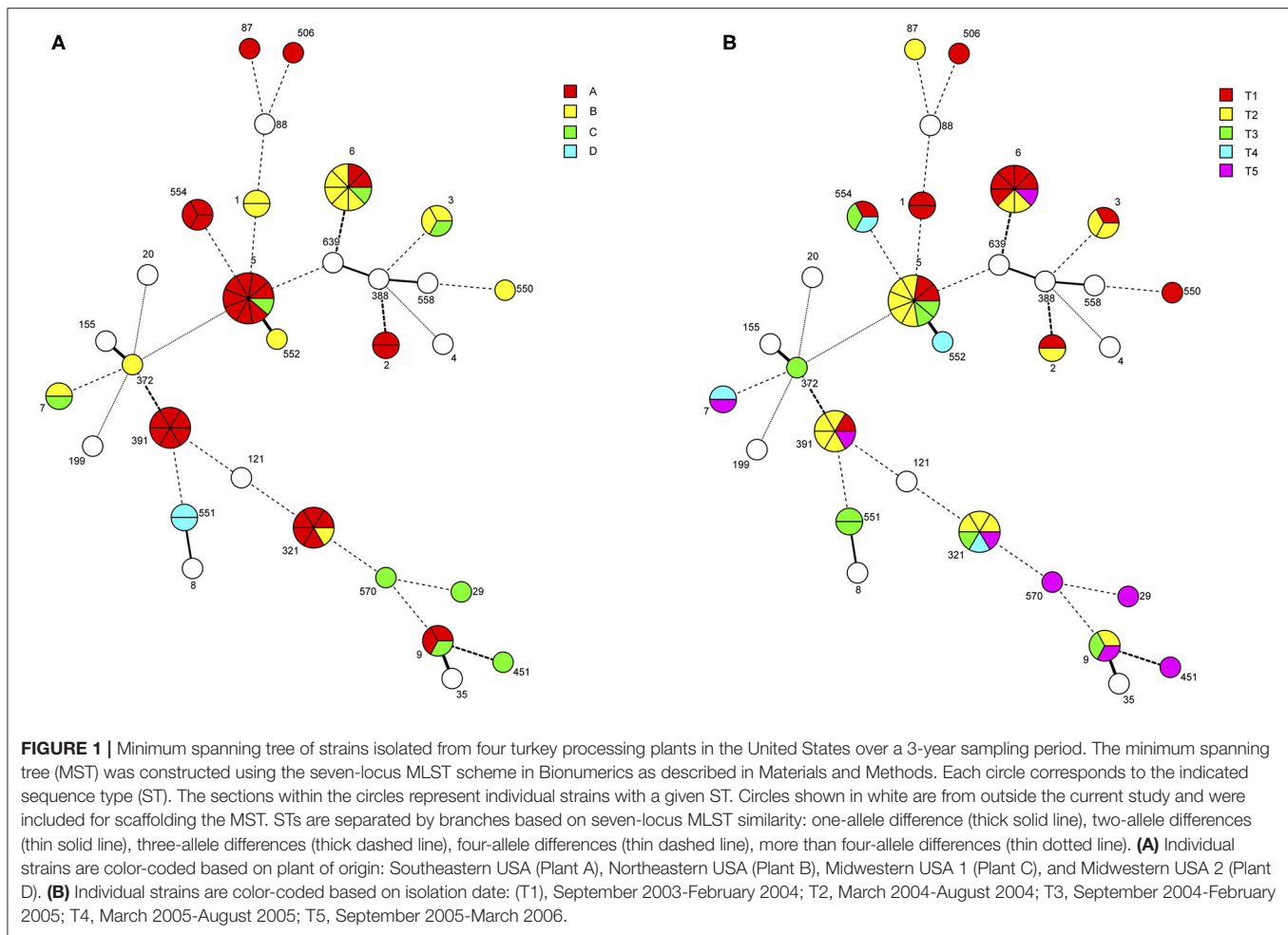
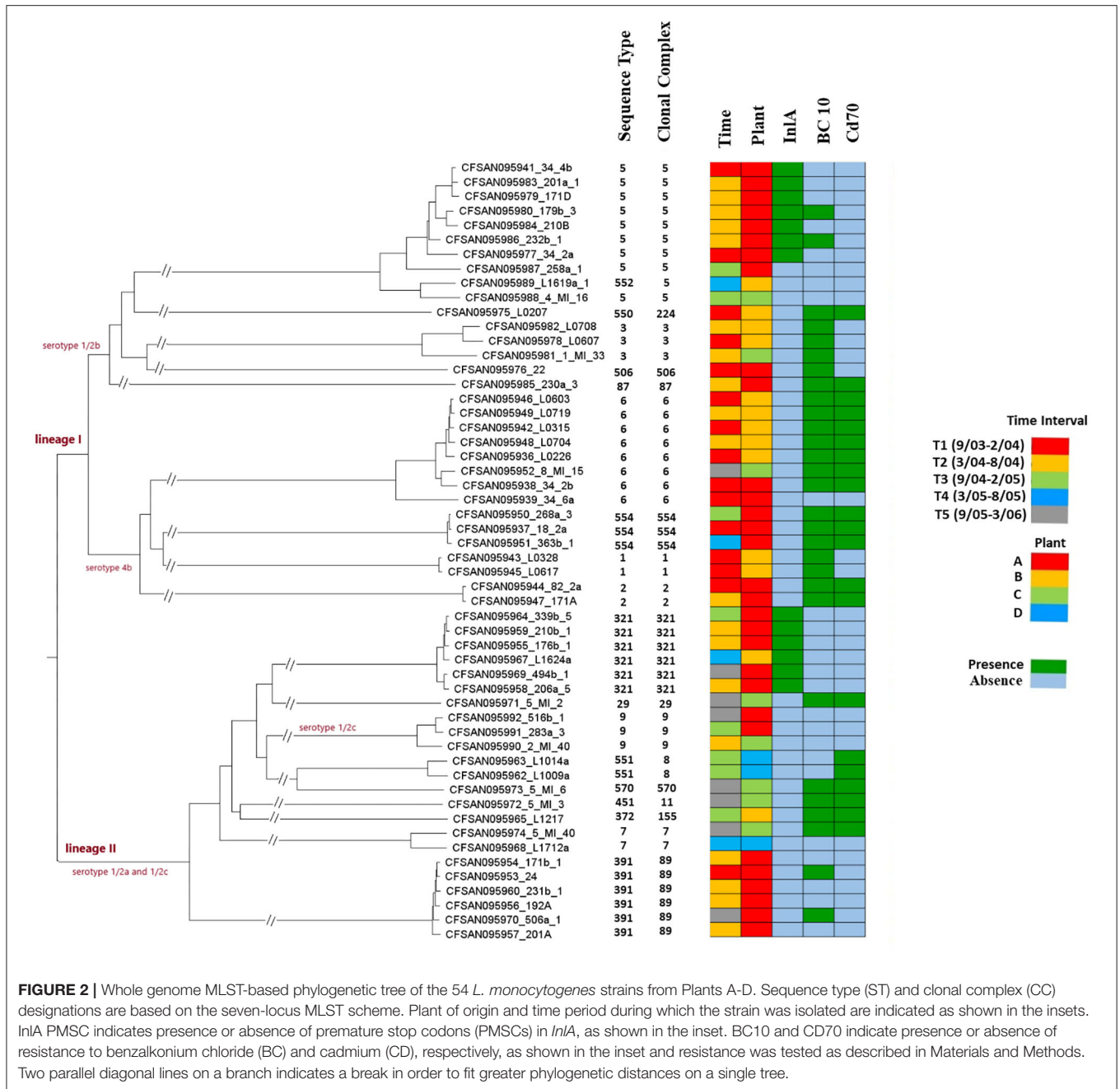


Figure 2). However, the possibility of repeated introduction should also be considered.

Both sequenced *L. welshimeri* strains (266a-1 and 34-3a) were BC and cadmium resistant, harboring the same *bcrABC* alleles found in the majority of *L. monocytogenes* strains and also *cadA2*, which frequently accompanied *bcrABC* in *L. monocytogenes* in the current panel (**Supplementary Table 1**) as well as in larger surveys of *L. monocytogenes* from the turkey processing plants (Mullapudi et al., 2008). Previous findings showed the potential of *bcrABC* to be transferred via conjugation from *L. welshimeri* to *L. monocytogenes* upon co-selection for cadmium resistance (Katharios-Lanwermeyer et al., 2012). These two *L. welshimeri* strains were isolated from two different floor drains of the same facility more than 1 year apart, had the same ST (**Supplementary Table 1**) and were closely related (79 wgMLST allele differences). Even though repeated introduction cannot be excluded, these findings may also suggest that persistence in the FPE is not unique to *L. monocytogenes* but can also be exhibited by non-pathogenic *Listeria* spp. FPE persistence in *L. welshimeri* adds further relevance to the potential transfer of *bcrABC*-mediated BC resistance from non-pathogenic *Listeria* spp. to *L. monocytogenes* within the same FPE.

Genome comparator-based analysis was employed to assess the possibility of strain dissemination among different plants. As discussed above, the five ST321 strains from Plant A belonged to the same SNP cluster (PDS000000366.424) as the single ST321 strain from Plant B (**Supplementary Table 1**). The phylogenetic tree revealed two clusters, with the Plant B strain being in the same cluster as three ST321 strains from Plant A and (as will be discussed below) harboring the same uncommon premature stop codon in *inlA* (**Figure 2**). Even though independent introduction events to Plants A and B from a common source cannot be excluded, such findings constitute suggestive evidence for cross-plant dissemination. Equipment relocation, for instance, was shown to mediate strain transfer between two different food processing plants (Fagerlund et al., 2016).

Evidence of cross-plant dissemination among other STs was uncommon. In the case of ST6, the phylogenetic analysis revealed three groups, one of which consisted of strains from Plant A and C (**Figure 2**). These two strains differed by 88 wgMLST alleles (**Supplementary Table 2**) and were both unaffiliated with a SNP cluster (**Supplementary Table 1**). Thus, if a cross-plant dissemination or independent introduction from a common source event had taken place, significance divergence appears



to have ensued, potentially reflecting early introduction events or differences in the ecology of the respective facilities. Cross-plant comparisons with strains of ST3, ST5, and ST9 found a range of 94–357 wgMLST allele differences, with the largest difference found for strains of ST5 (Supplementary Table 2). On the phylogenetic tree the ST5 strains partitioned in several sub-clusters (Figure 2), in agreement with the allelic diversity revealed via genome comparator analysis with cgMLST or wgMLST (Supplementary Table 2). ST5 strains from Plant A partitioned clearly apart from ST5 from Plant C (Figure 2). Interestingly, the ST5 strain from Plant C was closest to the ST552

strain from Plant B (Figure 2). ST5 and ST552 belong to the same clonal complex (CC) in the eight-locus MLST scheme, i.e., CC5 (Figure 2). The observed relatedness but also diversification between the ST5/ST552 strains from Plants B and C (Figure 2) may suggest earlier acquisitions e.g., via cross-plant transfer, and subsequent divergence, as discussed above for ST6 from Plant A and C.

For ST 3 and 9, the phylogenetic tree also reflects plant-specific diversification and does not provide evidence for cross-plant transfer (Figure 2). The ST3 strains from Plant B clustered together and separately from the ST3 strain from

Plant C (**Figure 2**). Similarly, ST9 strains from Plant A grouped separately from ST9 from Plant C (**Figure 2**). Additional strains from the different plants would need to be analyzed to further assess the potential for cross-plant transfer or other common-source acquisition events for these STs.

The Use of Allelic Variation to Derive Plant-Specific Signatures

While *L. monocytogenes* is known to persist for extended periods of time in FPEs, less is known about how strains adapt and diversify in a specific FPE as compared to others. Processing facilities can exert certain common selective pressures but no two are identical, with different food commodities, environmental surface types, facility age and complexity, cleaning and sanitation regimens, and FPE microbial community composition, among the possible differences (Carpentier and Cerf, 2011; Ferreira et al., 2014; Mazaheri et al., 2021). These differences can play major roles in genotypic and phenotypic diversification of strains between different processing plants. In addition, silent substitutions are expected to accrue due to genetic drift as populations evolve in physical isolation from each other in different FPEs. This can lead to genetically-related strains harboring plant-specific signatures that may be used to distinguish strains from different plants. Several STs were isolated in multiple processing plants in the current study (**Table 1**; **Figures 1A, 2**), giving us a unique opportunity to look for plant-specific genetic traits in these strains. ST7 was not included in this analysis as the panel included only one strain each from two different plants (**Figure 2**; **Supplementary Table 1**).

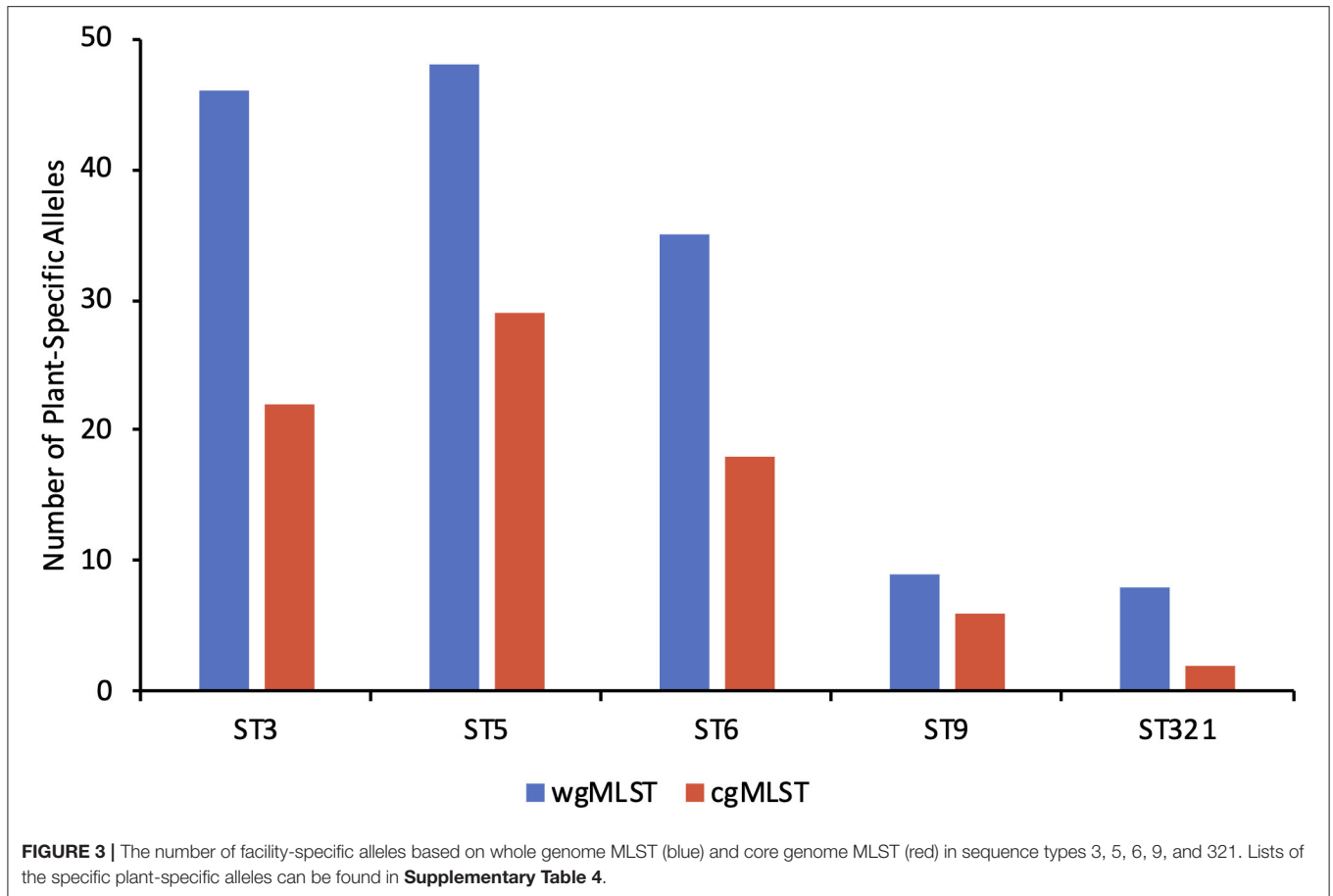
Whole genome MLST (wgMLST) was utilized with FPE-derived (Plants A-D) strains of ST3, 5 (and the closely-related strain of ST552), 6, 9, and 321 to identify putative plant-specific alleles. This analysis identified 387 wgMLST loci with plant-specific alleles, with 219 of these belonging to the core genome (1,748 core genes) (**Supplementary Table 3**). In order to further assess the potential of allelic variation at these loci to discern among different plants (e.g., Plant A vs. B), the FPE-derived strains were compared not only among themselves but also with several (7–16) other strains of the same ST, including strains from human listeriosis and USDA-FSIS surveillance. This led to a total of 181 wgMLST loci (96 from the core genome) found to have plant-specific alleles within a given sequence type, with 46, 93, 35, 9, and 8 plant-specific alleles being detected in ST3, 5 (and 552), 6, 9 and 321, respectively (**Figure 3**; **Supplementary Table 4**). Interestingly, eight of these loci, i.e., *lmo0061* (FtsK/SpoIIIE family protein, putative EssC/YukB component of Type VII secretion system), *lmo0530* (hypothetical protein), *lmo0829* (pyruvate-flavodoxin oxidoreductase), *lmo1291* (peptidoglycan O-acetyltransferase YrhL), *lmo1499* (murein endolytic transglycosylase MltG), *lmo1574* (DNA polymerase III alpha subunit), *lmo2370* (aminotransferase), and *oatA* (O-acetyltransferase), were found to be variable between plants in multiple STs, and *lmo1499* was especially noteworthy in exhibiting a plant-specific allele in three of the five STs (**Supplementary Tables 3, 4**). This suggests that these seven loci may be especially prone to responding to plant-specific selective

pressures, potentially leading to adaptive mutations and making them excellent candidates for plant-specific signatures.

ST5 (and the related ST552) and ST6 were the most predominant STs in this study that were encountered in multiple plants, and much of our focus was therefore directed to them. For CC5 (ST5 and 552), the ST552 strain from Plant B had more plant-specific alleles ($n = 46$) than the ST5 strains from Plant A ($n = 33$) or Plant C ($n = 16$), i.e., alleles shared among all CC5 strains from the relevant plant but not encountered in CC5 strains from the other plants (**Supplementary Table 4**). Interestingly, however, of the 16 Plant C-specific alleles, 100% were novel alleles designated as “new” in the genome comparator outputs, having not yet been deposited in the PasteurMLST database, while only 8/33 (24%) of the Plant A-specific alleles and 38/46 (83%) of the Plant B-specific alleles were novel. This suggests that a potentially novel ST5 strain may have been introduced into Plant C, which would be supported by this strain not being affiliated with a SNP cluster (**Supplementary Table 1**). Allelic content in *lmo0883* (transmembrane protein) appears to be a promising plant-specific marker for these strains, as all eight ST5 Plant A strains harbor the same novel allele (new#1) regardless of their SNP cluster designations. On the other hand, the ST5 strains from Plant C harbored a different novel allele (new#2), while a previously-identified allele was harbored by the ST552 (CC5) strain from Plant B and all other ($n = 16$) ST5 strains in the comparison panel, regardless of source (**Supplementary Table 4**).

Besides the ST5 and 552 (CC5) strains, ST6 also gave us the opportunity to compare across strains from three different turkey processing plants. In Plant A, the two ST6 strains were isolated from the same sample (sample 34) but were notably different from each other (271 allele differences by wgMLST) as also shown in the phylogenetic tree (**Figure 2**) and discussed further below, making it difficult to identify any ST6 Plant A-specific alleles (**Supplementary Table 2**). Nonetheless, we identified 17 and 21 alleles specific for ST6 strains in Plant A and B, respectively, with three loci (*lmo2306*, *lmo2307*, and *lmo2323*) having specific alleles for both plants (**Supplementary Table 4**). All three loci are annotated as bacteriophage A118-like proteins. The high allelic diversity found among ST6 strains at these loci suggests a high mutation frequency, with each locus exhibiting six to seven novel alleles just among the ST6 strains included in this analysis, and some strains lacking these loci entirely (**Supplementary Table 4**).

In addition to the six loci (*lmo0829*, *lmo1291*, *lmo1499*, *lmo1574*, *lmo2370*, and *oatA*) that were found to be variable between plants in multiple STs, there were many others where specific alleles were found in all strains from one plant but rarely in other sources (**Supplementary Table 3**). The occasional detection of these alleles in strains from other sources may potentially help with tracking strain movement. An example of this is ST5, where among our FPE-derived strains allele 33 at *lmo2561* appeared specific to Plant C (SK197). This allele was otherwise rare in our comparison panel, found only in one other ST5 strain, of clinical origin (PNUSAL002668) (**Supplementary Table 3**). Of the ST5 strains in this analysis, SK197 exhibited the least number of wgMLST allele differences ($n = 155$) from PNUSAL002668, even though the two strains do



not belong to the same SNP cluster (**Supplementary Table 2** and data not shown).

Similar findings were obtained with ST321, for which strains from Plant A shared the same allele at *LMRG_01558* while different alleles at this locus were found in ST321 genomes from Plant B or any of the other sources included in this analysis (**Supplementary Table 4**). Our panel of ST321 strains from other sources included six from a cold-smoked salmon processing plant in the United States, with three strains from each of the two clusters of ST321 identified in that facility (Harrand et al., 2020). The five ST321 strains from Plant A differed from each other by 16–114 wgMLST alleles while the cold-smoked salmon strains differed from each other by 25–189 wgMLST alleles, indicating somewhat higher diversity (**Supplementary Table 2**). This could be caused by the longer time period between these strains (17 years) (Harrand et al., 2020) as compared to the Plant A ST321 strains (2 years). Interestingly, some of the Plant A and cold-smoked salmon plant ST321 strains were more closely related to each other than to their ST321 counterparts from the same plant (**Supplementary Table 2**). The six cold-smoked salmon plant strains had 252 shared alleles that differed from their counterparts in Plants A or B (**Supplementary Table 3**). However, none of these alleles were unique to the cold-smoked salmon plant, as they were encountered in multiple strains from other sources (**Supplementary Table 4**).

One unexpected finding was that strains of diverse STs from Plant C had a higher incidence of new alleles based on BIGSdb wgMLST among their plant-specific alleles, with 66/84 (78.6%) of the Plant C-specific alleles being novel as compared to 8/34 (23.5%) and 61/80 (76.3%) for Plants A and B, respectively (**Supplementary Table 4**). This indicates higher diversification of strains from Plant B and C than from Plant A. This may reflect longer persistence and possibly greater or different selective pressures in Plants B or C, leading to the establishment of a greater number of mutations than in Plant A, or possibly the introduction of novel strains in this particular facility, as speculated earlier for ST5 in Plant C.

Premature Stop Codons in *inlA* Are Clonally Distributed and May Serve as FPE-Specific Markers

Internalin A (*inlA*) premature stop codon (PMSC) mutations leading to truncations and failure of InlA to anchor to the cell wall have been previously shown to be a frequent feature of serotype 1/2a, 1/2b, and 1/2c strains from processing plants, while remarkably uncommon in serotype 4b (Nightingale et al., 2005; Ward et al., 2010; Kathariou et al., 2017; Hurley et al., 2019). In addition, they are uncommon, regardless of serotype, in *L. monocytogenes* from natural environments, e.g., water, or from

clinical sources (Gorski et al., 2016; Kathariou et al., 2017; Hurley et al., 2019). Truncation of *InlA* is associated with attenuated virulence in animal models (Nightingale et al., 2005; Kathariou et al., 2017; Disson et al., 2021). We found that *inlA* PMSCs were ST-specific and not randomly distributed among the sequenced FPE strains. Specifically, they were only encountered in seven of the nine strains of ST5 (serotype 1/2b; T635) and, as previously reported (Brown et al., 2021), in all six ST321 strains (serotype 1/2a; T730) (**Supplementary Table 1; Figure 2**).

Food-derived ST5 isolates from Poland and China were previously reported to harbor *inlA* PMSCs (T535, T606, or T753) (Chen et al., 2020a; Kurpas et al., 2020). Our panel of nine ST5 strains consists of eight from Plant A and one from Plant C. The T635 PMSC was noted in seven of the Plant A strains, while the remaining Plant A strain and the ST5 strain from Plant C, as well as the closely-related ST552 (CC5) strain from Plant B, encoded full-length *InlA* (**Supplementary Table 1; Figure 2**). This T635 *InlA* PMSC appears to be novel, not previously reported in a study of 501 *L. monocytogenes* strains from processing facilities and RTE foods, despite 11 different *InlA* truncation locations identified among 243 strains (Ward et al., 2010), or in other surveys (Nightingale et al., 2005; Chen et al., 2018; Hurley et al., 2019; Kurpas et al., 2020). Thus, it appears to be a unique and apparently widespread signature in ST5 strains from Plant A, raising the possibility that ST5 strains harboring this particular *inlA* PMSC may be attributable to this particular FPE.

The ST321 strains from Plant A and Plant B harbored the T730 *inlA* PMSC, as also reported previously (Brown et al., 2021). ST321 isolates from a variety of sources have been previously reported to harbor *inlA* PMSCs, but in other locations. Specifically, T699 was found to be a common *inlA* PMSC in ST321 strains from the cold-smoked fish processing plant in the United States discussed earlier (Harrand et al., 2020), as well as meat and vegetable processing plants in Ireland (Hurley et al., 2019). This T699 *inlA* PMSC was also found in three USDA-FSIS ST321 strains (Accession Nos. PDT000101443.2, PDT000162336.2, and PDT000208198.2) from ready-to-eat (RTE) meat products. As also noted with the T635 *inlA* PMSC in ST5, the T730 *inlA* PMSC in ST321 was not detected in previous surveys (Nightingale et al., 2005; Ward et al., 2010; Chen et al., 2018; Hurley et al., 2019; Kurpas et al., 2020). The T635 and T730 *inlA* PMSCs identified in our ST5 and ST321 isolates, respectively, may be associated with the specific facility type, i.e., turkey processing plants, potentially conferring fitness advantages to ST321 strains in these FPEs. Other, apparently widespread *inlA* PMSCs, such as T699, may offer potential fitness benefits in other types of FPEs.

Selected Plant-Specific Genomic Markers in ST321

ST321 strains constitute an especially attractive model system to showcase plant-specific genomic markers, because, in addition to the six strains from the turkey FPEs, ST321 strains have been investigated extensively from an unrelated type of FPE, i.e., a salmon processing plant (Harrand et al., 2020), as discussed above. In addition, ST321 is emerging as a dominant ST among

food and FPE-derived isolates of *L. monocytogenes* (Hurley et al., 2019; Harrand et al., 2020; Matle et al., 2020; Cooper et al., 2021) and is noteworthy for high prevalence of BC resistance (Brown et al., 2021; Cooper et al., 2021). Therefore, ST321 strains from the turkey and salmon FPEs (Harrand et al., 2020) and other sources were investigated further for genomic content in a specific chromosomal hotspot located between *lmo301* and *lmo305* (Lee et al., 2012). A conserved type IV Mrr restriction-modification (RM) system was detected in this chromosomal hotspot in all five ST321 strains from Plant A and all six from the salmon FPE, as well as in the three USDA-FSIS strains mentioned above. Interestingly, however, the ST321 strain from Plant B lacked this RM system at this genomic location and instead harbored a gene putatively annotated as a lipoprotein (data not shown). Thus, the absence of the type IV Mrr RM system at this genomic hotspot appears to be unique to ST321 from Plant B. Furthermore, we previously showed that the Plant B ST321 strain was susceptible to the panel of wide-host-range lytic phages that we employed, while all five ST321 strains from Plant A were resistant and lacked either *N*-acetylglucosamine or rhamnose from the teichoic acid of the cell wall, thus preventing phage adsorption (Brown et al., 2021). The extent to which the type IV Mrr RM system may also contribute to phage resistance in the plant A strains of ST321 remains to be elucidated. These phage-resistant strains also harbored the same SNP (nt599, A to T) in *lmo1084*, a dTDP-4-dehydrorhamnose reductase involved in rhamnose wall teichoic acid glycosylation (Brown et al., 2021). Thus, in addition to the absence of the type IV Mrr RM, this particular SNP in *lmo1084* may serve as an excellent plant-specific signature for ST321 originating from Plant A vs. Plant B (**Supplementary Table 3**).

Plant-Specific Prophages Can Supplement Other Genomic Features as Plant-Specific Traits

The *comK* prophage junction fragments and prophage sequence content have been previously shown to be promising tools to track *L. monocytogenes* to individual FPEs, potentially reflecting plant-specific adaptations (Verghese et al., 2011; Kwon et al., 2020). STs encountered in multiple processing plants (**Table 1**) were screened for possible plant-specific prophages. A 43-kb prophage was found in the Plant B ST321 strain L1624a that was absent from the ST321 strains from Plant A or any of other ST321 strains that were examined, including those from the salmon FPE and the USDA-FSIS strains. Analysis via NCBI BLAST revealed no close matches to this prophage, with the best match being *Listeriophage LP-101* (Accession KJ094023.1, query coverage 67%, nucleotide identity 98.23%), classified as a temperate *Siphoviridae* with a genome size of 43.7 kb. Thus, in addition to the absence of the type IV Mrr RM, the presence of this LP-101-like 43-kb prophage in ST321 may constitute an additional molecular trait that may suggest Plant B origin for ST321 strains. Interestingly, as discussed earlier the ST321 strains from Plants A and B belong to the same SNP cluster (PDS000000366.424) (**Supplementary Table 1**), in spite of the difference in the plant-associated molecular traits. This, along

with their shared and unique *inlA* PMSC (T730), suggests that these strains likely have a recent common ancestor and then diverged in response to specific FPE-related selective pressures in Plant B.

Plant-specific differences in prophage content were also noted with ST5 strains, with two distinct prophages found only in the ST5 strain from Plant C (SK197), but absent from all eight ST5 strains of Plant A and all 12 strains of meat origin in Ireland (Hurley et al., 2019). These two prophages identified in the ST5 strain from Plant C are 40-kb and 43-kb in size. The 40-kb prophage matches best (Accession DQ003642.1, query coverage 64%, nucleotide identity 95.23%) with A006 (38-kb temperate *Siphoviridae* phage) while the 43-kb prophage matches best with LP-101 (Accession KJ094023.1, query coverage 70%, nucleotide identity 96.63%), which is 43 kb as discussed above. In contrast to STs 5 and 321, no plant-specific prophages were found among ST6 strains which were also derived from multiple plants (A, B, C) or other STs isolated from more than one plant. In comparison to prophages for which gain or loss in food processing facilities is commonly observed in *L. monocytogenes* (Muhterem-Uyar et al., 2018; Harrand et al., 2020; Yang et al., 2020), SNPs are stable and likely to be encountered in all genomes, and can therefore better provide permanent plant-specific signatures to distinguish between strains from different plants. Nonetheless, our findings suggest that for certain STs, such as ST321, prophage presence and content, as well as the hotspot-associated type IV Mrr RM system discussed above, are promising genomic traits for plant-specific differentiation.

Adaptive Traits Encountered in Different FPEs

Phage resistance can be mediated by multiple mechanisms ranging from single-nucleotide polymorphisms (SNPs) in relevant genes to dedicated systems including RM systems, bacteriophage exclusion (BREX) systems, clustered regularly interspaced short palindromic repeats (CRISPR) and prophages (Kim et al., 2012; Di et al., 2014; Goldfarb et al., 2015; Bondy-Denomy et al., 2016; Brauge et al., 2018; Hupfeld et al., 2018; Ofir et al., 2018). SNPs in specific genes have been previously shown to confer phage resistance through loss of wall teichoic acid substituents under laboratory selection for phage resistance as well as in FPE-derived strains (Denes et al., 2015; Brown et al., 2021), and can serve as candidates for plant-specific signatures.

As described earlier, the FPE strains in our panel were analyzed for their resistance profiles to a panel of four wide-host-range lytic phages (A511, P100, 20422-1, and 805405-1) (Kim et al., 2008; Klumpp and Loessner, 2013). Phage resistance was encountered in several STs and the resistance profile tended to be the same for strains from the same plant, with the notable exception of ST5, 391 and 554, where multiple resistance profiles were noted among strains from Plant A (Supplementary Table 1). As described previously, in ST321 phage resistance was encountered in all five strains from Plant A but not in the ST321 strain from Plant B (Brown et al., 2021). The Plant A strains lacked either *N*-acetylglucosamine or rhamnose from the teichoic acid of the cell wall and

did not share a common SNP conferring phage resistance (Brown et al., 2021). Phage resistance profiles of ST3 and ST7 (serotype 1/2b and 1/2a, respectively) also differed between plants (Supplementary Table 1).

Since phage resistance appears to be common among serotype 1/2a, 1/2b, and 1/2c strains from FPEs (Kim et al., 2008), its general usefulness as a facility-specific trait is likely to be limited. This is also reinforced by the fact that phage resistance is rare in serotype 4b, with the notable exception of the temperature-dependent resistance exhibited by ST6 strains (Kim et al., 2008, 2012). Nonetheless, phage resistance data can contribute to our currently limited understanding of the distinct adaptive traits of strains of a certain ST in response to FPE-specific selective regimes which, among other factors, can involve the abundance and composition of the phage community.

Additional phenotypic traits that were investigated included resistance to BC and to the heavy metal cadmium. As discussed above, 43% of the *L. monocytogenes* strains in our panel were resistant to BC, and BC resistance was always accompanied by resistance to cadmium; the reverse was not true, and we identified several BC-susceptible but cadmium-resistant strains (Figure 2; Supplementary Table 1). All BC-resistant strains in the panel harbored the *bcrABC* cassette originally identified on plasmid pLM80 (Elhanafi et al., 2010; Dutta et al., 2013). Two cadmium resistance determinants, *cadA1* and *cadA2*, were detected among the cadmium-resistant strains (Supplementary Table 1). When present, *bcrABC* was accompanied by *cadA1* ($n = 8$), *cadA2* ($n = 6$) or both *cadA1* and *cadA2* ($n = 8$) (Supplementary Table 1). All 26 FPE strains harboring *cadA1* shared the same novel allele (new#1) (Supplementary Table 1). Two different *cadA2* alleles were found among the 24 FPE strains, with most ($n = 22$) strains harboring the same allele and the remaining two harboring an alternative allele, with only one SNP difference. Both of the latter strains (#22 and 34-6a) were from Plant A but belong to different STs, ST506 and ST6, respectively (Supplementary Table 1), and the shared *cadA2* allele that they harbor may thus reflect acquisition of this determinant within this specific facility. Collectively, *cadA* alleles are extremely conserved and do not have the diversity required to yield plant-specific signatures.

Of the strains harboring *bcrABC*, all but one had the same allele for both *bcrA* and *bcrB*, while 3 alleles were detected among these strains for *bcrC* (Supplementary Table 1). The *bcrC* alleles appeared to be ST-specific and not associated with individual processing plants (Supplementary Table 1). Even though these resistance determinants are of limited use as plant-specific markers, information on their presence and variation can enhance our understanding of the FPE ecology and adaptations of *L. monocytogenes*.

As indicated earlier, the two sequenced *L. welshimeri* strains (266a-1 and 34-3a) appeared to be persistent in Plant A and were resistant to BC and cadmium, harboring the same *bcrABC* alleles found in *L. monocytogenes* strains, as well as *cadA2*, which frequently accompanied *bcrABC* in *L. monocytogenes* in the current study (Supplementary Table 1) as well as in larger surveys of *L. monocytogenes* isolates from these turkey processing plants (Mullapudi et al., 2010). These *L. welshimeri* strains also

harbored the *cadA2* allele encountered in the majority of *cadA2*-positive *L. monocytogenes*.

Truncations and Internal Deletions of Major Virulence Determinants

Investigation into potential variation in *Listeria* pathogenicity island-1 (LIPI-1) genes (Vázquez-Boland et al., 2001) revealed that *prfA*, *plcA*, *hly*, *mpl*, *plcB*, and *orfX* as well as *htp*, which is tightly controlled by PrfA (Chico-Calero et al., 2002), encoded full-length proteins in all 54 strains (**Supplementary Table 1**). However, conserved internal deletions (nt 854–959) in *actA* were found in diverse strains, including those of ST1, 5, 552, and 391 (**Supplementary Table 1**). This 105-nt internal deletion kept *actA* in frame but shortened it by 35 amino acids (AA), from 639 to 604 AA. In strains with full-length *actA*, such as EGD-e, a 35-AA sequence (PPPTDEELRLALPETPMLLGFNAPATSEPSSEFFP) is found twice in tandem but only once in strains containing this internal deletion. This same internal deletion was reported in ST1 isolates from ruminant rhombencephalitis (Dreyer et al., 2016). FPE isolates harboring *actA* internal deletions were also identified in all 12 meat-derived ST5 isolates from Ireland (Hurley et al., 2019), as well as in ST1 and ST5 isolates from illegally-imported food products in Europe (Rychli et al., 2018). Our analysis of several additional strains of ST1 ($n = 9$), ST5 ($n = 16$), and ST391 ($n = 4$) from diverse sources, including F2365 (ST1) and 2011L-2858 (ST5), indicates that this internal deletion and *actA* shortening is a clonal feature (**Supplementary Table 5**). Genome Comparator using PasteurMLST BIGSdb showed ST-specific *actA* allele conservation among all analyzed strains of ST1 (allele 56), ST5 (allele 120), and ST391 (allele 126) (**Supplementary Table 5**).

Major virulence determinants outside of LIPI-1 include *inlA* and *inlB*. As discussed earlier, PMSCs in *inlA* were found to be conserved (T730) in all ST321 strains from Plants A and B, and were also encountered and conserved (T635) in seven of the eight ST5 strains from Plant A (**Supplementary Tables 1, 5**). Interestingly, shortening of *inlB* was also found in the same seven Plant A ST5 strains that had PMSCs in *inlA* (**Supplementary Table 5**). This *inlB* shortening was due to internal deletions of nt 1,068–1,209 causing shortening from 630 to 583 AA (**Supplementary Table 1**). The deletion was conserved in all seven strains, and these strains harbored an identical *inlB* allele (allele 170) which was also encountered in several other strains of ST5 from other sources (**Supplementary Table 5**).

The concurrent presence of PMSCs in *inlA* and internal deletions in *inlB* in these Plant A strains, together with the internal deletion in *actA*, suggest selection for FPE adaptations that may promote environmental persistence while also potentially causing attenuated virulence. It was noteworthy that the ST5 strains with concurrent PMSCs in *inlA* and internal deletions in *inlB* also tended to be resistant to all four lytic wide-host-range phages that were tested (**Supplementary Table 1**). These and other aspects of environmental adaptation of these strains remain to be further investigated. In addition to the

above-discussed strong association of *inlA* PMSCs with FPE and food origin in serogroup 1/2 strains (Kathariou et al., 2017), ActA truncation in strains of diverse STs has also been found to be more commonly encountered in FPE and food-derived isolates (190/810, 23.5%) than clinical isolates (10/332, 3.0%) (Painset et al., 2019). Such mutations in major virulence factors in FPE-derived strains highlight the specialization of *L. monocytogenes* to the FPE at the potential cost of virulence.

FPE strains were also screened for the two pathogenicity islands LIPI-3 and LIPI-4. LIPI-3 was detected in 18/31 lineage I strains, specifically the serotype 4b strains of ST1, 6, and 554 and the serotype 1/2b strains of ST3, 506 and 550, and not among any of the 23 lineage II strains (**Supplementary Table 1**), in agreement with the previously-reported strong association of LIPI-3 with lineage I (Cotter et al., 2008; Kathariou et al., 2017). Within the Lineage I FPE strains, presence of LIPI-3 was conserved among strains of the same ST (**Supplementary Table 1**). Despite LIPI-3 being associated with lineage I, it was not detected among any of the ST5/ST552 (CC5) strains from our study (**Supplementary Table 1**), similarly to findings from other investigations of ST5 (Hurley et al., 2019; Chen et al., 2020a). LIPI-4 was uncommon in our panel with only two of the 54 strains harboring LIPI-4, i.e., the serotype 1/2b strains 230a-3 and #22 of ST87 and 506, respectively (**Supplementary Table 1**). LIPI-4 rarity is in agreement with the fact that our panel lacked major clones that have been known to harbor it, such as ST4 and ST382 (Maury et al., 2016; Moura et al., 2016; Kathariou et al., 2017; Chen et al., 2020b).

WGS Reveals Diversity of Strains From the Same FPE Sample, Even Among Strains of the Same Serotype and ST

FPEs are commonly contaminated by multiple strains and clones of *L. monocytogenes* (Hurley et al., 2019; Stoller et al., 2019; Yang et al., 2020), and selective enrichments are typically used to isolate *L. monocytogenes* from environmental samples (Johnson, 1998). However, this also creates a challenge in ascertaining how many colonies should be isolated from a *Listeria*-positive selective enrichment. While many colonies are likely to result from identical daughter cells, others may be distinct, resulting from the presence of multiple *Listeria* strains in the same sample (Ryser et al., 1996).

Among the 54 strains analyzed here, we noted examples where highly diverse strains were obtained from the same environmental swab. Specifically, samples 34, 201, and 210 yielded several distinct strains (**Supplementary Table 1; Figure 2**). Of special note was sample 34 from Plant A, which yielded four distinct strains of *L. monocytogenes*, two of ST5 (serotype 1/2b) and two of ST6 (serotype 4b), as well as one strain of *L. welshimeri*. The pronounced diversity in this sample, including different *L. monocytogenes* serotypes and two different *Listeria* species, highlights the importance of isolating multiple colonies from a single enrichment. Furthermore, standard multiplex PCR-based serotyping cannot reveal diversity among strains of the same serotype designation.

Even more noteworthy was the finding that diversity may remain hidden even upon further genotyping such as afforded by the seven-locus MLST scheme. For instance, the two serotype 4b strains from sample 34 (34-2b and 34-6a) were both of ST6 but WGS analysis revealed that they differed by 271 alleles based on wgMLST, with 116 of these belonging to the core genome. One of these strains, 34-6a, was in fact the most distant to all other ST6 strains from Plant A, B, or C combined (**Supplementary Table 2, Figure 2**). This strain belongs to SNP cluster PDS000025549.3 together with three other strains, all from human listeriosis in the United States in 2017 and 2018, while strain 34-2b was not affiliated with a SNP cluster (**Supplementary Table 1**). In addition to their noticeable genomic divergence, these two ST6 strains from sample 34 differed in their BC and cadmium resistance profiles (**Supplementary Table 1**) with 34-6a being resistant to both BC and cadmium while 34-2b was susceptible to both compounds (**Supplementary Table 1**). Both strains were resistant to all tested wide-host-range lytic phages at 25°C (**Supplementary Table 1**), reflecting the temperature-regulated RM system unique to ST6 (Kim et al., 2012), but 34-6a was previously found to be resistant to wide-host-range lytic phages when tested at 37°C, while 34-2b was susceptible at this temperature (Kim et al., 2008). Even though a previous analysis of ST6 strains indicated clustering of isolates from the same facility (Kwon et al., 2020) our data clearly show that this is not always the case, with pronounced divergence between ST6 strains even from the same environmental sample.

The two ST5 strains (34-2a and 34-4b) from sample 34 were more closely related, with identical T635 *inlA* PMSCs, common internal deletions in *inlB*, shared *bcrABC* and *cadA* alleles and also shared phage resistance profiles. However, they still differed by 170 alleles based on wgMLST, with 57 of these in the core genome and did not belong to the same SNP cluster (**Supplementary Tables 1, 2**). These strains were also placed in clearly distinct branches of the ST5/ST552 (CC5) clade in the phylogenetic tree (**Figure 2**). While one (34-2a) is not affiliated with a SNP cluster at all, highlighting its genetic novelty, the other (34-4b) belongs to a small three-strain SNP cluster (PDS000083092.1) containing only two additional Plant A strains of ST5 (**Supplementary Table 1**), and was placed together with these strains in the phylogenetic tree (**Figure 2**). Even though traditional tests such as multiplex PCR for serotype designations, seven-locus MLST and phage resistance profiles would not be sufficient to differentiate between 34-2a and 34-4b from each other, WGS clearly identified these as distinct strains.

Significantly, as noted above the ST6 strain 34-6a belongs to a SNP cluster which includes three human clinical strains, emphasizing the importance of not missing such a strain, should an epidemiological investigation need to be carried out with a specific FPE. Such findings illustrate the need to isolate and analyze via WGS multiple colonies from a selective enrichment. Not doing so will increase the risk of failing to detect clinically-relevant and potentially novel strains that may not cluster well with other members of the same ST (**Supplementary Table 1**).

CONCLUSIONS

Our study of 54 *L. monocytogenes* strains from turkey processing plants in the United States reveals the potential of WGS-based analysis of FPE-derived strains to identify facility-specific signatures and to inform aspects of FPE sample analysis. The value of WGS-based analysis was even further enhanced by integration of the WGS data with phenotypic assays of special relevance to the FPE ecosystem such as sanitizer resistance and resistance to lytic phages, with potential for novel insights on the FPE ecology and evolution of *L. monocytogenes*. We identified plant-specific alleles and other WGS signatures that may prove useful in attributing strains to the FPE of their origin. Additional investigations are needed to assess the potential biological relevance of those plant-specific alleles. We also demonstrated that a single FPE sample may harbor diverse strains whose genetic distinctness would go unnoticed with lower-resolution tools such as serotyping or even the seven-locus MLST scheme. Better understanding of such intra-sample diversity is critically needed not only for our overall understanding of *Listeria's* FPE ecology but also to help inform the number of *L. monocytogenes* isolates that may need to be characterized from a single *Listeria*-positive FPE sample. This would maximize the usefulness of environmental sampling not only for epidemiological investigations but also in monitoring contamination patterns and mitigation effectiveness within a specific FPE.

Strains of the same ST may harbor novel mutations and other genomic traits, including PMSCs in *inlA*, deletions in *inlB*, sanitizer resistance genes and prophages that may be used to differentiate among strains from different FPEs. While *InlA* truncations were found to be not randomly distributed throughout *L. monocytogenes* and common only in certain STs, the location of the PMSC may be unique to certain plants, though the extent to which such uniqueness may also reflect the specific type of FPE (i.e., turkey slaughter and processing plants) remains to be ascertained. The absence of a specific genetic feature may also serve as a plant-specific marker, as demonstrated by our finding that for certain STs the lack of specific accessory genome components, such as a type IV Mrr system and prophages, were unique to certain processing plants. These plant-specific genetic traits have the potential to be used to track *L. monocytogenes* movement through the food chain. Future work will be necessary to examine more sequenced strains, from diverse FPEs and regions, in order to further elucidate the distribution of plant-specific genetic traits in *L. monocytogenes*. The findings from the current study will be useful in designing and informing such future studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: PRJNA215355].

AUTHOR CONTRIBUTIONS

SK and PB: conceptualization. PB: formal analysis and writing—original draft. SK, ER, CL, L-AJ, and JE: funding acquisition. PB, SK, YC, RS, CS, and EB: investigation. SK: project administration. PB, YC, and SK: visualization. PB, SK, YC, RS, CP, L-AJ, JE, ER, CL, and CS: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work was partially supported by a National Alliance for Food Safety and Security grant (USDA) and by award

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- 2018-07464 from the USDA National Institute of Food and Agriculture.

ACKNOWLEDGMENTS

We would like to acknowledge the CFSAN GenomeTrackr sequencing team as well as Julie S. Sherwood and Sheri Kernodle for their technical assistance in this project. We thank Sangmi Lee for sharing her insights on the genomic hotspot region.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2021.742353/full#supplementary-material>

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