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Surveillance and genetic characterization of *Listeria monocytogenes* in the food chain in Montenegro during the period 2014–2022

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Introduction: *Listeria monocytogenes* is an ubiquitous foodborne pathogen that represents a serious threat to public health and the food industry.

Methods: In this study Whole Genome Sequencing (WGS) was used to characterize 160 *L. monocytogenes* isolates obtained from 22,593 different food sources in Montenegro during the years 2014–2022.

Results: Isolates belonged to 21 different clonal complexes (CCs), 22 sequence types (STs) and 73 core genome multilocus sequence types (cgMLST) revealing a high diversity. The most prevalent STs were ST8 ($n = 29$), ST9 ($n = 31$), ST121 ($n = 19$) and ST155 ($n = 20$). All isolates carried virulence genes (VGs), 111 isolates carried mobile genetic elements (MGEs) (ranging from 1 to 7 MGEs) and 101 isolates carried plasmids (ranging from 1 to 3 plasmids). All isolates carried the intrinsic resistance genes *fosX* and *lin*. None of the isolates carried acquired antimicrobial resistance genes (ARGs).

Discussion/conclusion: Continuous monitoring and surveillance of *L. monocytogenes* is needed for improving and ameliorating the public health.

KEYWORDS

Listeria monocytogenes, whole-genome sequencing, food products, antimicrobial resistance genes, Montenegro

1 Introduction

Listeria monocytogenes (*L. monocytogenes*) is the etiological agent of human and animal listeriosis (Chlebicz and Slizewska, 2018), with three major invasive clinical presentations in humans: bloodstream infection, infection of the central nervous system, and maternal foetal listeriosis (Hernandez-Milian and Payeras-Cifre, 2014). Strains of *L. monocytogenes* are often isolated from ready-to-eat (RTE) food, including meat, milk, dairy, fish and other seafood, but also ice cream, fresh frozen end processed vegetables, fruits, composite vegetables food and spices (Gambarin et al., 2012; Mateus et al., 2013). This pathogenic bacterium is ubiquitous in different habitats, such as: soil, water, vegetation, animal feed, farms and industrial environment (Kimura, 2006). As a contaminant of food production facilities, *L. monocytogenes* can be found in raw materials, in the environment of the facilities itself, on equipment and the final product. From all these spots, *L. monocytogenes* may spread throughout the facility via aerosols, personnel, food workflows and contaminated contact materials leading to its

persistent presence and frequent resistance to hygiene measures for cleaning and disinfection (Zuber et al., 2019). Along with the ability to adapt to different niches and tolerate adverse environmental conditions better than all other non-sporulating bacteria (Liu et al., 2002), the ability of *L. monocytogenes* to form biofilms (Lakicevic and Nastasijevic, 2016) is another property which leads to long-term persistence and repeated cross-contamination of food products (Ferreira et al., 2014).

Listeria monocytogenes monitoring programs using molecular sub-typing techniques have been helpful in identifying persistent isolates within whole food chains (Leong et al., 2017). Microbial whole-genome sequencing (WGS) provides identification, characterization and subtyping of a microorganism at the ultimate level of resolution (Ronholm, 2018) making it a superior molecular typing method for monitoring foodborne outbreaks. *L. monocytogenes* is differentiated into 13 serotypes and four phylogenetic lineages of which most human listeriosis cases are associated with *L. monocytogenes* from lineage I, while *L. monocytogenes* from lineage II is commonly found in natural and farm environments (Orsi et al., 2011; Pyz-Lukasik et al., 2022; Benjamin et al., 2023).

The abuse or misuse of antibiotics, in both human and veterinary medicine, has led to an increase in the number of multidrug-resistant strains in recent decades (Maung et al., 2023). Although *L. monocytogenes* is normally susceptible to many antibiotics, some multidrug-resistant strains of *L. monocytogenes* have been isolated from food and the environment (Maung et al., 2023), following its initial discovery in France in 1988 (Poyart-Salmeron et al., 1990). In the food industry, the resistance of *L. monocytogenes* strains to disinfectants complicates the control and eradication of the bacteria in environments where hygiene is crucial (Oloketuyi and Khan, 2017). This poses a significant challenge that many groups address on a daily basis. Even though major advances in this field have been made, it remains crucial to develop safe and effective disinfectants against *L. monocytogenes*.

Virulence factors, encoded by virulence genes (VGs), are the key factor for *L. monocytogenes* to adapt and spread in the environment (Disson et al., 2021; Quereda et al., 2021). *Listeria* Pathogenicity Islands (LPIs) are regions in the *L. monocytogenes* genomes playing a crucial role in virulence and pathogenicity (Quereda et al., 2021; Wiktorczyk-Kapischke et al., 2023). The four known LPIs (LPI-1 – LPI-4) contain genes involved in various stages of infection like adhesion, invasion, and evasion of the immune response of the host. The key virulence factors encoded by LPIs are internalins, listeriolysins and phospholipases (Wiktorczyk-Kapischke et al., 2023). LPI-1 is essential for the intracellular lifecycle of *L. monocytogenes* and encodes the virulence factors InlA, InlB and listeriolysin O (LLO). LPI-2 enables the movement within host cells and encodes ActA. LPI-3 is found in certain strains and responsible for the increased virulence (Pyz-Lukasik et al., 2022), carrying *lls* genes (*llsA*, *llsB*, *llsD*, *llsG*, *llsH*, *llsP*, *llsX*, and *llsY*) which encodes listeriolysin S (LLS), a hemolytic/cytolytic factor impacting potential virulence. LPI-4 encodes proteins involved in carbohydrate metabolism contributing to the ability of *L. monocytogenes* to survive and persist in the environment (Wiktorczyk-Kapischke et al., 2023). ST1 and ST4 *L. monocytogenes* have been previously reported of carrying both, LPI-3 and LPI-4 and are therefore regarded as hypervirulent clones (Shen et al., 2022). On the other hand, several types of Mobile Genetic Elements (MGEs) like transposons, plasmids and bacteriophages have

been identified, that contribute to resistance and enhanced virulence (Parra-Flores et al., 2022).

In this study, we utilized WGS to characterize *L. monocytogenes* isolates collected over an eight-year period (2014–2022) as part of pathogen monitoring in Montenegro's food industry with the aim to examine similarity and possible common origin sources between isolates. The absence of documented cases of human listeriosis in Montenegro underscores the importance for greater attention regarding this pathogen. Identification and association of identical/similar isolates circulating through the food chain will enhance monitoring efforts significantly. This approach can facilitate the establishment of more effective surveillance programs, helping food operators to successfully meet food safety regulations. Finally, these measures are crucial to improve consumer safety and public health.

2 Materials and methods

2.1 Origin and sample collection

A total of 22,593 different samples (environmental/food/other origin) were sampled from production facilities, retail shops and control at the borders. The samples were collected by analysing different food products of animal origin, i.e., raw milk and fresh meat during a period of 8 years (2014–2022), through programs of regular monitoring of the presence of *L. monocytogenes* in products on the market (8,698 samples), through programs of official controls (7,373 samples), but also within the framework of regular self-control by producers and food business operators (6,522 samples). The sampling that was done as part of regular monitoring programs as well as official control was carried out in accordance with the sampling plan in compliance with the principles of Commission Regulation (EC) no. 2073/2005 including the sampling of five units of the same production lot. The plan of sampling and testing of products carried out by manufacturers is most often also based on Commission Regulation (EC) no. 2073/2005. Tissue swabs were collected from different surfaces, including those in contact with food in production facilities ($n=798$), the surface around the facility ($n=55$) and workers' hand ($n=115$). Additionally, samples from raw milk ($n=14$) and fresh meat ($n=34$) were collected. For downstream analysis using Whole Genome Sequencing (WGS), one *L. monocytogenes* colony per positive sample was selected.

2.2 Cultural detection of *Listeria monocytogenes*

Samples were tested according to the EN ISO 11290-1 standard (ISO, 2022). Thus, samples were enriched (first in half Fraser broth at 30°C for 24h, then in Fraser broth at 35°C for 48h) and were then plated on Agar *Listeria* according to Ottaviani and Agosti (ALOA, Oxoid Ltd., Basingstoke, United Kingdom) and on Palcam agar (Oxoid Ltd., Basingstoke, United Kingdom) and incubated at 37°C for up to 48h. Colonies which retained typical *L. monocytogenes* appearance were transferred to Tryptone Soya Agar plates supplemented with 0.6% yeast extract (TSYEA, Oxoid, Basingstoke, United Kingdom) and incubated at 37°C for 24h. All isolates were identified with API *Listeria* system (BioMérieux SA, France) and all

were stored in Brain Heart Infusion Broth (BHI, Oxoid Ltd., Basingstoke, United Kingdom) with 15% glycerol at -80°C . The isolates were refreshed once, every year, and then returned to storage at -80°C .

2.3 DNA extraction and whole genome sequencing

For whole genome sequencing (WGS) bacterial isolates were grown on blood agar plates supplemented with 5% sheep blood (COS) (Biomerieux, Vienna, Austria) at $37^{\circ}\text{C}/24\text{h}$ /aerobiosis. High molecular weight DNA was isolated from overnight cultures using the KingFisher Apex System (ThermoFisher, Vienna, Austria) with the MagMAX Viral/Pathogen Ultra Nucleic Acid Isolation Kit (ThermoFisher, Vienna, Austria) following the manufacturer's instruction for Gram-positive bacteria with the following modifications: 2 hours of lysis with $60\mu\text{L}$ enzyme mix. DNA was quantified using Dropsense 16 (Trinean NV/SA, Gentbrugge, Belgium) and Qubit Flex Fluorometer (ThermoFisher, Vienna, Austria) with 1x dsDNA High Sensitivity (HS) kit (ThermoFisher, Vienna, Austria). Genomic libraries were prepared using the DNA Illumina Prep kit (Illumina, San Diego, United States) according to the manufacturer's instructions. Paired-end sequencing was performed on a NextSeq2000 instrument (Illumina, San Diego, United States) with a read length of $2 \times 150\text{bp}$ (Illumina, San Diego, CA, United States) aiming for a minimum coverage of 30-fold.

2.4 Sequence data analysis

Raw reads were *de novo* assembled using SPAdes (version 3.11.1) (Bankevich et al., 2012). Contigs were filtered for a minimum coverage of 5 and a minimum length of 200 base pairs using SeqSphere+ software v8.5.1 (Ridom, Münster, Germany).

Subtyping of isolates was conducted in SeqSphere+ software v8.5.1 with classical multilocus sequence typing (MLST) based on the seven housekeeping genes as described by Salcedo et al. (2003), Clonal Complex (CC) (Ragon et al., 2008), core genome (cg)MLST (Werner et al., 2015) using a cluster threshold of 10 allelic differences.

Information on serogroups was extracted from genome data based on the 5-plex PCR (*lmo118*, *lmo0737*, *ORF2110*, *ORF2829* and *prs*) as described (Michel et al., 2004; Lee et al., 2012).

NCBI AMRFinder+ v3.11.2. was used with the EXACT method at 100% setting together with the BLAST alignment at $>90\%$ of protein sequences against the AMRFinder+ database to detect antimicrobial resistance genes (ARG) (Michael et al., 2019). The Virulence Factor Database (VFDB) (<http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Listeria>; accessed on 6th June 2023) was used to detect VGs (Liu et al., 2022). Thresholds were set for the target scanning procedure to $\geq 85\%$ identity with the reference sequence and $\geq 99\%$ with the aligned reference sequence. Pathogenicity islands LIPI-3 and LIPI-4 were extracted from genome data. The CGE Mobile Element Finder v1.0.5. was used with the $<90\%$ identity and $>95\%$ alignment method to detect Mobile Genetic Elements (MGE) (Johansson et al., 2021). Chromosome & Plasmid Finder v1.0. through MOB-suite v3.1.4. available in SeqSphere+ v8.5.1. was used with the Mash Neighbor Distance >0.06 to detect plasmids (Robertson and Nash,

2018). Isolates of the most prevalent STs of *L. monocytogenes* of our study (ST8, ST9, ST121, ST155, ST204) were compared with those from pubMLST database¹ and the Austrian Reference Laboratory for Listeria, which currently includes more than 20,000 genomes.

3 Results

3.1 Detection of *Listeria monocytogenes*

Out of 22,593 samples tested, *L. monocytogenes* was detected in 160 (0.7%) of them. From each positive sample, a single colony of *L. monocytogenes* was isolated, resulting in a collection of isolates consisting of 147 isolates from Montenegro and 13 isolates from food products imported from other European countries. During 8 years of monitoring the presence of *L. monocytogenes* in the food chain, a total of 986 environmental swabs, 48 raw material samples and 21,559 RTE food samples were examined. From all 147 isolates with Montenegrin origin, 131 isolates were from food products from 19 different industries, five isolates were isolated from small farms (households) and 11 *L. monocytogenes* isolates were isolated from unknown companies from Montenegro between 2014 and 2015. In addition, 13 isolates were obtained from food imported from different European countries: France=1, Serbia=2, Italy=9 and Bosnia and Hercegovina=1 (Tables 1, 2). From the total of isolates included in the study two *L. monocytogenes* isolates were obtained from environmental swabs, eight *L. monocytogenes* isolates originated from raw material and 150 *L. monocytogenes* isolates originated from food. 80% of the isolates originated from meat (pork, beef, chicken, and mixed meat), 16% from dairy products (mozzarella, cow cheese, butter, fresh milk), 0.7%, from ready-to-eat fish, 0.7% from frozen vegetables, 0.7% from food contact surfaces in production plants, and 1.8% were of unknown origin.

3.2 Whole genome sequence based subtyping, description of clusters, and description of serogroups

WGS based characterization of the 160 *L. monocytogenes* isolates revealed a high diversity. Isolates belonged to four different serogroups [IIa ($n=105$), IIb ($n=10$), IIc ($n=36$) and IVb ($n=9$)], 21 different clonal complexes (CCs), 22 different sequence types (STs) and 73 cgMLST complex types (CTs). One hundred twenty-two isolates belonged to a total of 23 cluster and 38 isolates were singletons (Figure 1; Table 2). Number of isolates per industry ranged from one isolate to 34 isolates (Table 1).

Listeria monocytogenes isolates belonging to serogroup IIa encompassed 13 different STs (ST7, ST8, ST14, ST26, ST31, ST37, ST101, ST121, ST124, ST155, ST204, ST321, and ST451), isolates belonging to serogroup IIb encompassed 4 different STs (ST3, ST489, ST517, and ST736), isolates belonging to serogroup IIc encompassed 2 different STs (ST9 and ST580), and *L. monocytogenes* isolates

¹ https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_isolates&page=profiles

TABLE 1 Information on diversity of industries/producers, years, and sequence types (STs) in the *L. monocytogenes* isolates included in this study.

Industry (No. of isolates)	Years	Sequence type	Prevalence per industry (%)
Meat industry 1 (23)	2015	ST9	ST4 (4%)
	2016	ST9, ST121	ST7 (4%)
	2017	ST8, ST9	ST8 (13%)
	2018	ST4, ST9	ST9 (48%)
	2019	ST7, ST8	ST121 (31%)
	2021	ST8, ST9, ST121	
Meat industry 2 (34)	2016	ST8, ST9, ST37, ST121	ST8 (50%)
	2019	ST31, ST101, ST580	ST9 (10%)
	2020	ST8, ST121, ST155, ST451, ST580	ST31 (6%)
	2021	ST8	ST37 (3%)
	2022	ST8	ST101 (6%)
			ST121 (6%)
		ST155 (6%)	
		ST451 (3%)	
		ST580 (10%)	
Meat industry 3 (9)	2017	ST9, ST736	ST8 (22%)
	2020	ST8	ST9 (22%)
	2021	ST736	ST736 (56%)
Meat industry 4 (1)	2021	ST1	ST1 (100%)
Meat industry 5 (6)	2019	ST9, ST121, ST321	ST9 (50%)
	2020	ST9	ST121 (33%) ST321 (17%)
Meat industry 6 (12)	2015	ST9	ST8 (18%)
	2016	ST8, ST9, ST451	ST9 (37%)
	2017	ST121	ST37 (9%)
	2019	ST9	ST121 (9%)
	2020	ST8, ST37, ST489	ST451 (9%) ST489 (17%)
Meat industry 7 (1)	2019	ST155	ST155 (100%)
Meat industry 8 (4)	2015	ST7, ST9	ST7 (25%)
	2019	ST8	ST9 (50%) ST9 (25%)
Meat industry 9 (10)	2016	ST1, ST321	ST1 (10%)
	2019	ST8, ST121, ST321	ST8 (30%) ST121 (40%) ST321 (20%)
Meat industry 10 (2)	2018	ST121	ST37 (50%)
	2019	ST37	ST121 (50%)
Meat industry 12 (1)	2016	ST3	ST3 (100%)
Meat industry 13 (2)	2016	ST8	ST8 (50%)
	2017	ST121	ST121 (50%)
Meat industry 15 (3)	2016	ST8, ST37, ST451	ST8 (34%)
			ST37 (33%)
			ST451 (33%)

(Continued)

TABLE 1 (Continued)

Meat industry 16 (1)	2016	ST8	ST8 (100%)
Meat industry 17 (3)	2015	ST2	ST2 (100%)
Milk industry 1 (2)	2020	ST101	ST101 (100%)
Milk industry 2 (9)	2016	ST8, ST204	ST8 (11%)
	2017	ST204	ST37 (11%)
	2018	ST37, ST204	ST204 (78%)
Milk industry 3 (7)	2016	ST155	ST155 (100%)
	2017	ST155	
	2018	ST155	
Milk industry 4 (1)	2017	ST7	ST7 (100%)
Small farms (5)	2017	ST1	ST1 (20%)
	2018	ST7, ST121, ST204	ST7 (20%)
	2019	ST26	ST26 (20%)
ST121 (20%)			
		ST204 (20%)	
Unknown industries (11)	2014	ST9, ST101, ST517, ST736	ST1 (9%) ST2 (9%) ST9 (37%) ST101 (9%) ST124 (9%) ST451 (9%) ST517 (9%) ST736 (9%)
	2015	ST1, ST2, ST124, ST451	
Imported (13)			
IMP 1	2017 Bosnia	ST7	ST7 (8%)
IMP 2	2020 France	ST37	ST14 (8%)
IMP 3 and IMP 4	2018–2019 Serbia	ST14, ST155	ST37 (8%)
IMP 5	2022 Italy	ST155	ST155 (77%)

belonging to serogroup IVb encompassed 3 different STs (ST1, ST2, and ST4).

The most prevalent STs were ST8 ($n = 29$), ST9 ($n = 31$), ST121 ($n = 19$), ST155 ($n = 20$, including 9 isolates assigned to the Italian hotdog outbreak), and ST204 ($n = 9$) from the period 2015–2020 (Figure 2). The industries with the highest prevalence of isolates were Meat IND 1 ($n = 23$), Meat IND 2 ($n = 34$) and Meat IND 6 ($n = 12$).

L. monocytogenes isolates from Meat IND 1 ($n = 23$) encompassed five different STs (Table 1; Figures 2, 3). ST9 was the most prevalent with twelve isolates (52%) being detected every year from 2015–2022 (except 2019). All isolates belonged to cluster 2 (Figure 1). ST121 was the second most prevalent with seven isolates (31%). ST121 was the most prevalent type in the year 2016.

Listeria monocytogenes isolates from Meat IND 2 ($n = 34$) encompassed nine different STs (Table 1; Figures 2, 3). ST8 was the most prevalent with sixteen isolates (50%). ST8 was detected every year from 2016 to 2022 (except in 2019). All isolates belonged to cluster 1 (Figure 1).

L. monocytogenes isolates from Meat IND 6 ($n = 12$) encompassed six different STs (Table 1; Figures 2, 3). ST9 was the most prevalent

TABLE 2 Information on *L. monocytogenes* isolates belonging to different clusters included in this study.

Cluster	No. of isolates	ST	CT	Source	Industry	Year	Country
1	18	8	69	Fermented beef sausage	Meat IND 1	2017	Montenegro
			69, 73, 1358, 5250, 5503, 16828	Smoked pork sausage, prosciutto, pancetta	Meat IND 2	2016–2022	Montenegro
			16783	Prosciutto	Meat IND 6	2020	Montenegro
			16783	Pancetta	Meat IND 13	2016	Montenegro
2	11	9	16782	Fermented beef sausage	Meat IND 1	2016–2022	Montenegro
3	9	155	2760	Chicken hot dog	IMP 5	2022	Italy
4	8	204	16788	Mozzarella	Milk IND 2	2016–2018	Montenegro
5	7	121	2134, 16793	Fermented beef sausage	Meat IND 1	2016–2021	Montenegro
6	7	155	1234, 8675, 16789	Cow cheese, butter	Milk IND 3	2016–2018	Montenegro
7	7	8	5503	Smoked pork sausage	Meat IND 3	2020	Montenegro
				Fresh pork meat	Meat IND 8	2019	
				Smoked pork sausage	Meat IND 9	2019	
8	6	736	16577	Pork meat	Unknown	2014	Montenegro
					Meat IND 3	2017–2021	
9	5	9	354, 3779	Smoked pork sausage, pancetta	Meat IND 6	2015–2019	Montenegro
			1691	Smoked pork sausage	Meat IND 8	2015	
10	5	9	17054	Pancetta	Meat IND 2	2016	Montenegro
			1698	Smoked pork sausage	Meat IND 8	2015	
			1704	Fresh pork meat, unknown	Unknown	2014	
11	5	580	15473	Pork meat	Meat IND 2	2019–2020	Montenegro
12	5	101	3186	Prosciutto	Meat IND 2	2019	Montenegro
				Cow cheese, fresh milk	Milk IND 1	2020	
				Smoked pork sausage	Unknown	2014	
13	4	9	358	Smoked pork sausage	Meat IND 2	2016	Montenegro
				Smoked pork sausage	Meat IND 3	2017	
14	4	2	8598	Smoked pork sausage, unknown	Meat IND 17	2015	Montenegro
			8598	Prosciutto	Unknown	2015	
15	4	121	8919	Smoked pork sausage	Meat IND 2	2016	Montenegro
				Fresh bacon, smoked pork sausage	Meat IND 9	2019	
16	3	321	197	Mixed frozen meat	Meat IND 5	2019	Montenegro
				Pork meat	Meat IND 9	2016, 2019	
17	2	155	3856	Smoked pork sausage	Meat IND 2	2020	Montenegro
18	2	121	2907, 16784	Pancetta, prosciutto	Meat IND 5	2019	Montenegro
19	2	121	5750	Smoked pork sausage	Meat IND 10	2018	Montenegro
					Meat IND 13	2017	
20	2	9	285	Pancetta	Meat IND 5	2019	Montenegro
21	2	7	16794	Cow meat	Milk industry 4	2017	Montenegro
				Sour cream	IMP 1	2017	Bosnia and Herzegovina
22	2	489	16785	Pork meat	Meat industry 6	2020	Montenegro
23	2	31	6240	Pork meat	Meat industry 2	2019	Montenegro

with five isolates (37%) detected in 2015, 2016 and 2019. Four isolates belonged to cluster 9. One ST9 isolate differed by 1,129 alleles from cluster 9 (Figure 1).

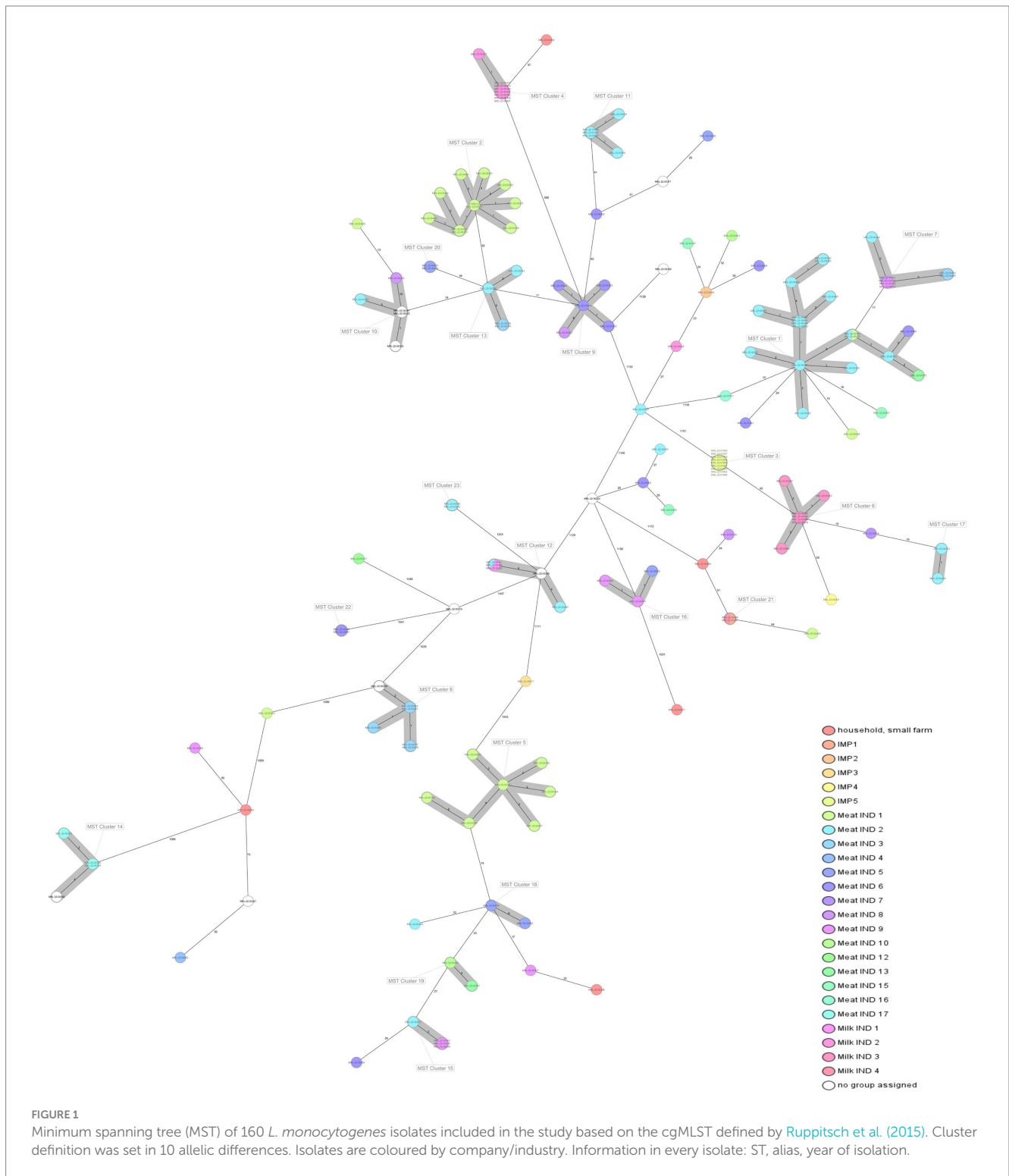
Listeria monocytogenes isolates from Milk IND 1, 2, 3 and 4 encompassed five different STs (Table 1; Figures 2, 3) from years 2016–2020. ST155 was the most prevalent with seven isolates (35% of the

ST155) in Milk IND 3. All isolates belonged to cluster 6 (Figure 1). ST204 was the most prevalent with nine isolates (100% of the total ST204) in Milk IND 2 and one small farm. All isolates belonged to cluster 4 (Figure 1).

3.2.1 *Listeria monocytogenes* ST8 (n = 29)

Twenty-nine *L. monocytogenes* ST8 isolates (18% of the total) were obtained from ten industries in Montenegro during the period 2016–2022. ST8 isolates were the most prevalent in Meat IND 2

(n = 16, 55,2% of ST8), Meat IND 13 (n = 1, 50%) and Meat IND 16 (n = 1, 100%). *L. monocytogenes* ST8 isolates were distributed to cluster 1 (n = 18), cluster 7 (n = 7) and four singletons. Cluster 1 comprised one isolate from Meat IND 1 (3.4% of the ST8), fifteen isolates from Meat IND 2 (48%), one isolate from Meat IND 6 (3.4%) and one isolate from Meat IND 13 (3.4%) with a maximum of eight allelic differences within the cluster (Figure 1). Cluster 7 comprised seven isolates differing by a maximum of three alleles. One isolate was obtained from Meat IND 2 (3.4%), two isolates from Meat IND 3



(7%), one isolate from Meat IND 8 (3.4%) and three isolates from Meat IND 9 (10%) (Figure 1). Cluster 1 and cluster 7 isolates differed by a minimum of 11 allelic differences (Figure 1). The four ST8 singletons were isolated from Meat IND 1 from 2021, Meat IND 6 from 2016, Meat IND 15 from 2016 and Meat IND 16 from 2016 (Figure 1; Supplementary Figure S1). They differed from each other by a minimum of 20 and a maximum of 35 alleles and from cluster 1 and cluster 7 isolates by a minimum of 12 and 19 alleles, respectively.

ST8 isolates from Meat IND 2 (cluster 1) were differing by 12 alleles from a salmon isolate from Poland (CC8/ST8/CT1269) (accession no. SRR975366) (Moura et al., 2016) and by 18 alleles from a human isolate from France (CC8/ST8/CT2873) (accession no. ERR1100940) (Maury et al., 2016; Moura et al., 2016).

3.2.2 *Listeria monocytogenes* ST9 (n = 31)

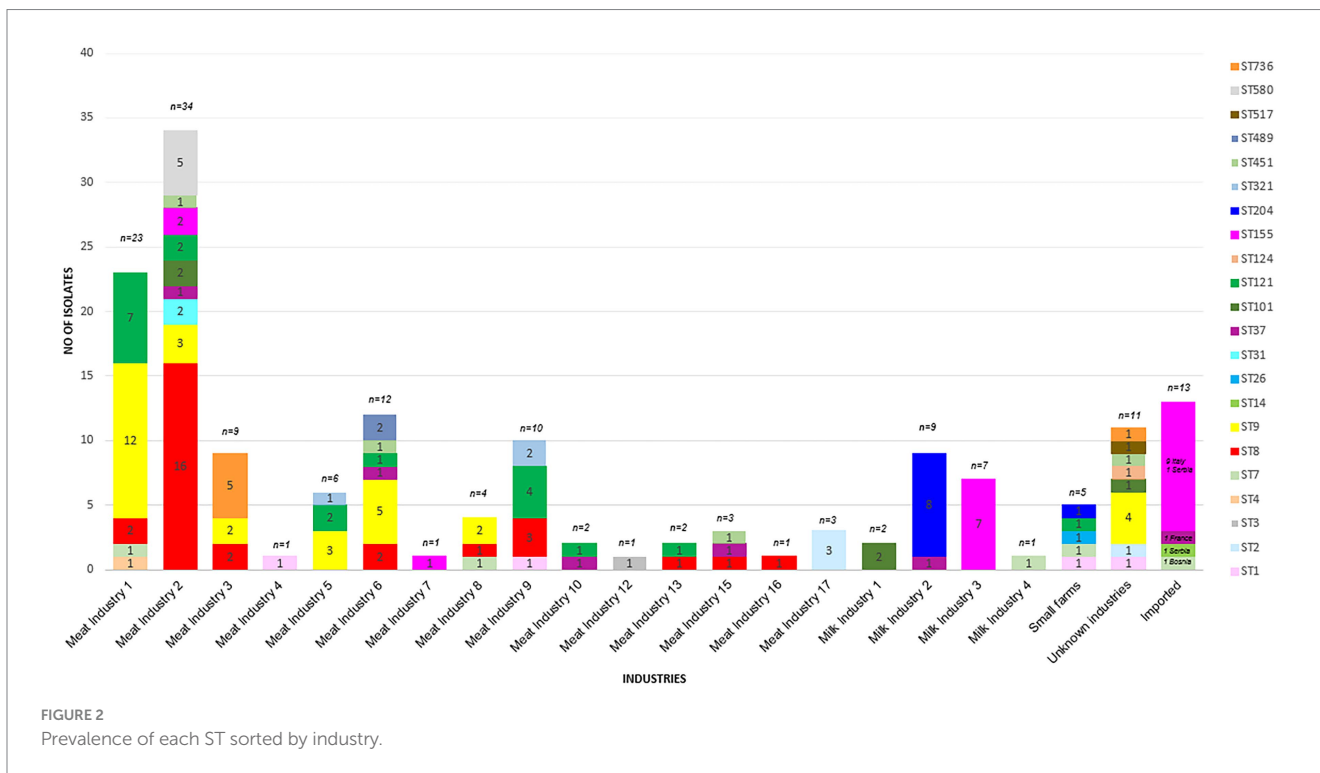
Thirty-one *L. monocytogenes* ST9 isolates (19% of the total) were obtained from ten different industries in Montenegro from the period 2014 to 2022. ST9 isolates were the most prevalent type in Meat IND 1 (n = 12, representing the 38% of ST9), Meat IND 5 (n = 3, 10%), Meat IND 6 (n = 5, 16%), and Meat IND 8 (n = 2, 6%) (Table 1; Figure 2). *L. monocytogenes* ST9 isolates were distributed to five clusters (cluster 2, 9, 10, 13 and 20) and four isolates were singletons. Cluster 2 isolates (n = 11, 35.48% of ST9) were obtained from beef meat from Meat IND 1 from 2015 to 2022, whereas isolates from the other clusters were derived from pork meat. Cluster 9 isolates (n = 5, 16.12% of ST9) were obtained from Meat IND 6 and Meat IND 8, with a maximum of eight allelic difference. Isolates within Cluster 10 (n = 5, 16.12% of ST9) were obtained from Meat IND2 (n = 1, 3.22%), Meat IND 8 (n = 1, 3.22%) and from and unknown industries (n = 3, 9.67%), with a maximum of ten allelic differences within the cluster. Cluster 13 isolates (n = 4; 12.90% of ST9) were obtained from Meat IND 2 and Meat IND 3 with a maximum difference of nine alleles. Isolates in Cluster 20

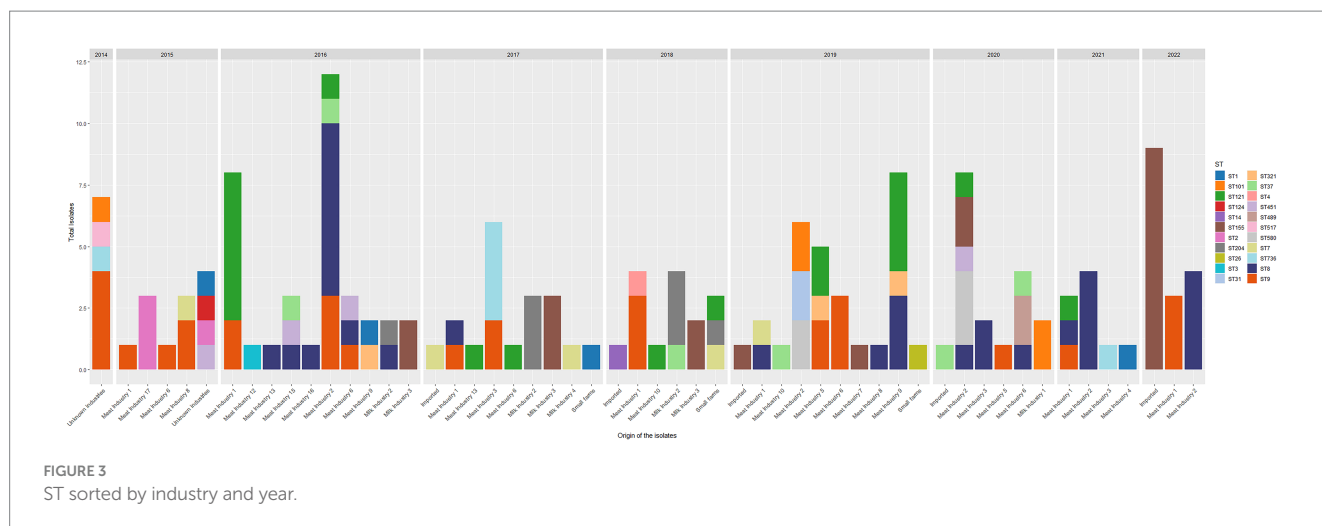
(n = 2, 6.45% of ST9) derived from Meat IND 5 showed no allelic difference in their core genome.

A ST9 singleton (MRL-22-00345) from Meat IND 6 differed by 18 and 23 alleles from two human isolates from France, (CC9/ST9/CT7213) (accession no. ERR1100975) and (CC9/ST9/CT7272) (accession no. ERR1100950) (Maury et al., 2016; Moura et al., 2016), respectively. ST9 isolates from Meat IND 6 (cluster 9) differed by 50 alleles from a human isolate from France (CC9/ST9/CT7208) (accession no. ERR1100972) (Maury et al., 2016; Moura et al., 2016). ST9 isolates from Meat IND 2 (cluster 13) differed by 25 alleles from a human isolate from France (CC9/ST9/CT7211) (accession no. ERR1100973) (Maury et al., 2016; Moura et al., 2016). Strains with the same cgMLST profile (CC9/ST9/CT354) and (CC9/ST9/CT3779), mainly obtained from meat samples, were found in the database of the Austrian National Reference Laboratory (NRL) for *Listeria* (personal communication).

3.2.3 *Listeria monocytogenes* ST121 (n = 19)

Nineteen *L. monocytogenes* ST121 isolates (12% of the total) were obtained from seven industries and one small farm from 2016 to 2021. *L. monocytogenes* ST121 were distributed to four clusters (cluster 5, 15, 18 and 19) and four singletons. Cluster 5 (n = 7, representing 37% of ST121) comprised isolates obtained from Meat IND 1 from the years 2016 (n = 6) and 2021 (n = 1) differing by a maximum of six alleles (Table 1; Figure 2). Cluster 15 (n = 4; representing 21% of ST121) isolates were obtained from Meat IND 2 in 2016 (n = 1) and from Meat IND 9 in 2019 (n = 3, representing 16% of ST121) with a maximum of three allelic differences. Cluster 18 (n = 2) isolates were obtained from Meat IND 5 in 2019. Cluster 19 (n = 2; representing 10% of ST121) isolates were obtained from Meat IND 10 in 2018 and Meat IND 13 in 2019. Four ST121 singletons were obtained from three industries (Meat IND 2, 6, 9) and a small farm differing by 23 to 61 alleles from each other and by 17 to 51 alleles from the clusters (Figure 1; Supplementary Figure S2).





ST121 isolates from Meat IND 5 (cluster 17) differed by 9, 21 and 68 alleles from French isolates originating from human and food-associated sources (CC121/ST121/CT2907) (accession no. 1100947), (CC121/ST121/CT7267) (accession no. ERR1100919) and (CC121/ST121/CT7212) (accession no. 1100974), respectively, (Maury et al., 2016; Moura et al., 2016).

3.2.4 *Listeria monocytogenes* ST155 (n = 20)

Twenty *L. monocytogenes* isolates ST155 were obtained from three industries in Montenegro, one industry from Serbia and one from Italy from 2016 to 2022. ST155 isolates were distributed to three clusters (cluster 3, 6 and 17) and two isolates were singletons. Cluster 3 (n=9, representing 45% of ST155) comprised identical isolates obtained from chicken sausages from industry IMP 5 from Italy in 2022. Cluster 6 (n=7, representing 35% of ST155) isolates were obtained from Milk IND 3 from 2016 and 2018 differing by a maximum of three alleles. Cluster 17 (n=2; 10% of ST155) isolates were obtained from pork meat from Meat IND 2 in 2020 showing one allelic difference. Two singletons were obtained from industry IMP 4 (Serbia, 2018) and beef meat from meat IND 7 in 2019 differing by a minimum of 62 alleles from each other (Figure 1; Supplementary Figure S3).

ST155 isolates from Milk IND 3 (cluster 6) differed by 32, 42 and 51 alleles from one isolate from Finland (CC155/ST155/CT15) (accession no. NC_017547) (den Bakker et al., 2013; Maury et al., 2016), two isolates from France [one from human (CC155/ST155/CT2885) (accession no. ERR1100937) (Maury et al., 2016; Moura et al., 2016) and one from salmon (CC155/ST155/CT7342) (accession no. ERR10849967) (Moura et al., 2024)].

A clinical isolate from Serbia (unpublished, personal communication) from the year 2015 (CC155/ST155/CT2760) available through the NRL for *Listeria* database differed by 5 alleles from cluster 3 food isolates (Italian outbreak; EpiPulse ID: 2022-FWD-00053). In addition, nine clinical isolates from Serbia and eight isolates from Austria isolated from fish (CC155/ST155/CT2842) from the NRL for *Listeria* database clustered with a singleton from our study (MRL-22-00397, CC155/ST155/CT2842), which was obtained from smoked salmon (personal communication).

3.2.5 *Listeria monocytogenes* ST204 (n = 9)

A total of nine *L. monocytogenes* ST204 isolates were obtained from Milk IND 2 (n=8, representing 88% of ST204) and one small farm in Montenegro (n=1, 12%). All Milk IND 2 isolates belonged to cluster 4 differing by a maximum of one allele within the cluster, whereas the small farm isolate differed by a minimum of 37 alleles from cluster 4 (Figure 1; Supplementary Figure S4).

3.2.6 Other *Listeria monocytogenes* STs

Six ST736 isolates (3.75% of the total) obtained from Meat IND 3 from 2017 to 2021 and from an unknown industry in 2014 belonged to cluster 8. Five ST580 isolates (3.125% of the total) from Meat IND 2 belonged to cluster 11. Five ST101 isolates (3.125% of the total of isolates) obtained from Milk IND 1 in 2020, Meat IND 2 in 2019, and an unknown industry in 2014 a belonged to cluster 12 (Table 2; Figure 1). Twenty-three isolates were singletons obtained from different sources, industries, and years (Table 3). Singletons belonging to ST1 (n=4, 2.5% of the total), ST37 (n=6, 3.75%) and ST451 (n=4, 2.5%) were the most prevalent and were obtained from different sources and from different industries (Table 3).

Some singletons obtained from pork meat with less prevalent STs from our study showed a close genetic relationship with strains from the Austrian NRL for *Listeria* database (unpublished, personal communication). Singleton CC1/ST1/CT16036 (MRL-22-00463) was identical to two Austrian strains obtained from ice-cream. Singleton CC7/ST7/CT7998 (MRL-22-00330) differed by 4 and 9 alleles, from two Austrian isolates obtained from sausage and sheep meat. Singleton CC37/ST37/CT5456 (MRL-22-00449) differed by one allele to four Austrian strains obtained from meat and meat products. Singleton CC11/ST451/CT3790 (MRL-22-00353) clustered with 40 Austrian strains which were obtained from meat and meat products. Singleton CC517/ST517/CT5481 (MRL-22-00319) clustered with 44 Austrian isolates, which were obtained from meat and meat products.

Cluster 16 isolates (CC321/ST321/CT197) from our study differed by nine alleles from a clinical isolate (accession no. SRR13744450) from Queens, United States of America.

Detailed information about isolates included in this study are available in the supplements (Supplementary Table S1).

TABLE 3 Information on *L. monocytogenes* singletons isolates included in this study.

Sample ID	ST	CT	Source	Industry	Year	Country			
MRL-22-00393	1	16797	Cow cheese	Small farm	2017	Montenegro			
MRL-22-00463		16036				Pork meat	Meat IND 4	2021	Montenegro
MRL-22-00327		16803				Pork meat	Unknown	2015	Montenegro
MRL-22-00368		17053				Pork meat	Meat IND 9	2016	Montenegro
MRL-22-00337	3	16787	Pork meat	Meat IND 12	2016	Montenegro			
MRL-22-00403	4	17052	Beef meat	Meat IND 1	2018	Montenegro			
MRL-22-00330	7	7998	Pork meat	Meat IND 8	2015	Montenegro			
MRL-22-00429		16798	Beef meat	Meat IND 1	2019	Montenegro			
MRL-22-00400		3879	Cow cheese	Small farm	2018	Montenegro			
MRL-22-00351	8	4172	Smoked pork sausage	Meat IND 16	2016	Montenegro			
MRL-22-00361		8350	Smoked pork sausage	Meat IND 6	2016	Montenegro			
MRL-22-00468		16829	Fermented beef sausage	Meat IND 1	2021	Montenegro			
MRL-22-00367		8506	Smoked pork sausage	Meat IND 15	2016	Montenegro			
MRL-22-00317	9	16576	Smoked pork sausage	Unknown	2014	Montenegro			
MRL-22-00445		5099	Smoked pork sausage	Meat IND 5	2020	Montenegro			
MRL-22-00345		15203	Smoked pork sausage	Meat IND 6	2016	Montenegro			
MRL-22-00406		3755	Smoked pork sausage	Meat IND 1	2018	Montenegro			
MRL-22-00417	14	16799	Beef meat	IMP 3	2019	Serbia			
MRL-22-00431	26	16781	Cow cheese	Small farm	2019	Montenegro			
MRL-22-00449	37	5456	Pork meat	Meat IND 6	2020	Montenegro			
MRL-22-00347		17051	Pork meat	Meat IND 15		Montenegro			
MRL-22-00396		16805	Cow cheese	Milk IND 2		2016	Montenegro		
MRL-22-00413		14979	Pork meat	Meat IND 10		2018	Montenegro		
MRL-22-00459		3311	Mixed frozen vegetables	IMP 2		2019	Montenegro		
MRL-22-00358		16792	Pork meat	Meat IND 2		2020	France		
MRL-22-00427	121	16804	PVC swab	Meat IND 9	2019	Montenegro			
MRL-22-00398		17050	Cow cheese	Small farm	2018	Montenegro			
MRL-22-00448		16798	Smoked pork sausage	Meat IND 2	2020	Montenegro			
MRL-22-00389		16801	Smoked pork sausage	Meat IND 6	2017	Montenegro			
MRL-22-00336	124	16790	Pork meat	Unknown	2015	Montenegro			
MRL-22-00397	155	2842	Smoked salmon	IMP 4	2018	Serbia			
MRL-22-00416		5044	Fresh beef meat	Meat IND 7	2019	Montenegro			
MRL-22-00404	204	16802	Cow cheese	Small farm	2018	Montenegro			
MRL-22-00324	451	16578	Ham	Unknown	2015	Montenegro			
MRL-22-00455		3999	Pork meat	Meat IND 2	2020	Montenegro			
MRL-22-00353		3790	Pork meat	Meat IND 6	2016	Montenegro			
MRL-22-00365		16800	Pork meat	Meat IND 15	2016	Montenegro			
MRL-22-00319	517	5481	Pork meat	Unknown	2014	Montenegro			

3.3 Mobile genetic elements and plasmids

One hundred eleven out of 160 isolates carried at least one MGE. Nine isolates (5.6% of the total) carried over five MGEs. The most prevalent MGE was the unit transposon *Tn5422*, which was found in eighty-four isolates (52.5% of the total) followed by the insertion sequences *ISLmo1* and *ISLmo9* found in forty-three (26.8% of the total) and forty-seven (29.3% of the total) isolates, respectively (Supplementary Table S1). All *L. monocytogenes* ST8, ST9 and ST121

isolates carried MGE *Tn5422*. *ISLmo1*, *ISLmo7*, *ISLmo8*, and *ISLmo9* were present in 32, 39, 39, and 55% of *L. monocytogenes* ST9, respectively. All ST204 and two ST155 isolates carried *ISLmo1*. The remaining eighteen ST155 isolates and ST1, ST2, ST4, ST7, ST451, ST489, ST517, ST736 isolates carried no MGEs. ST3, ST9, ST31, and ST580 carried 5–7 MGEs (Supplementary Table S1).

One hundred one out of 160 *L. monocytogenes* isolates carried at least one plasmid. The plasmid sizes ranged from 25.6–432.1 kb. All *L. monocytogenes* isolates belonging to ST3, ST8, ST9, ST31, ST121,

ST321, ST517 and ST580 carried plasmids (Supplementary Table S1; Supplementary Figure S5). *L. monocytogenes* isolates belonging to those ST profiles were obtained from pork or beef sausages from different meat industries. Seven out of twenty ST155 isolates (35% of ST155), one out of four ST451 isolates (0.6%) and one out of six ST736 isolates (3.75%) carried plasmids (Supplementary Table S1). All ST155 plasmid carrying isolates were obtained from butter or cow cheese from Milk IND 3. ST451 and ST736 plasmid carrying isolates were obtained from pork meat from unknown and Meat IND 3, respectively.

3.4 Antimicrobial genes, pathogenicity islands, and virulence genes

The *fosX* and *lin* genes were detected in all 160 *L. monocytogenes* isolates included in this study.

All known Listeria VGs were detected in the Montenegrin isolates (Supplementary Table S1). Eight out of 160 *L. monocytogenes* isolates (5% of the total) carried pathogenicity island LIPI-3. Specifically, four ST1 (2.5% of the total) isolates, one ST3 (0.6%) isolate, one ST4 (0.6%) isolate and two ST489 (1.25%) isolates obtained from seven different industries carried LIPI-3. Two out of 160 isolates carried LIPI-4 (1.25% of the total). Specifically, one ST4 (0.6%) isolate and one ST517 (0.6%) isolate obtained from different type of meat and different industries carried LIPI-4. One ST4 (0.6%) isolate obtained from beef meat from Meat IND 1 carried both, LIPI-3 and LIPI-4. *L. monocytogenes* ST1 isolates lacked the *actA*, *ami*, *aut*, *gtcA*, *inlF*, *inlJ*, *inlK*, and *vip*. *L. monocytogenes* ST2 lacked the *ami*, *aut*, *gtcA*, *inlF*, *inlJ*, *inlK*, *lapB*, and *vip*. *L. monocytogenes* ST7, ST37, ST204 and ST321 lacked *vip*. *L. monocytogenes* ST8, ST9, ST155, ST451 and ST580 had the complete spectra of VGs. One ST101 isolate lacked VGs *aut*, *fbpA*, *iap/cwhA*, *inlF*, *inlA*, *inlJ*, *inlP*, *lplA1*, *plcB*, and *prfA*, all other ST101 isolates carried all known VGs. *L. monocytogenes* ST121 lacked *actA*, *iap/cwhA*, *inlF*, and *inlJ*.

Briefly, one hundred eighteen out of 160 *L. monocytogenes* isolates carried *InlF*, one hundred twenty *L. monocytogenes* isolates carried *InlJ*, one hundred twenty-five *L. monocytogenes* isolates carried *ActA*, one hundred forty-five *L. monocytogenes* isolates carried *aut*, one hundred forty-six isolates carried *ami* and *InlK*, one hundred fifty-one *L. monocytogenes* isolates carried *GtcA*, one hundred fifty-two *L. monocytogenes* isolates carried *lapB*, one hundred fifty-seven *L. monocytogenes* isolates carried *InlA*, *InlB* and *InlP*, one hundred fifty-eight *L. monocytogenes* isolates carried *PdgA* and *LplA1*, one hundred fifty-nine *L. monocytogenes* isolates carried *FbpA*, *Hyl*, *plcA*, *plcB*, *PrfA* and *Bsh*, one hundred sixty *L. monocytogenes* isolates carried *OatA* and *LntA*. *ClpC*, *ClpE*, *ClpP*, *InlC*, *lap*, *Mpl*, *LspA*, *LpeA*, *PrsA2*, and *Iap* genes were present in all *L. monocytogenes* isolates.

4 Discussion

4.1 Genetic diversity

Controlling *L. monocytogenes* in the food chain is an ongoing and important challenge due to its resilience in different environments. Over a period of 8 years, samples from food products and food

production environments of different origin and composition were examined, showing a prevalence rate of 0.7% among all samples analysed. In a similar study conducted in the USA from the period 2005–2017, the prevalence of *L. monocytogenes* in 150,000 RTE meat samples was 0.4% (Mamber et al., 2020), which is similar to the prevalence in RTE meat samples in our study (0.6%). The incidence of *L. monocytogenes* in RTE pork in our study (69%) is significantly higher compared to similar studies (Gamboa-Marin et al., 2012; Wang et al., 2018), where percentages ranged from 13.5–33.9%. The presence of *L. monocytogenes* in RTE milk products was 12%, which is consistent with findings from other studies (Chen et al., 2020). The occurrence in RTE fish was 0.5%, which is lower than reported in recent studies (Korsak et al., 2012; European Food Safety Authority, and European Centre for Disease Prevention and Control, 2017).

In our study, all isolates belonged either to lineage I or II, both of which are known sources of listeriosis outbreaks, as previously described (Fretz et al., 2010; Orsi et al., 2011; Ruppitsch et al., 2015; Gelbíčová et al., 2018; Pietzka et al., 2019; Matle et al., 2020; Benjamin et al., 2023). Genetic characterization of *L. monocytogenes* isolates from Montenegro is crucial for microbiological safety because both, lineage I and lineage II *L. monocytogenes* strains pose a serious threat to public health.

Based on the WGS characterization, the 160 isolates of our study were grouped into four serogroups (IIa, IIb, IIc, and IVb), being serogroup IIa the most prevalent (66.4%), followed by serogroup IIc (21.7%), serogroup IIb (6.2%) and lastly, serogroup IVb (5.6%). Similar findings were reported by Korsak et al. (2012), where 471 *L. monocytogenes* strains isolated from different foods had the following serogroup distribution: IIa (54.4%), IIc (25.5%), IIb (12.5%) and IVb (7.6%). Serogroup IIa was isolated from a wide variety of milk and meat products from several industries mainly from Montenegro, but also from other countries such as France, Spain, Serbia, and Bosnia. These results are in concordance with previous studies characterizing *L. monocytogenes* from different food sources, which showed the higher prevalence of serogroup IIa in comparison to others (Clémentine et al., 2016; Pyz-Lukasik et al., 2022). Some of the characteristics that may contribute to a higher prevalence of serogroup IIa in the food and food processing environment are a more efficient biofilm formation and predominance in biofilms of mixed cultures (Youwen et al., 2009), a greater resistance to bacteriocins (Korsak et al., 2012) and the possession of a great number of plasmids (60% of *L. monocytogenes* serogroup IIa isolates of our study carried at least one plasmid) that confer resistance to toxic metals and possibly other compounds found in the environment (Orsi et al., 2011). Nevertheless, not only group IIa is a contaminant in food since previous studies also reported that these four groups (groups IIa, IIb, IIc, and IVb) are involved in listeriosis worldwide (Gorski, 2021). For instance, the study carried out by Espinosa-Mata et al. (2022) reported that serogroup IVb was the most prevalent among *L. monocytogenes* isolates coming from cheese, which is in the opposite direction from our study since our isolates coming from cheese belongs mainly to serogroup IIa.

The most prevalent STs among our isolates were ST8, ST9, ST121 and ST155, which is in concordance with other studies (Cabal et al., 2019; Shen et al., 2022). In our study, we observed the spreading of the same ST in different meat and dairy industries during different years. ST9 is a common food associated (Althaus et al., 2014; Martín et al., 2014; Maury et al., 2016, 2019) one in

most European countries and worldwide (Bespalova et al., 2021). ST9 has been shown to persist in the same facility for more than 9 years successfully overcoming hygienic barriers within the factory (Annette et al., 2020). A possible hypothesis for the occurrence of the exact same ST9 clone in the two industries in Montenegro is that the strain entered the facilities with the raw meat imported from Spain (personal communication).

Although there are no reports on cases of human listeriosis in Montenegro, the fact that hypervirulent ST8 strains are among the most prevalent STs in food samples in this study is of concern. ST8 isolates were detected in almost all meat and dairy industries. A previous study showed that in Germany, thirteen out of thirty-nine listeriosis cases caused by *L. monocytogenes* ST8 were detected at health-care facilities where patients had consumed ready-to-eat meat products from the same manufacturer (Lachmann et al., 2021). *L. monocytogenes* ST8 and ST121 are described to contain plasmids that contribute to a higher tolerance to high temperature, salinity, acidic environments, oxidative stress and disinfectants (Naditz et al., 2019), contributing to their ubiquity and persistence capabilities. These results agree with ours, since all ST8 and ST121 carried plasmids. In our study ST121 is predominantly associated with the meat food industry, which does not contradict other studies where ST121 is predominantly associated with food plant environments (Schmitz-Esser et al., 2015). The *L. monocytogenes* ST155 isolates examined in this study were isolated from milk products and chicken meat from Montenegro and Italy, respectively, as well as from an unknown source from Serbia. Previous studies have documented the involvement of ST155 in clinical cases (Zhao et al., 2021) and outbreaks (Stessl et al., 2022) in recent years. Other STs reported as hypervirulent, such as ST1, ST2, and ST4 were detected in nine isolates coming mainly from meat products but one isolate was derived from cowmilk cheese. Detection of the hypervirulent ST1, ST2 and ST4 strains (Guidi et al., 2021) in our study represents a public health concern. Our ST1 strains differed by a minimum of 40 alleles to ST1 strains from recent studies (Maury et al., 2016; Guidi et al., 2021). National surveillance data from France and the Netherlands indicate this clonal complex as predominant in systemic infections in humans with neurological forms of listeriosis (Maury et al., 2016; Koopmans et al., 2017).

In our study ST101 strains were detected in products from two different industries (Milk IND 1 and Meat IND 2), located within the same industrial yard and being part of the same production brand. While the production procedures of these two industries are separated, our findings suggest that cross-contamination of products with this ST101 strain consistently present in the environment is likely occurring. Recognizing the environment as a key source of *L. monocytogenes*, Linke et al. (2014), underscored the significance of the environment as a primary source of *L. monocytogenes*, as shown in their two-year study of soil and water samples, which revealed ST101 as one of the dominant strains (Linke et al., 2014). In the Listeria SEQuencing (LiSEQ) project ST101 isolates were linked with milk/milk products (Painset et al., 2019). The detection of two identical ST7/CT16794 isolates in 2017, one from fresh cow's milk from Montenegro and the other from sour cream from Bosnia might be explained by the import of milk contaminated with listeria from Bosnia and Serbia (personal communication). Along with the identification of ST7 strains in clinical and food samples, it has been linked to listeriosis cases in

cattle and sheep (Annette et al., 2020; Zhao et al., 2021). Among the 20 different STs isolated from meat products ST7 has been reported, along with others like ST1, ST2, ST3 and ST155 to persist in industrial plants for extended periods. The consistent isolation of isolates with no allelic differences from spatially distant industries suggests a possible contamination originating from the raw material. Notably, the only isolate (ST37) detected in frozen vegetables during the entire testing period was detected in a product imported from France in 2020. This same ST was also found in isolates coming from pork from various meat industries and in cow milk cheese. In addition to a frequent isolation from clinical samples, ST37 has been isolated from meat, fish as well as dairy products (Alvarez-Molina et al., 2021; Kubicová et al., 2021; Maćkiw et al., 2021). ST37 has also been isolated from mallard, pheasant and teal feces, moose carcass and wild boar (Fredriksson-Ahoma et al., 2022). In Austria in 2017, ST37 was the fifth most frequent ST in the category food including meat, dairy products, and vegetables (Cabal et al., 2019).

Several *L. monocytogenes* types of our study were found to be closely related to *L. monocytogenes* strains several other European countries (den Bakker et al., 2013; Maury et al., 2016; Moura et al., 2016, 2024). This highlights the broader European scenario of *L. monocytogenes* dissemination and the conservation of isolation sources across different countries, which is influenced by the international trade of food. These findings underscore the importance of stringent control measures for *L. monocytogenes* strains in the global food chain to prevent cross-border contamination and ensure food safety.

4.2 Virulence genes and pathogenicity islands of *Listeria monocytogenes* isolates

LIPIs are specific regions in the genome of *L. monocytogenes* that encode virulence factors playing a crucial role for its pathogenicity (Quereda et al., 2021; Wiktorczyk-Kapischke et al., 2023). Those virulence factors, as previously said, are key factors in the adaptation and spread of *L. monocytogenes* in the environment (Disson et al., 2021; Quereda et al., 2021; Fredriksson-Ahoma et al., 2022). de Melo Tavares et al. (2020) showed that *L. monocytogenes* lineage II did not carry LIPI-3, which is in contrast with our study since three *L. monocytogenes* lineage II in our study carried LIPI-3, although the LIPI-3 carrying *L. monocytogenes* were in the majority lineage IVb. Shen et al. (2022) and Lake et al. (2021) showed that *L. monocytogenes* CC4 was a carrier of LIPI-4, which is in accordance with our findings. However, in our study the other strain with LIPI-4 was *L. monocytogenes* ST517, for which there is no information whether it normally has LIPI-4. Furthermore, numerous studies agree that *L. monocytogenes* ST9 and ST121 do not carry pathogenicity islands of any kind (Shen et al., 2022), which also agrees with our results, since none of them carried neither LIPI-3 nor LIPI-4. Recently, Zhang et al. (2024) described *L. monocytogenes* isolates from RTE carrying LIPI-3 and LIPI-4, as well as ST3 isolates carrying LIPI-3, which is in concordance with the results of our study.

A special feature of *L. monocytogenes* lineage II is the presence of *vip*, *inlF* and *inlK* which has been described only to be present in this lineage and not in others (Pyz-Lukasik et al., 2022), which is in

concordance with our results since, for instance all our *L. monocytogenes* lineage IV isolates were missing these genes (*vip*, *inlF*, *inlK*) in addition to the lack of others such as *inlJ*, *gtcA*, *ami*, and *aut*.

Several studies showed that *aut* is not present in *L. monocytogenes* lineage IVb (Cabanes et al., 2004) which agrees with our findings since in all of them *aut* gene was absent. On the other hand, the study carried out by Pyz-Lukasik et al. (2022) suggests that *vip* gene is only present in pathogenic *Listeria* species, while it is absent in non-pathogenic *Listeria*. Some studies revealed that CC9 *L. monocytogenes* is highly associated with the presence of *vip* gen (Wang et al., 2019) which agree with our results (present in eighty-seven isolates). In contrast, some studies reported *L. monocytogenes* ST9 strains (which is one of the STs included in CC9) as hypo virulent. This would contrast with the above and with our results since, if *L. monocytogenes* ST9 is hypo virulent it should have no association with *vip* and instead in our study they do.

4.3 Antimicrobial resistance genes, plasmids, and mobile genetic elements

All our isolates carried *lin* and *fosX* ARGs, which is in concordance with other studies (Olaimat, 2018; Hanes and Huang, 2022), *lin* and *fosX* are considered as natural/ intrinsic resistance factors of *L. monocytogenes* conferring resistance to lincosamides and fosfomycin (Olaimat, 2018; Fredriksson-Ahoma et al., 2022; Hanes and Huang, 2022). No acquired ARGs were detected.

Previous studies reported that the most prevalent MGEs found in *L. monocytogenes* were *ISLmo3*, *ISLmo5*, *ISLmo7*, *ISLmo9*, *ISLmo8*, *ISS1N*, *cn_8625_ISS1N* and *cn_12410_ISS1N* (Parra-Flores et al., 2022), which agrees with our findings since all were found in our strains. Tn5422 is a natural transposon of *L. monocytogenes* which can generate deletions being probably the reason for the size diversity of the *L. monocytogenes* plasmids (Lebrun et al., 1994). On the other hand, Tn6188 is structurally related to Tn554 from *Staphylococcus aureus*, which suggests a common origin or horizontal gene transfer within these both species (Müller et al., 2013). In the same study they found that this Tn6188 was present in some *Listeria monocytogenes* strains coming from food and food processing environments predominantly from serovar 1/2a. These results are in concordance with our findings.

ST1, ST2, ST3, ST155 and ST204 have been described as plasmid-carriers clones coming from food (Wagner et al., 2020; Mafuna et al., 2021; Schmitz-Esser et al., 2021). In contrast, in our study *L. monocytogenes* ST1, ST2, ST3, ST155 and ST204 strains did not carry any plasmids. On the other hand, ST101, ST124, ST489 were not yet reported in the literature as plasmid carriers, which is in concordance with our results.

5 Conclusion

Monitoring the presence of *L. monocytogenes* over a longer period is a reliable indicator of this pathogen's opportunities in the food chain. Our results reveal a high genetic diversity and variability of *L. monocytogenes* in the Montenegrin food chain, underscoring the importance of ongoing surveillance. This data is crucial for

improving and ameliorating the public health and food safety sector. Whole-genome sequencing (WGS) allows researchers the tracking of the trajectory and persistence of *L. monocytogenes* among other pathogens, and the identification of genetic markers of resistance and virulence. The combination of WGS and seeking the epidemiological link provides a comprehensive view of the pathogen's spread and impact. Furthermore, the Austrian reference database for *L. monocytogenes*, alongside other extensive and continuously updated pathogen databases, has allowed us to verify that many of our isolates share a close genetic relationship with isolates of the same sequence type (ST) widely distributed across different European countries. These findings reinforce the relevance of our study in a broader European context, not only at the level of Montenegro, establishing relevant connections in the field of food safety and public health.

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 in the article/Supplementary material.

Author contributions

BD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. AP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. AM: Conceptualization, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Visualization, Writing – review & editing. WR: Conceptualization, Formal analysis, Funding acquisition, Investigation, Resources, Software, Supervision, Writing – original draft, Writing – review & editing. IZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1418333/full#supplementary-material>

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