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Unraveling the emergence and population diversity of *Listeria monocytogenes* in a newly built meat facility through whole genome sequencing

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ABSTRACT

The food processing environments of a newly opened meat processing facility were sampled in ten visits carried out during its first 1.5 years of activity and analyzed for the presence of *Listeria monocytogenes*. A total of 18 *L. monocytogenes* isolates were obtained from 229 samples, and their genomes were sequenced to perform comparative genomic analyses. An increase in the frequency of isolation of *L. monocytogenes* and in the diversity of sequence types (STs) detected was observed along time. Although the strains isolated belonged to six different STs (ST8, ST9, ST14, ST37, ST121 and ST155), ST9 was the most abundant (8 out of 18 strains). Low (0 and 2) single nucleotide polymorphism (SNP) distances were found between two pairs of ST9 strains isolated in both cases 3 months apart from the same processing room (Lm-1267 and Lm-1705, with a 2 SNPs distance in the core genome; Lm-1265 and Lm-1706, with a 0 SNPs distance), which suggests that these strains may be persistent *L. monocytogenes* strains in the food processing environment. Most strains showed an in silico attenuated virulence potential either through the truncation of InlA (in 67% of the isolates) or the absence of other virulence factors involved in cell adhesion or invasion. Twelve of the eighteen *L. monocytogenes* isolates contained a plasmid, which ranged in size from 4 to 87 Kb and harbored stress survival, in addition to heavy metals and biocides resistance determinants. Identical or highly similar plasmids were identified for various sets of *L. monocytogenes* ST9 isolates, which suggests the clonal expansion and persistence of plasmid-containing ST9 strains in the processing environments of the meat facility. Finally, the analysis of the *L. monocytogenes* genomes available in the NCBI database, and their associated metadata, evidenced that strains from ST9 are more frequently reported in Europe, linked to foods, particularly to meat and pork products, and less represented among clinical isolates than other *L. monocytogenes* STs. It also showed that the ST9 strains here isolated were more closely related to the European isolates, which clustered together and separated from ST9 North American isolates.

1. Introduction

Listeria monocytogenes is a major foodborne pathogen causing the disease listeriosis among humans (Haase et al., 2014). *L. monocytogenes* strains have been classified in four different lineages, with lineage I (including serotypes 1/2b and 4b, the serotypes most frequently associated with human listeriosis cases) and lineage II (comprising serotypes 1/2a and 1/2c, commonly found in food and food processing environments) being the most widely spread (Orsi et al., 2011). Indeed, from the total of thirteen *L. monocytogenes* serotypes described, these four serotypes are responsible for 90% of the worldwide listeriosis outbreaks (Braga et al., 2017).

Listeriosis cases are characterized by the onset of encephalitis, meningitis, diarrhea, spontaneous abortion and septicemia as the most frequent clinical symptoms (Ruppitsch et al., 2015). The incidence of listeriosis has grown continuously but slowly in the European Union (EU), from 0.41 cases per 100,000 population in 2012 (EFSA, 2014) to 0.47 cases per 100,000 population in 2018 (EFSA, 2019), and listeriosis is a major threat in the EU as this zoonotic disease has very high hospitalization (97%) and mortality (16%) rates (EFSA, 2019). Pregnant women, infants, elderly and immuno-compromised individuals form the risk-group for listeriosis (Dortet et al., 2019; WHO Food Safety Department, 2004).

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Several food products have been associated with listeriosis outbreaks, including cheese, fish, meat, fruits and vegetables (Alvarez-Ordóñez et al., 2018). According to the Rapid Alert System for Food and Feed (RASFF), in 2019, fish and fish products, milk and milk products, meat and meat products (other than poultry), poultry meat and poultry meat products, and prepared dishes and snacks were the top 5 food categories linked to *Listeria* alerts in the EU (RASFF - Food and Feed Safety Alerts, 2020).

Multiple studies have shown that specific clonal complexes are more often retrieved from food and can persist longer in food-processing environments (Ortiz et al., 2016). The ability of *L. monocytogenes* to grow and spread at low temperature conditions (Saldivar et al., 2018), its capability to form protective biofilms (Dygico et al., 2020), and its tolerance to high salinity levels (Aalto-Araneda et al., 2020) and low pH (NicAogáin and O'Byrne, 2016), among other stress conditions, contributes not only to its ubiquitous nature, but also to its persistence in food processing environments. Thus, the timely detection of persistent *L. monocytogenes* strains and their elimination through efficient sanitation processes are a priority for food industries, which are facing a pathogen which is difficult to eradicate. In fact, *L. monocytogenes* can develop resistance against sanitation agents through biofilm formation (Roedel et al., 2019) or the acquisition of resistance genes harbored in mobile genetic elements (MGE) (Kropac et al., 2019). For instance, the resistance of *L. monocytogenes* to quaternary ammonium compounds (QACs), such as benzalkonium chloride (BAC), present in some biocidal formulations used in industrial sanitation processes, has been increasingly studied. Tolerance mechanisms against BAC have been identified in persistent *L. monocytogenes* isolates, including efflux pumps, like the one encoded by *emrE* (Kovacevic et al., 2016), transporters, such as the one encoded by Tn6188_qac (*ermC*) (Müller et al., 2013), or resistance cassettes (e.g., *bcrABC*) in plasmids (Dutta et al., 2013).

Pulsed field gel electrophoresis (PFGE) has been the gold standard procedure for tracking outbreaks of listeriosis as well as for identifying events of *L. monocytogenes* persistence in food processing environments (Halpin et al., 2010), with standardized protocols and data interpretation approaches being available to facilitate data sharing between countries (Roussel et al., 2017). However, the advances in whole genome sequencing (WGS) technologies in the last decades have led to the co-existence of both techniques (Laksanalamai et al., 2012). The continuous decrease in the costs of next generation sequencing (NGS) is favoring the routine implementation of WGS in outbreak investigation and surveillance of foodborne pathogens in foods and food processing environments, taking also into account the increased yield of information that can be obtained from WGS data (Jackson et al., 2016; Van Walle et al., 2018, 2019). Indeed, WGS allows to obtain the classic multilocus sequence typing (MLST) profile, based in the case of *L. monocytogenes* on the sequence of seven housekeeping genes, to assign the strain to a given sequence type (ST), consequently facilitating the study of phylogenetic relationships among isolates (Stessl et al., 2014). Moreover, recent advances in sequencing capabilities and approaches for genome data analysis have generated more discriminative typing methods, such as core genome MLST (cgMLST) analyses, which expand the principle of MLST to a higher number of genes/*loci* (up to 2000 or more) that are present in the majority of the strains of the species under study. Two cgMLST schemes have been already implemented for *L. monocytogenes* in commercial bioinformatics software, i.e., the scheme by Ruppitsch et al (Ruppitsch et al., 2015) in SeqSphere® software, based on 1701 *loci*, and the scheme by Moura et al (Moura et al., 2016) in BioNumerics® software, based on 1748 *loci*. In addition, WGS may also provide useful information on other relevant strain features, such as antimicrobial resistance, virulence potential or stress tolerance (Oniciuc et al., 2018).

The aim of this study was to monitor the colonization by *L. monocytogenes* of a newly opened meat processing plant by sampling food processing environments in ten sampling visits carried out during its first 1.5 years of activity and characterize by WGS all the *L. monocytogenes* isolates recovered, in order to get insights into the persistence of this important foodborne pathogen in industrial settings.

2. Materials and methods

2.1. Environmental sampling and L. monocytogenes isolation

The survey at the meat processing facility started on July 2017. The first sampling visit (considered as day 0) was carried out just after the building works finished, before any meat processing activity or sanitation procedure had taken place. The following samplings (visits 2–10) were performed 7, 14, 21, 35, 70, 143, 268, 436 and 562 days thereafter. The first tests of the equipment and process chain functioning started on day 7, and pig carcasses were for the first time processed on day 14. Swab samples were collected in the early morning hours, before the beginning of the production process and after regular sanitation activities had been conducted. In total, 229 swab samples (27 to 31 swab samples per sampling point) were taken along the course of the survey. Different surfaces were sampled (drains, floors, walls, equipment, tables, meat, trays and knives) at various processing rooms (R) along the facility (Fig. 1). R1 was the reception and chilling room for carcasses, while R2 included two cold storage (0–2 °C) chambers for carcasses, with both rooms being considered as "pre-cutting" rooms. R3 and R4 were cutting rooms for white and Iberian pork, respectively, and R5 included various cold storage (0–2 °C) rooms for different types of meat pieces. R6 was a small room for pig trotters' processing and R7 was the packaging room. R5, R6 and R7 were consider as "post-cutting" rooms. The surfaces sampled were randomized. Thus, the vast majority of swab samples were taken at different sites within the facility. Just a minority of sites from small processing rooms like R6 were sampled more than one time throughout the study.

HydraSponge sterile swabs (3 M, USA), pre-moistened with neutralizing buffer, were used for swabbing. When enough surface was available (e.g. floors, walls, etc), approx. 1 m^2 was swabbed. For other types of surfaces, where this was not possible, individual units (e.g. 1 drain or 1 knife) were swabbed. Swabs were kept at approx. 4 °C in a cooling box containing ice packs and transported to the laboratory within two hours for sample processing. A first general pre-enrichment step of the swabs was carried out in 100 mL of Buffered Peptone Water (BPW, Merck, Germany) for 24 h at 37 °C. Then, a first selective enrichment was undertaken by inoculating 100 μL of this suspension in 10 mL of Half Fraser broth (Merck), which was incubated for 24 h at 30 °C. Subsequently, a second selective enrichment was performed by inoculating 100 μL in 10 mL of full Fraser broth (Merck) and incubating it for 48 h at 30 °C. Finally, 100 μL of this suspension was spread onto agar Listeria according to Ottaviani and Agosti (ALOA) (Merck) plates, which were incubated for 48 h at 37 °C.

Identity confirmation of those colonies with the characteristic morphology of *L. monocytogenes* (blue-green colonies surrounded by an opaque halo) was performed by Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS, Microflex LT model, Bruker-Daltonics) following the protocol detailed by Barbuddhe et al. (2008). Briefly, 2–3 presumptive *L. monocytogenes* isolates from each ALOA agar plate were surface inoculated on BHI (Merck) agar plates. Subsequently, one isolated colony was picked with a sterile inoculation loop and placed on a well of the MSP96 Bruker steel plate, and 1 μL of matrix solution (HCCA, Bruker-Daltonics) was added to each well and allowed to dry at room temperature in a laminar flow cabinet for 5 min. In all occasions, the Bruker BTS standard sample (mass calibration standard showing a typical *Escherichia coli* DH5 alpha peptide and protein profile plus additional proteins) was included as reference. Finally, spectra were acquired and queried against the Bruker-Daltonics MALDI Biotyper Compass library database using the MALDI Biotyper software.

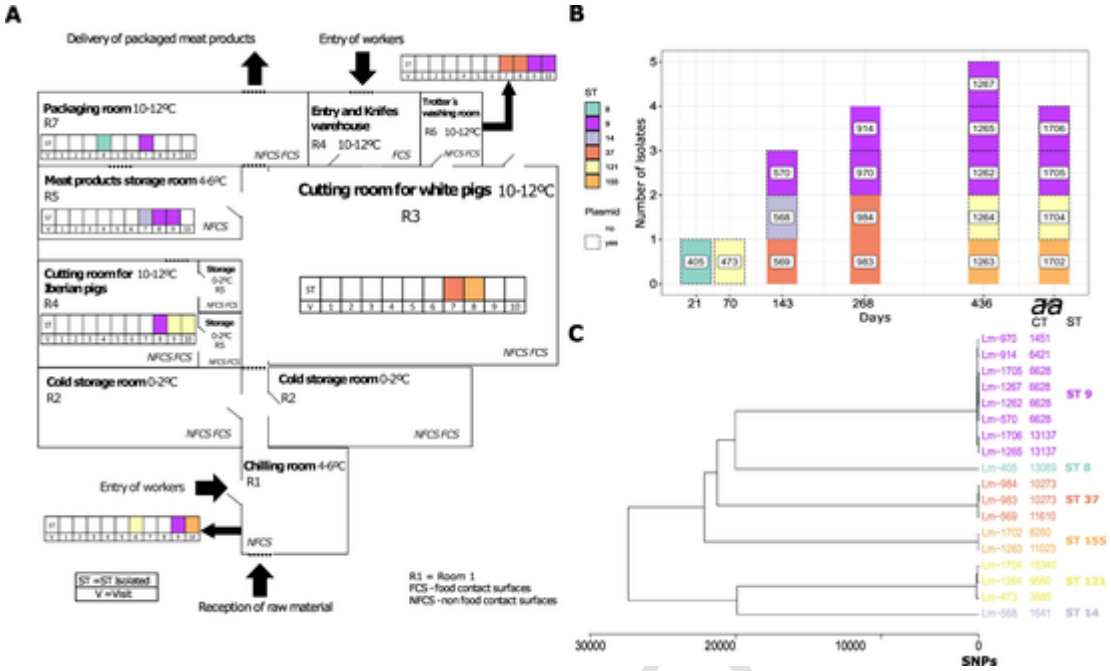

Fig. 1. Distribution and similarity of *L. monocytogenes* strains.A: Representation of the different rooms of the facility. Each room was coded as R1 to R7. The temperature of the room is shown, together with information about the type of surfaces sampled, the sequence types (ST) isolated, and the sampling visits (V) at which each *L. monocytogenes* strain was isolated. Rooms were separated from corridors or other rooms by closed doors. Apart of these barriers, disinfection foot baths were available at both entries of workers.B: Emergence of *L. monocytogenes* isolates over the sampling period. The color legend corresponds to the ST, and the discontinuous outer-line shows the presence of a plasmid.C: SNPs average distance matrix obtained from hierarchical clustering of Table S2 using UPGMA linkage criteria.

2.2. WGS, assembly and annotation

Genomic DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Germany), following the manufacturer's instructions. The quality of the extracted DNA was determined by capillary electrophoresis using a Fragment Analyzer (Genome DNA 50 Kb analysis kit, AATI), and DNA concentration was measured using a fluorometric assay (Qubit dsDNA High-Sensitivity assay kit, Thermo Fisher). Library preparation was carried out using the TruSeq DNA PCR-Free kit (Illumina), following the manufacturer's instructions, with a previous DNA fragmentation using Covaris E220 to obtain approx. 50 bp fragments. Then, a quality control step was again performed using the Fragment Analyzer and Qubit. Finally, a qPCR was performed to prepare samples for sequencing using the Complete kit Roche Light Cycler 480 (Kappa). WGS was performed in an Illumina HiSeq 1500 platform, using 150 bp paired-read sequencing.

The quality of the sequences was analyzed by FastQC version 0.11.9 software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adapters were trimmed using Trim Galore version 0.6.5 software (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), followed by a second quality check with FastQC. Clean reads were assembled with PLACNETw (https://castillo.dicom.unican.es/upload/) (Vielva et al., 2017). After a manual pruning of the networks produced by the platform, contigs were divided into plasmid and chromosomal components, and analyzed separately. Assembly quality was checked using QUAST version 5.0.2 (http://quast.sourceforge.net/quast.html) (Gurevich et al., 2013) specifying the flag "--gene-finding", selecting as reference genome the strain *L. monocytogenes* EGD-e. Details about the sequencing and assembly quality can be consulted in Supplementary Table S1. Genome annotation was performed using Prokka version 1.14.5 (https://github.com/tseemann/prokka) (Seemann, 2014).

2.3. MLST and cg-MLST analysis

In silico MLST determination was performed from the assemblies using mlst version 2.19.0 (https://github.com/tseemann/mlst) and the 7 loci scheme (Ragon et al., 2008) available at BIGSdb-Lm (https://bigsdb.pasteur.fr). Additionally, isolates were also typed using the cgMLST scheme of 1748 loci (Moura et al., 2016) through the BIGSdb-Lm web interface.

Finally, a core genome of the 18 *L. monocytogenes* strains isolated from the plant was generated following the methodology described by Lanza et al. (2014). Briefly, genes present in 90% of the isolates with a 90% of identity and coverage were extracted with CD-HIT-EST version 4.8.1 (Li and Godzik, 2006), and the core genes of each strain were concatenated and aligned with progressive Mauve version 2.4.0 (Darling et al., 2010). After applying the SNP export tool of Mauve GUI, a tabular list of SNPs was extracted and a SNPs distance matrix was constructed with the number of SNPs shared by every pair-wise combination of strain core genomes (Supplementary Table S2). The SNPs matrix was used as a distance matrix to perform hierarchical clustering analysis with the base R function *hclust*, measuring Euclidean distance and selecting UPGMA as linkage criteria under R version 3.5.3. The dendrogram representation was done with the *plot* function.

2.4. Antibiotic resistance, virulence, and stress survival genes

The Antibiotic Resistance Gene-Annotation (ARG-ANNOT) (Gupta et al., 2014), Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2016), NCBI AMRFinderPlus (Feldgarden et al., 2019) and ResFinder ("Center for genomic epidemiology, Resfinder," n.d.) databases were downloaded in order to perform local BLASTn (Altschul et al., 1990) searches through ABRicate software v0.9.9 (https://github.com/tseemann/abricate).

Searches of virulence factors were performed against the DNA sequences of the core set A from the Virulence Finder Database (VFDB) (Chen et al., 2005, 2016), downloaded in April 2020, which in-

cluded 3228 sequences. Virulence factors were detected by BLASTn (Altschul et al., 1990), using a 90% cut-off value for both identity and coverage, and with the option "*-subject_besthit -max_target_seqs 1"* enabled to retain just the best match for the best alignment of each query sequence. Truncations or loss-of-function mutations in the genes were manually checked in CLC workbench genomic viewer 20, using "finding open reading frames" from extracted gene nucleotide sequences and *L. monocytogenes* EGD-e as a reference genome. Additionally, the genes *vip, inlF, inlA, bsh, ami, inlJ, prfA, hly, actA* and *inlB* were aligned with Bowtie2 (Langmead et al., 2019; Langmead and Salzberg, 2012) to those gene sequences from the *L. monocytogenes* EGD-e genome. SAM files were transformed into BAM files, sorted and indexed with SAMtools (Li et al., 2009) in order to visually inspect each gene sequence with Integrative Genomics Viewer (Robinson et al., 2011) to corroborate the results.

For the detection of the *Listeria* Pathogenicity Islands (LIPI) 1, 3, and 4, a similar approach was followed. The contigs were queried against databases composed by the gene sequences extracted from the coordinates of reference genomes available in the VFDB web (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Listeria). LIPI-2 was not analyzed, as this island has been only detected in *L. ivanovii* (Domínguez-Bernal et al., 2006). For LIPI-1 and the Stress Survival Islets (SSI) 1 and 2, supplementary analyses were performed by using the fastANI tool (Jain et al., 2018) over these elements with the objective of comparing their average nucleotide identity (ANI) against their reference sequences, which correspond to the *loci lmo0200-lmo0206* (LIPI-1) and *lmo0444-lmo0448* (SSI-1) of *L. monocytogenes* EGD-e, and *lin0464-lin0465* (SSI-2) of *L. innocua* CLIP 11262. In addition, the integrity of the Coding Sequences (CDS) was visually checked with CLC main workbench 20.

In order to identify genetic determinants which could confer resistance to food processing-related stresses and/or sanitation agents, BLASTx (Altschul et al., 1990) comparisons against proteins in the Antibacterial Biocide & Metal Resistance Genes Database (BacMet) ("BacMet Database - Home," n.d.) version 2.0 (155,512 sequences, last update on 2018/03/11) were performed by using DIAMOND (Buchfink et al., 2014) and CDS regions as query. Coverage and identity thresholds were set this time at 80% due to the low number of sequences related to *Listeria* genus (n = 180) at the BacMet database, and the *"-subject_besthit -max_target_seqs 1"* option was selected to retain the best match.

2.5. Plasmid analysis

Paired-end pre-processed reads were uploaded to PLACNETw (Vielva et al., 2017) for plasmids detection and characterization of relaxase (MOB) gene families and replicase genes (REP).

The Mob-typer software version 1.0 (https://github.com/jrober84/mob-typer) was employed for the prediction of mobility, as well as for the detection and identification of important genomic sites, such as the origin of replication, the mobility genes (MOB), used for plasmid classification, or the mating pair formation system (MPF). Amino acids fasta files were submitted to MOBscan (Garcillán-Barcia et al., 2020) to subtype the MOBs, and these sequences were manually compared to the motifs described by Garcillán-Barcia et al. (2009). Nucleotide sequences from MOBtyper (419 sequences) were merged with PlasmidFinder (Carattoli et al., 2014) REP databases (507 sequences), and employed as a database to carry out BLASTn searches in order to improve the replicase characterization.

Additionally, the sequences of 53 reference plasmids carried by *L. monocytogenes* isolates were downloaded from the NCBI Plasmid_Annotation Report (https://www.ncbi.nlm.nih.gov/genome/browse/#!/plasmids/159/) in order to compare them with those from our study. Nonetheless, the sequences of the plasmids belonging to the strain NCTC7974 were removed from the analysis as these presumptive plasmids (with sizes ranging from 394 to 906 Kb) did not have REP or MOB proteins and did not share CDSs in common with other *L. monocytogenes* plasmids. The reference plasmids were also analyzed using MOBtyper and MOBscan. For plasmids pLM33 and pCFSAN022990, as MOBtyper detected a MOB_P protein that MOBscan was unable to detect, a specific search using hidden Markov model profiles was done with the suspect MOB CDS. The CDS protein fasta files of all plasmids from the *L. monocytogenes* isolates recovered from the facility and of the reference plasmids downloaded from NCBI were merged and clustered by CD-HIT (Fu et al., 2012; Li and Godzik, 2006) to obtain a CDS database without redundant sequences which contains clusters of protein sequences which met an identity and coverage cut-off of 80%.

The ".clstr" output file from CD-HIT was used to construct a presence/absence matrix of CDSs, using binary distances and the UPGMA algorithm to perform a hierarchical clustering, which was plotted by using the R-package *ggtree* (Yu, 2020). The BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011) software was chosen to compare plasmids belonging to the same clusters.

2.6. Analysis of ST9 genomes

All fasta and GenBank files available in the NCBI "Genome" database belonging to *L. monocytogenes*, according to the document Genome assembly and annotation report (https://www.ncbi.nlm.nih.gov/genome/?term=Listeria+monocytogenes), were downloaded on the 28th February 2020, together with biosample attributes (which included geographic location, serovar, host and source) using *in house* R scripts. Manual pruning of the metadata associated with each genome was performed as follows. Geographic origin, when available, was relabeled with the continent where the strain was isolated. The source and host information served to create three different categories. From the most general to the most detailed one, the first category differentiates the origin of the isolate (food, environmental, clinical, or missing/unknown), the second category splits the clinical isolates depending on the host (animal or human) and the food/environmental isolates depending on the food production sector (meat, fish, dairy products, fruits and vegetables, Ready-To-Eat [RTE], or missing/unknown), and the third category details the specific type of foodstuff or animal species (e.g., butter, pork). Smoked salmon and salami, which were among the most frequently reported sources, were considered as RTE.

The ST of all *L. monocytogenes* genomes (n = 3286) was determined following the procedure previously described, and those genomes belonging to isolates from the ST9 (n = 360) were selected for further phylogenetic analyses, together with the genomes from the ST9 isolates recovered from the meat processing facility in the current study (n = 8). A BLAST database was constructed with all the sequences of the 1748 *loci* from the Moura cgMLST scheme (Moura et al., 2016), which were downloaded from BIGSdb (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_listeria_seqdef&page=downloadAlleles). The ST9 genomes were queried against this database and the best hit per *loci* was saved after setting 100% identity and 90% coverage cutoff values. The resulting presence/absence matrix, with genomes on rows and loci numbers and genes on columns, was clustered by calculating binary distances and using the UPGMA clustering algorithm. This hierharchical clustering was plotted by using the R-package *ggtree* (Yu, 2020). Additional comparisons of ST9 isolates versus non ST9 isolates were performed just with the 3286 isolates present at the NCBI database, with statistical tests being performed using *rstatix* R package.

2.7. Data availability

The raw fastq files and isolate information were submitted to the NCBI database under BioProject accession number PRJNA659909, comprising BioSamples from SAMN15945271 to SAMN15945280 and SRA accession numbers from SRR12560250 to SRR12560267.

3. Results

3.1. Environmental sampling and L. monocytogenes isolation

From the total of 229 samples analyzed, 18 *L. monocytogenes* isolates were obtained from 13 different sampling sites at various time points, which represents a 7.9% prevalence. *L. monocytogenes* was detected for the first time at the fourth sampling visit (day 21), one week after the first pig carcasses were processed (day 14) (Fig. 1A, B). Afterwards, an increase in the frequency of isolation of *L. monocytogenes* was observed. Indeed, while the prevalence of *L. monocytogenes* during the first five sampling visits, which were carried out during the first 35 days of activity of the meat processing plant, was 1.2%, this figure dramatically increased to 11.6% in the last five sampling visits, which covered a much larger period of time. Most *L. monocytogenes* strains were isolated from non-food contact surfaces (drains and floors), which showed a prevalence of isolation of 11.9%. Only two *L. monocytogenes* strains (Lm-914 and Lm-970) were isolated from food contact surfaces (cutting tables in both cases), which showed a 1.1% prevalence. The only rooms where *L. monocytogenes* was never detected throughout the course of the study were the carcass cold storage rooms (R2) (Table 1; Fig. 1), where the limited transit of workers and/or the absence of cutting/processing activities may have acted as barriers for the colonization by this pathogenic microorganism.

WGS-based MLST analysis revealed that all *L. monocytogenes* isolates belonged to one of the following six STs: ST8, ST9, ST14, ST37, ST121 and ST155 (Table 1). ST9 was the most abundant (8 out of 18 strains), followed by ST37 and ST121 (3 out of 18 strains each), ST155 (2 out of 18 strains), and ST8 and ST14 (1 out of 18 strains each). All strains belonged to either serotype 1/2a (ST8, ST14, ST37, ST121 and ST155 strains) or 1/2c (ST9 strains). Each of the strains from ST8, ST14, ST121 and ST155 belonged to a different core-genome type (CT), according to the scheme of Moura et al. On the other hand, the strains from ST37 (Lm-983 and Lm-984) both belonged to CT10273 and several ST9 strains also were from the same CT (Lm-570, Lm-1262, Lm-1267 and Lm-1705, from CT6628; Lm-1265 and Lm-1706, from CT13137) (Table 1). In fact, a low SNP distance in the core genome specifically built with the genomes of the eighteen isolates, composed of 2520 CDSs and 2,313,399 bp, was observed among the strains from these three CTs (Fig. 1C) (Supplementary Table S2). For instance, no SNPs in the core genome were found between Lm-983 and Lm-984, or Lm-1265 and Lm-1706, and only 2 SNPs were detected between the isolates Lm-1267 and Lm-1705. These last two pairs of strains were isolated 3 months apart from the same processing room (R5 for Lm-1267 and Lm-1705, and R6 for Lm-1265 and Lm-1706), suggesting that they may be persistent *L. monocytogenes* strains in the food processing environment.

Table 1
L. monocytogenes isolated over sampling.

Genome Identifier	Isolation surface	Isolation room	Isolation date	In silico serotype	MLST (ST)	cgMLST (CT)	Plasmid size (Kb)	Plasmid genes	MOB	REP	MPF	Mobility
Lm-405	Drain	7	2017-08-07	1/2a	8	13,089	87	86	MOB_{P2}	Rep-26	MPFT	Conjugative
Lm-473	Floor	1	2017-09-26	1/2a	121	3885	61	62	MOB_{P2}	Rep-26	MPFT	Conjugative
Lm-568	Floor	5	2017-12-13	1/2a	14	1641	4 [a]	6	MOB_{V1}/MOB_{T} [b]	Rep-52	–	Mobilizable
Lm-569	Floor	6	2017-12-13	1/2a	37	11,610						
Lm-570	Floor	7	2017-12-13	1/2c	9	6628	58	61	MOB_{P2}	Rep-25	–	Mobilizable
Lm-914	Table	4	2018-04-16	1/2c	9	6421						
Lm-970	Table	4	2018-04-16	1/2c	9	1451	25	25	–	Rep-25	–	Non-mobilizable
Lm-983	Drain	6	2018-04-16	1/2a	37	10,273						
Lm-984	Drain	3	2018-04-16	1/2a	37	10,273						
Lm-1262	Drain	1	2018-10-09	1/2c	9	6628	58	61	MOB_{P2}	Rep-25	–	Mobilizable
Lm-1263	Drain	3	2018-10-09	1/2a	155	11,023			MOB_{T} [b]			
Lm-1264	Drain	4	2018-10-09	1/2a	121	9560	61	59	MOB_{P2}	Rep-26	MPFT	Conjugative
Lm-1265	Floor	6	2018-10-09	1/2c	9	13,137	63	71	MOB_{P2}	Rep-25	–	Mobilizable
Lm-1267	Floor	5	2018-10-09	1/2c	9	6628	69	73	MOB_{P2}	Rep-25	–	Mobilizable
Lm-1702	Drain	1	2019-01-22	1/2a	155	8260						
Lm-1704	Drain	4	2019-01-22	1/2a	121	18,340	61	59	MOB_{P2}	Rep-26	MPFT	Conjugative
Lm-1705	Drain	5	2019-01-22	1/2c	9	6628	69	73	MOB_{P2}	Rep-25	–	Mobilizable
Lm-1706	Drain	6	2019-01-22	1/2c	9	13,137	63	70	MOB_{P2}	Rep-25	–	Mobilizable

MOB type corresponds to the relaxase family, REP type corresponds to the replicase family, MPF corresponds to the mating pair formation family.
[a] Plasmid-Lm-568 was in high copy-number (approximately 40 copies).
[b] MOBT were found in bacterial chromosomes.

3.2. Antimicrobial resistance, virulence and stress survival genes

Only four antimicrobial resistance genes, present in all of the 18 *L. monocytogenes* strains, were detected (Fig. 2), i.e., *norB*, conferring quinolone resistance, and *mprF*, which protects against cationic peptides (both of them detected with the CARD database), *lin* (involved in lincomycin resistance and detected with ARG-ANNOT and CARD), and *fosX* (involved in fosfomycin resistance and detected with ResFinder and NCBI AMRFinderPlus db).

Although the presence of the majority of virulence genes, pathogenicity islands, and stress survival genes and islets was shared across all *L. monocytogenes* isolates, certain specific determinants were absent in some of the strains (Fig. 2). Thus, for instance, *inlF*, encoding an internalin which is involved in cell adhesion and invasion, was not found in the ST14 and ST121 isolates, and *vip*, involved in cell invasion, was absent in the isolates from ST8 and ST37. In relation to the InlA, which facilitates the entry of *L. monocytogenes* into host cells, twelve of the eighteen *L. monocytogenes* isolates, from ST9, ST37, ST121 and ST155, harbored a truncated version (Fig. 2). Although none of the strains carried the pathogenicity islands LIPI-3 or LIPI-4, all of them harbored a DNA fragment of 9 Kb corresponding to the pathogenicity island LIPI-1, composed of six genes (i.e., *prfA*, *mpl*, *plcA*, *plcB*, *actA* and *hly*) which encode virulence factors that act during the cell invasion process. No truncations or loss-of-function mutations were found in the *prfA* gene, the most important virulence regulator in *L. monocytogenes*, or in *hly* and *actA*.

Five different genes related to stress survival were found through searches in the BacMet database in all the isolate's chromosomes, i.e., *perR* (a peroxide operon regulator), *fabL* (involved in triclosan resistance), *gadC* (a transporter which improves survival upon exposure to acid pH stress), and *lde* and *mdrL* (efflux pumps responsible for expelling harmful substances from the cell).

SSI-1, an islet of 8.7 Kb composed of five genes (*lmo0444, lmo0445, pva, gadD1* and *gadT1*) whose expression favors the growth of *L. monocytogenes* under low pH and/or high salinity conditions, was found in 11 out of the 18 isolates, belonging to ST8, ST9, and ST155. SSI-2, a small stress survival islet composed of two genes (*lin0464 and lin0465*) showing homology to genes from the non-pathogenic species *L. innocua* encoding a transcriptional regulator and a Pflp protease that act by protecting the cell upon exposure to alkaline and/or oxidative stress (Harter et al., 2017), was exclusively found in the three ST121 strains (Fig. 2). Average nucleotide identity analyses of LIPI-1, SSI-1 and SSI-2 showed high levels of homology with their reference sequences, with the lowest ANI value being 99.17% for LIPI-1, 99.93% for SSI-1 and 98.55% for SSI-2 (Supplementary Table S3).

3.3. Plasmid analysis

Twelve of the eighteen *L. monocytogenes* isolates contained a plasmid (Table 1). The plasmids ranged in size from 4 Kb (for the strain Lm-568, from ST4) to 87 Kb (for the strain Lm-405, from ST8). All the plasmids, except a 25 Kb plasmid harbored by Lm-970, were mobilizable or conjugative. MOB and REP typing of the plasmids showed a low diversity of different plasmid types. Indeed, all plasmids, except the small 4 Kb plasmid of Lm-568, were from the MOB_{P2} subfamily and either contained a Rep-25 or Rep-26 replication initiation protein, together with a mating pair formation system in the case of those plasmids harboring a Rep-26 type protein (Table 1). Neither virulence determinants nor antimicrobial resistance genes were found in the twelve plasmids when searching against the VFDB, NCBI AMRFinderPlus db, CARD, ARG-ANNOT and ResFinder databases.

A hierarchical clustering classification based on the presence/absence of 510 non-redundant CDSs in the 48 reference *L. monocytogenes* plasmids from the NCBI database and the 12 plasmids recovered in the current study showed the presence of three main clusters of plasmids associated with *L. monocytogenes* ST9 strains (clusters A, B and C, Fig. 3), all of them belonging to the MOB_{P2} subfamily and containing a Rep-25 replication initiation protein. It also grouped all the conjugative plasmids, except one (PLMIV) (cluster D, Fig. 3). Within them, a subcluster was formed by all the plasmids associated with strains of *L. monocytogenes* ST121 (cluster E, Fig. 3). Plasmid and host biosample accession numbers associated with the plasmid names analyzed at Fig. 3 can be consulted in Supplementary Table S4.

Globally, 38 plasmids were mobilizable (63%) and 13 conjugative (22%), while only 9 plasmids (15%) did not carry the needed machinery to be transferred (Fig. 3). The Rep-26 replication initiation protein was present in all the conjugative plasmids from cluster D. Regarding MOB_P subtyping, MOB_{P2} was the most abundant, being present in 47 plasmids (78%), while MOB_L and MOB_{V1} were found in only 2 plasmids, with the latter one being associated with plasmids of size <5 Kb (P-Lm-568 and pLmN12–0935) (Fig. 3). There were 9 plasmids with

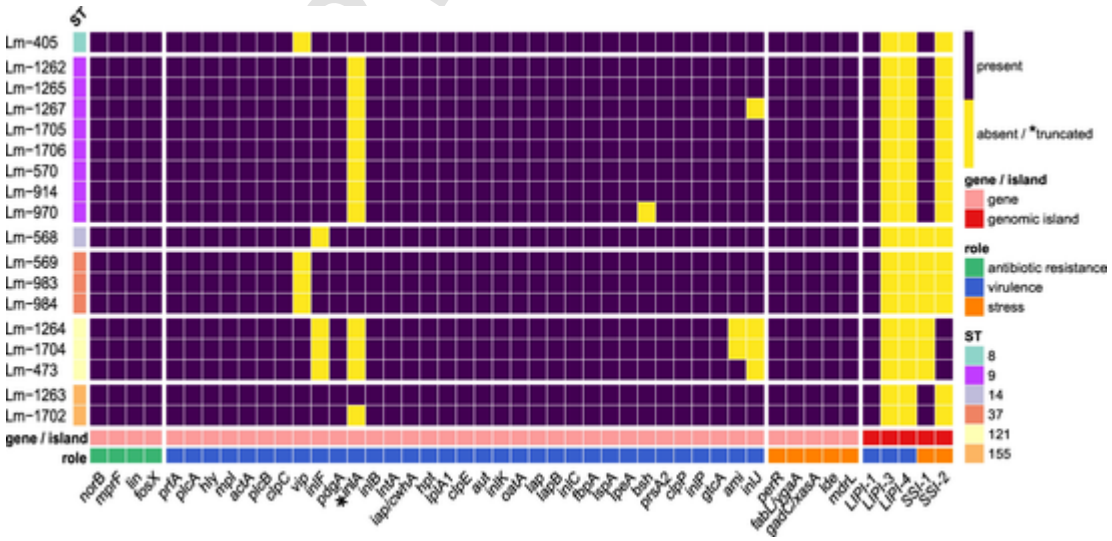

Fig. 2. Antibiotic resistance, virulence, and stress survival determinants.Presence/absence of antibiotic resistance and virulence genes with a 90% coverage and identity threshold against their reference sequence, together with the presence/absence of stress survival genes with a coverage and identity threshold of 80%.*all the isolates carry the *inlA* gene; the yellow color indicates the presence of a loss-of-function mutation leading to a truncated InlA protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

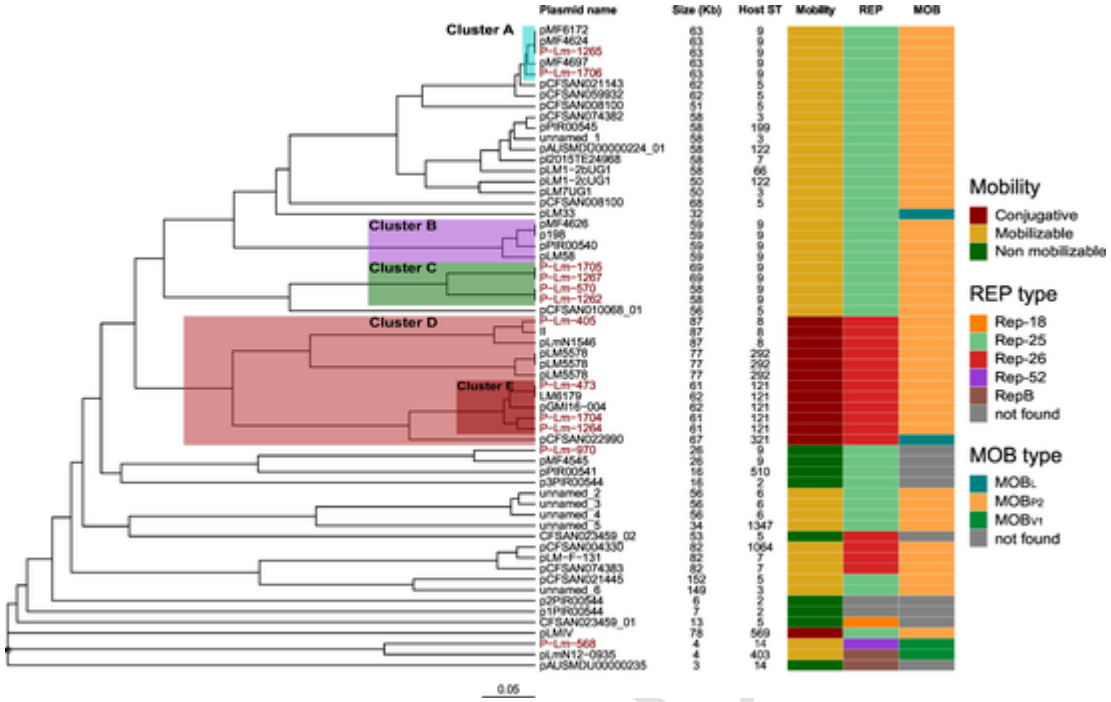

Fig. 3. Plasmid clustering.Dendrogram representation of the hierarchical clustering measuring binary distance based on the absence/presence of 510 clusters of similar CDSs. The size of the plasmid is shown in kilobase-pairs together with the information on the host ST. Mobility was predicted by MOBtyper. Type of REP protein and MOB protein are represented with color scales. Specific groups of plasmids have been highlighted following three criteria: the plasmid was hosted by a ST9 strain (cluster A, B and C); the plasmid was predicted as conjugative (cluster D); the plasmid was hosted by a ST121 strain (Cluster E).

no MOB detected, six of them also of small size, ranging from 1 to 12.5 Kb, and high copy number, and three of them (P-Lm-970, pMF4545 and CFSAN023459_02) of larger size, in the range of 25–100 Kb, and characterized as non-mobilizable.

The main clusters of plasmids shared as common features two genes of resistance to cadmium (*cadA* and *cadC*). In addition, with the exception of plasmids from cluster C, all of them carried genes encoding a FIC domain protein involved in cellular invasion, as well as a Clp protease that helps *L. monocytogenes* escape detection by the host immune system. On the other hand, different clusters of plasmids harbored a range of different genetic determinants potentially linked to persistence in food processing environments, through metal, biocide or stress resistance mechanisms (Table 2, Fig. 4). Plasmids in cluster A (including P-Lm-1265 and P-Lm-1706) harbor genes encoding a glycine ABC transporter and a FAD oxidoreductase, potentially associated with the response to osmotic and oxidative stress, respectively, and genes of resistance to benzalkonium chloride (*bcrABC* cassette). Plasmids from cluster B carry different genes related to the protection against heavy metals, like a gene encoding a heavy metal translocating ATPase, or *mco* and *copB*, involved in copper resistance, genes of response to oxidative stress conditions, such as a gene encoding a FAD dependent oxidoreductase, together with *fetB,* and *yhdK*, encoding a response regulator involved in the cell envelope stress response in Gram positive bacteria. Cluster C is formed by P-Lm-570, P-Lm-1262, P-Lm-1267 and P-Lm-1705 and comprises two different sets of plasmids that are distinguished by the presence in two of them (P-Lm-1267 and P-Lm-1705) of a 9 Kb fragment that contains arsenic resistance genes and the *umuC* gene, encoding a protein involved in the repair of damages caused by ultraviolet light (Fig. 4). Plasmids from cluster C also contain the genes *zosA* (encoding a zinc transporter), *rapA* (encoding a protein involved in the response to salt stress), and *crcB_1* and *crcB_2* genes (associated with fluoride tolerance). Plasmids from cluster E, which includes P-Lm-473, P-Lm-1264 and P-Lm-1704, harbor a gene encoding a CPBP metalloprotease involved in resistance to bacteriocins.

4. Discussion

The prevalence of *L. monocytogenes* observed throughout the 1.5 year-long survey here conducted (7.9%) is in line with that reported in previous similar studies monitoring the occurrence of *L. monocytogenes* in food processing environments (Alvarez-Ordóñez et al., 2018; Madden et al., 2018), despite the use of BPW instead of Half Fraser broth at the first enrichment step of the isolation procedure, which could have resulted in a reduced recovery of *L. monocytogenes* throughout the study. As expected, *L. monocytogenes* was not detected in the first sampling visits, when the meat processing activities had not yet started or a few pig carcasses were processed to test the new equipment and processing lines. From then onwards, the frequency of isolation sharply increased along time, which evidences that *L. monocytogenes* is well equipped to thrive and compete in the harsh environments of food processing plants, as it has been previously reported (Bucur et al., 2018). In fact, non-food contact surfaces and, in particular, drains, with prevalences of 11.9% and 14.9%, respectively, were revealed as important reservoirs of *L. monocytogenes* in the meat processing plant. Drains have been previously implicated as relevant harborage sites of *L. monocytogenes* in food production facilities, which frequently lead to cycles of recontamination (Hoelzer et al., 2011; Zhao et al., 2006). This could be due to the fact that drains extract run-off from food processing activities, including organic matter and low concentrations of chemical agents used during cleaning and disinfection, and they are sometimes not properly designed or well-maintained (Dzieciol et al., 2016).

All *L. monocytogenes* strains isolated belonged to serotype 1/2a or 1/2c and, therefore, to lineage II. Lineage II strains have an increased genomic plasticity and are predominantly isolated from a broad diversity of sources, including foods and food processing environments, than strains from other lineages (Gray et al., 2004). Also, the STs detected (ST8, ST9, ST14, ST37, ST121 and ST155) are among the most prevalent STs isolated from foods and food processing environments (Fagerlund et al., 2016; Pasquali et al., 2018). The most prevalent ST was

Table 2
Relevant features for environmental persistence conveyed by plasmids of different clusters.

		Cluster A	Cluster B	Cluster C	Cluster E
Metal resistance	Arsenic			*arsA, arsC, arsD and acr3* *	
	Cadmium	*cadA, cadC*	*cadA, cadC*	*cadA, cadC*	*cadA, cadC*
	Copper		*copB, mco*	*copB, copY*	
	Zinc			*zosA*	
	General		Heavy metal translocator		
Stress response	Bacteriocin				CPBP metalloprotease
	Cell wall		*yhdK*		
	Osmotic	Glycyne ABC transporter			
	Oxidative	FAD oxidoreductase	FAD oxidoreductase, *fetB*		
	Saline			*rapA*	
	Temperature	Clp protease	Clp protease		Clp protease
	Ultraviolet light			*umuC**	
Biocides resistance	Benzalkonium chloride	*bcrA, bcrB* and *bcrC* cassette			
	Fluorides			*crcB_1, crcB_2*	

This summary table has been extracted after comparison of BRIG output files, * genes present in a 9 Kb fragment that differentiates two kinds of plasmids in cluster C.

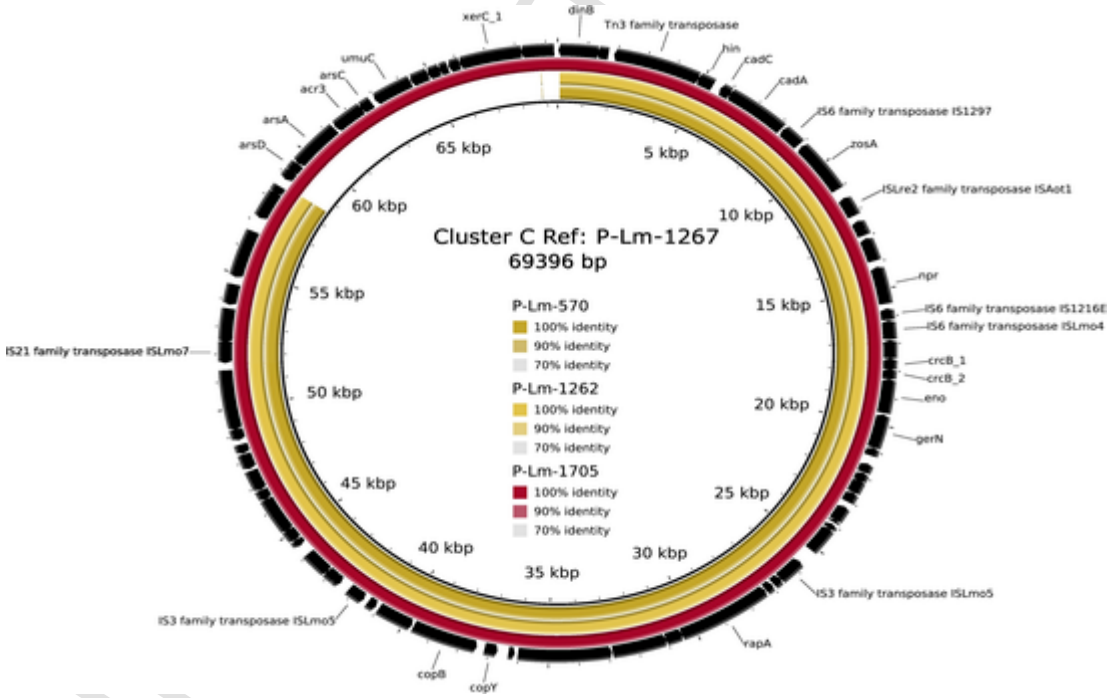

Fig. 4. Cluster C BRIGG comparison.BRIGG output comparing three plasmids of cluster C (P-Lm-570, P-Lm-1262 and P-Lm-1705) against the reference P-Lm-1267. The external black ring and labels correspond to the CDSs detected by Prokka. Gaps in colored rings indicate the absence of specific genes as compared to the reference plasmid sequence.

ST9 (44% of all the strains isolated from the facility belonged to it), which, together with ST121, has been frequently detected in food processing environments, often related to pork products and associated with episodes of persistence in the processing environment (Fagerlund et al., 2018; Li et al., 2018). Remarkably, this ST has been previously associated with foodborne cases of listeriosis (Chen et al., 2019; Fagerlund et al., 2018; Li et al., 2018; Martín et al., 2014), although it is more commonly linked to contamination of food and food processing environments. Indeed, Maury and co-workers have defined

ST9 and ST121 as hypovirulent clones adapted to food processing environments (Maury et al., 2016).

In the current study, two pairs of nearly identical ST9 isolates were obtained, in two consecutive samplings separated 3 months apart, from the same processing rooms, R5 (Lm-1267 and Lm-1705) and R6 (Lm-1265 and Lm-1706). These two ST9 strains could be considered as persistent strains in the food processing environment.

It is worth highlighting that a detailed analysis of the ST9 genomes available in the NCBI database, and their associated metadata, evidenced that ST9 strains are frequently linked to meat, and specifically to pork products, suggesting that this ST is particularly adapted to the micro-environments prevailing in meat and pork processing plants. Isolates from ST9 in the NCBI database have been mainly reported in Europe and North America (Supplementary Fig. S1A). Interestingly, a hierarchical clustering based on the presence/absence of 1748 *loci* of the cgMLST scheme by Moura et al. showed that *L. monocytogenes* ST9 strains were grouped mainly according to their geographic origin, with most of the North American and European genomes being located in different branches of the tree (Fig. 5). All ST9 genomes from the strains isolated in the current study (red dots, Fig. 5) grouped together with most European ST9 genomes downloaded from the NCBI database. Regarding their isolation source, ST9 strains are more frequently isolated from food samples (59%) than strains from other STs (34%) (*Z*-test *p*-value < 0.001; Fig. S1B), while other STs are more frequently associated with clinical samples (25%) than ST9 strains (6%) (Z-test p-value < 0.001; Supplementary Fig. S1B). Among those strains isolated from food samples, ST9 strains are more frequently isolated from meat (33%) and RTE products (42%) than other STs (19% and 31%, respectively), while other STs are more frequently associated with fruits and vegetables (14%) than ST9 strains (2%) (Z-test p-value <0.001; Supplementary Fig. S1C). Some RTE products, such as deli pork or salami-like products are among the main food sources from which ST9 has been previously isolated (Nastasijevic et al., 2017; Stessl et al., 2014).

In nature, *L. monocytogenes* strains exhibit considerable diversity concerning virulence-associated phenotypes, with the occurrence of both hypervirulent and hypovirulent clones (Maury et al., 2016). In our survey, most of the *L. monocytogenes* strains recovered from the facility showed an attenuated in silico virulence potential either through the truncation of InlA (in 67% of the isolates) or the absence of other virulence factors involved in cell adhesion or invasion. Studies on *inlA* have shown that mutations leading to premature stop codons naturally occur in *L. monocytogenes* and are associated with a hypovirulent phenotype. Of note, increasing evidence suggests that hypovirulent *L. monocytogenes* strains and strains with *inlA* premature stop codons are more frequently isolated from foods and food processing environments than from clinical samples and other isolation sources (Ferreira da Silva et al., 2017; Tamburro et al., 2010). These findings suggest that, in *L. monocytogenes*, virulence potential can cause fitness costs in environmental reservoirs. This has been previously observed in a number of pathogens, for which environmental isolates are seldom as virulent as clinical isolates, pointing out that traits needed for survival and persistence in the environment are traded off with traits linked to virulence so that an investment in one trait leads to a corresponding decrease in another (Maury et al., 2019; Mikonranta et al., 2012).

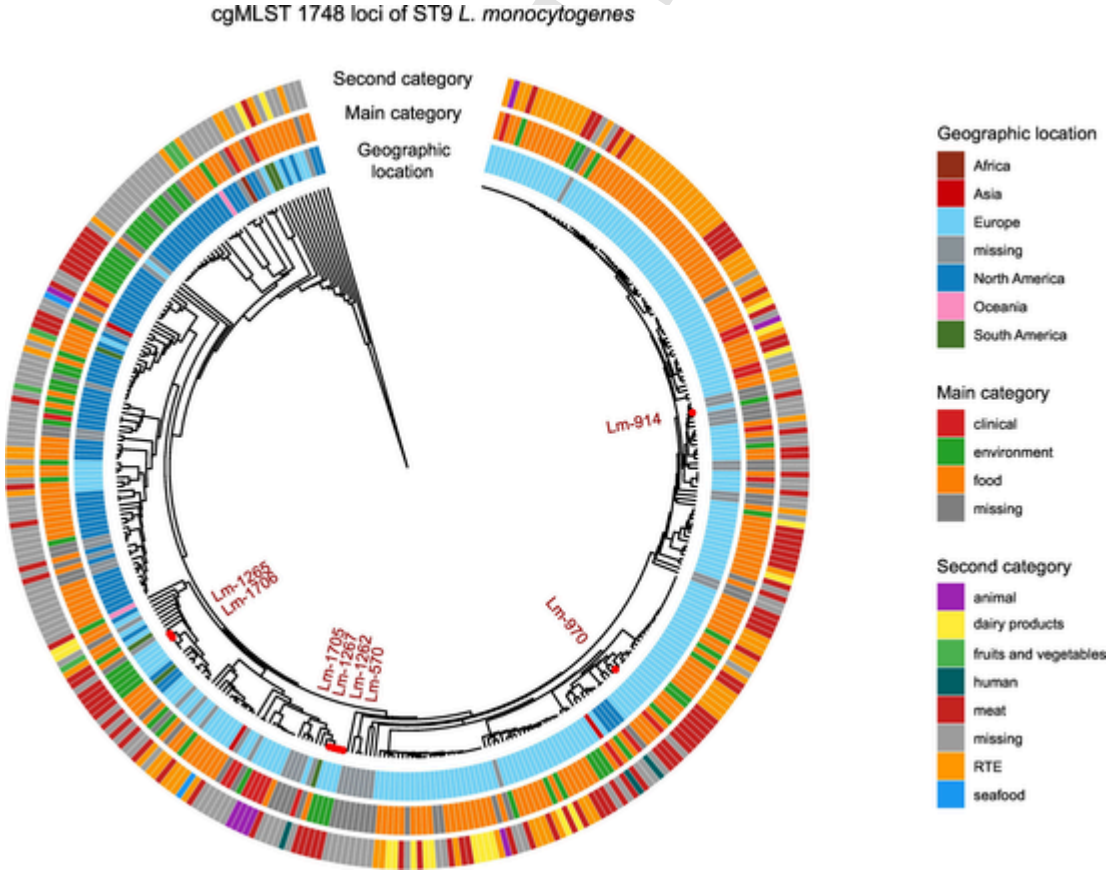

Fig. 5. ST9 dendrogram.Allelic distance clustering representation of the 360 ST9 genomes of *L. monocytogenes* available at the NCBI database, plus the eight ST9 genomes obtained from the strains isolated from the meat processing facility sampled (red points), using the 1748 *loci* cgMLST scheme from Moura et al. The first ring shows the continent of origin of the isolates, the second ring shows a general categorization of the source of isolation (food, environmental, clinical, or missing/unknown), and the third ring subclassifies the clinical isolates depending on the host (animal or human) and the food/environmental isolates depending on the food production sector (meat, fish, dairy products, fruits and vegetables, Ready-To-Eat [RTE], or missing/unknown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Most of the *L. monocytogenes* isolates contained one of the two known stress survival islets, SSI-1 or SSI-2, and/or plasmids carrying genes associated with resistance to stress conditions, heavy metals or biocides, which may be responsible for the survival and development of *L. monocytogenes* under the harsh conditions prevailing in food processing plants. SSI-1 is one of the many genetic determinants that help explain the ubiquity of *L. monocytogenes* in such a wide range of temperatures, pH or salinity conditions, as this element encodes mechanisms which favor the growth of *L. monocytogenes* at high acidity and/or high salinity conditions (Fagerlund et al., 2016). SSI-2 seems to be spread among ST121 isolates (Harter et al., 2017), as it was the case in our study, and offers protection against alkaline pH conditions.

The frequency with which plasmids were detected on the strains isolated in this study (in 67% of the isolates) is in agreement with the observations made by other authors (Hingston et al., 2019). Plasmids hosted by members of the *Firmicutes* phylum (e.g., *Staphylococcus, Enterococcus* or *Listeria*) usually share a pool of identical or homologous genetic determinants (Lanza et al., 2015). Remarkably, plasmids found in the genus *Listeria* commonly are indigenous plasmids harboring determinants of stress survival and metal and biocides resistance genes, while plasmids from other distant bacterial species more frequently contain antimicrobial resistance or virulence genes, which are uncommon in *L. monocytogenes* plasmids probably due to their high fitness cost (Baquero et al., 2020).

Among the plasmids here identified, several belonged to groups of plasmids of similar sizes and content, and they were associated with ST9 (clusters A and C) or ST121 (cluster E) *L. monocytogenes* strains. The high level of specialization that makes ST9 and ST121 some of the STs more prevalently found at food processing facilities (Chen et al., 2019; Martín et al., 2014) might be, at least in part, a consequence of the higher disposition of this STs to harbor plasmids which encode persistence determinants involved in resistance to stress, biocides and heavy metals.

Identical or highly similar plasmids were identified in the current study for various sets of *L. monocytogenes* ST9 isolates (e.g., P-Lm-1265 and P-Lm-1706; P-Lm-1267 and P-Lm-1705; P-Lm-570 and P-Lm-1262), which suggests a clonal expansion and persistence of plasmid-containing ST9 strains in the processing environments of the meat facility. Remarkably, the plasmids P-Lm-1267 and P-Lm-1705 differed from P-Lm-570 and P-Lm-1262 only in a 9-Kb DNA fragment, and the four of them were hosted by strains from the same CT (CT 6628) which were separated by a very low SNP distance in their core genome (from 2 to 19 SNPs). It is unknown whether an evolution occurred from the Lm-570 strain, isolated in December 2017, with the acquisition by descendant and persistent strains (like those isolated on October 2018 and January 2019) of such 9-Kb DNA fragment in situ, in the environment of the processing plant, or whether highly similar plasmid-containing strains accessed the processing plant independently and at different time points throughout the survey.

The main plasmid clusters here detected in ST9 and ST121 isolates harbored genetic determinants of resistance to biocides, such as the *bcrABC* cassette (Elhanafi et al., 2010) in plasmids of cluster A, involved in resistance to benzalkonium chloride (Cherifi et al., 2018; López-Alonso et al., 2020; Ortiz et al., 2016), or the *crcB_1* and *crcB_2* genes in plasmids of cluster C, implicated in resistance to fluorides (Liu et al., 2017), which may favor the persistence of these strains in food processing environments. They also contained several genes related to heavy metal resistance, involved in the cellular response to arsenic, zinc, cadmium or copper, which may allow *L. monocytogenes* to compete better in particular environments (Parsons et al., 2019). Finally, these plasmids clusters also carried genes associated with the response to stress, including determinants involved in the bacterial adaptation to high salinity conditions or oxidative stress, DNA repair systems specifically associated with the repair of damages caused by UV light, and mechanisms of defense to bacteriocins (detailed in Table 2), what agree with previous results reported by other research groups (Naditz et al., 2019; Nicolaou et al., 2013). Supplementary Table S5 shows the information collected through the execution of a questionnaire to the food business operator regarding the cleaning and disinfection practices followed at the facility and other aspects related to the plant characteristics and management. A range of different agents, containing phosphoric acid, sodium hydroxide, sodium hypochlorite, and peracetic acid as active compounds, are routinely used at the processing plant. The management team did not communicate any change in sanitation practices or the introduction of corrective measures along the study to tackle *L. monocytogenes* contamination.

To conclude, WGS has allowed to gain insights on the diversity of *L. monocytogenes* strains colonizing a newly opened meat processing facility over 1.5 years, providing also information on their possible persistence in the processing environments. A detailed analysis of *L. monocytogenes* plasmids detected at the facility and those retrieved from the NCBI database revealed groups of similar plasmids occurring among prevalent and persistent *L. monocytogenes* STs (ST9, ST121) which may contribute to their wide spread in food industries through stress, metal and biocides resistance traits. Finally, a broader comparison with *L. monocytogenes* genomes available in public databases allowed to corroborate the relevance of ST9 strains in meat processing industries and evidenced the need of undertaking further research activities focused on the characterization of this ST (and other persistent STs) in order to reduce their impact in food safety.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109043.

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Declaration of competing interest

The authors declare no conflicts of interests.

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