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Unified Sampling Across European Food Processing Environments Reveals Complex Genetic and Contextual Factors Underlying Listeria monocytogenes Persistence

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Unified Sampling Across European Food Processing Environments Reveals Complex Genetic and Contextual Factors Underlying *Listeria monocytogenes* Persistence

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Abstract

Listeria (L.) monocytogenes is a major foodborne pathogen that can persist in food processing environments for months or even years, posing a risk for product contamination and listeriosis outbreaks. Despite routine environmental monitoring and studies investigating the factors underlying persistence, genetic and phenotypic traits do not fully explain why some strains are able to persist. In this study, 1,538 environmental swabs across Europe (Austria, Greece, and Spain) were collected to assess distribution and persistence. Overall, 22.0% of samples tested positive, particularly on non-food contact surfaces and in post-cleaning samples. We sequenced 316 isolates representing 18 sequence types (STs) and 69 cgMLST types. SNP clusters (≤10 SNPs) detected on ≥3 different sampling dates were classified as persistent. In total, 177 isolates belonged to persistent clusters. An additional 37 historical isolates from facility D (2020-2023) were analyzed with the same criteria and formed a persistent SNP cluster. Persistent SNP clusters were detected in all facility types. Pangenome-wide analysis of the accessory genome (2,268 gene families) identified 541 gene families associated with persistence or non-persistence, including 339 associated with persistence and 202 with non-persistence. These were frequently annotated with functions in DNA replication, recombination and repair, transcription regulation and mobile genetic elements, whereas gene families annotated with metabolism, nutrient processing and intracellular trafficking were associated with non-persistence. Virulence and environmental stress tolerance gene profiles were driven more by ST type rather than persistence classification. The high detection rate in this study shows that *L. monocytogenes* is widespread and persistent

clusters occur under highly dynamic and heterogeneous conditions present across food production environments. In summary, our findings underscore the need to integrate genomic, ecological and environmental data to predict and control persistence.

Keywords

- *Listeria monocytogenes*
- Persistence
- Food Processing Environments
- Environmental sampling
- Whole genome sequencing
- cgMLST
- Pangenome Analysis
- Genomic diversity

Introduction

Listeria monocytogenes is the causative agent of listeriosis, a foodborne illness currently considered the most serious under EU surveillance (European Centre for Disease Prevention and Control, 2025). In 2023, the EU/EEA countries reported 2,933 confirmed listeriosis cases with 340 deaths, which is the highest annual number recorded since listeriosis surveillance began in 2007. Because *L. monocytogenes* is abundant in various natural, agricultural, and even urban environments, it is highly adapted to diverse environmental conditions and exhibits strong resilience to environmental stressors (Chandler-Khayd et al., 2023; Liao et al., 2021; Raschle et al., 2021; Sauders et al., 2012; Stea et al., 2015; Vivant et al., 2013). These environments (e.g. soils and fresh waters) may serve as a source for transmission into the food chain via raw materials (animals, vegetables, or fruits), moving vehicles (transportation trucks or forklifts), or workers introducing the pathogen into food processing facilities.

Once introduced into the food processing environment, some *L. monocytogenes* strains can survive for months or even years despite hygiene measures applied by industry professionals, as they often harbor disinfection resistance genes (Palaiodimou et al., 2021; Roedel et al., 2019). Such strains are referred to as persistent and can be repeatedly detected from the same food processing facility over an extended period (Harrand et al., 2020; Palma et al., 2020). Persistent *L. monocytogenes* in food processing environments present a continual threat of product contamination. Under favorable conditions in the final product, the bacterium can grow to cell

numbers sufficiently high to cause human infection and listeriosis outbreaks (Ferreira et al., 2014; Nüesch-Inderbinen et al., 2021; Olsen et al., 2005; Wenger et al., 1990).

Several studies have investigated the potential for genotype- and gene-level associations with persistence of *L. monocytogenes*, including links to specific lineages and clonal complexes (CCs). In the context of persistence, it is often observed that most of the isolates belong to Lineage II (Fagerlund et al., 2022; Orsi et al., 2011; Palaiodimou et al., 2021; Wagner et al., 2021). Multi-locus sequence typing (MLST) further divides these lineages into clonal complexes and sequence types (STs). Within Lineage II, CC9, CC8, and CC121 are frequently associated with persistence, while in Lineage I, CC2, CC5, CC6 and CC87 have been reported to be frequently associated with persistence in the food processing environment (Alteio et al., 2025; Hingston et al., 2017; Maury et al., 2019; Stasiewicz et al., 2015).

Beyond CC and ST patterns related to persistence, several genes have been investigated for their potential role in persistence in previous studies but have yet to be robustly linked. These genes include biocide tolerance genes (e.g. *bcrABC*, *qacH*) (Harrand et al., 2020; Palaiodimou et al., 2021), stress survival genes (e.g. *SSI-1*, *SSI-2*) (Centorotola et al., 2021; Chen et al., 2022), biofilm formation genes (e.g. *bapL*) (Castro et al., 2021), and mobile genetic elements such as plasmids (*e.g. pLM33 and pLM5578) (Gilmour et al., 2010; Kuenne et al., 2010)* and prophages (Castro et al., 2021; Schmitz-Esser et al., 2021). Plasmids, which are more prevalent in Lineage II, often carry cadmium resistance genes (e.g. *cadA1* and *cadA2)*, quaternary ammonium compounds (QACs) resistance genes (e.g. *bcrABC*), as well as heat shock proteins (e.g. *clpL)* (Anast et al., 2022; Chen et al., 2020; Hingston et al., 2019, 2017; Schmitz-Esser et al., 2021). Prophages also appear more

frequent in persistent than in non-persistent strains (Castro et al., 2021). Some researchers hypothesize a trade-off between survival traits and virulence potential, as persistent isolates are often associated with reduced virulence compared to clinical isolates (Alvarez-Molina et al., 2021; Maury et al., 2019; Mikonranta et al., 2012). The evolutionary dynamics by which these traits are gained or lost remains unclear, and some persistent *L. monocytogenes* strains caused listeriosis outbreaks in the past (Ferreira et al., 2014; Nüesch-Inderbinen et al., 2021; Orsi et al., 2008).

While these findings point out genomic associations of mobile genetic elements and environmental resistance genes with persistence, a comprehensive understanding of the biological mechanisms that underly the persistence phenomenon is still lacking (Alteio et al., 2025; Belias et al., 2022). Methodological inconsistencies contribute to this gap. Studies use different isolation strategies (e.g., different selection media) to obtain isolates (Bruhn et al., 2005; Pitti et al., 2025; Tuytschaever et al., 2026). Moreover, various typing methods are used, including Pulsed-Field Gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD), Ribotyping, Multivirulence-Locus Sequence Typing (MVLST), and more recently Whole Genome Sequencing (WGS). Consequently, cross-study comparisons are very difficult and may lead to variability in findings. Among these methods, WGS offers the highest resolution and has become the most widely used “gold standard” approach in this field of research in recent years (Pennone et al., 2022). Furthermore, whole genome sequencing of isolates will become mandatory in the EU for epidemiological investigations of foodborne illnesses (Costa et al., 2022). Gaining higher resolution provides detailed information about gene presence or absence, gene structure (e.g.

115 truncations), and enables a high-quality comparison of genomes based on the single-nucleotide
116 polymorphisms (SNP).

117 A major challenge is the lack of a universal definition for persistence in literature, which affects
118 research and practice. The criteria for identifying the “same” strain, such as frequency of isolation
119 and SNP differences across the whole genome, vary between studies. Previous studies applying
120 SNP cutoffs range between 10 to 25 SNPs to be considered identical (Koutsoumanis et al., 2024;
121 Pightling et al., 2018; van de Merwe et al., 2024). Some authors even propose using different SNP
122 cutoffs to describe levels of relatedness (Bolten et al., 2024). Using very stringent SNP cutoffs to
123 define persistence can be problematic because the mutation rate of *L. monocytognes* in food
124 processing environments is estimated to ~0.35 nucleotides per year (Harrand et al., 2020). This
125 estimate is based on one cluster belonging to ST321 and may differ in other STs or be highly
126 dependent on the environmental context. Moreover, sequencing errors can affect SNP calling.
127 Therefore, reliable classification of persistent isolates based on SNP differences depend on high
128 sequencing coverage, >50x (Pham et al., 2025; Stoler and Nekrutenko, 2021).

129 Persistence is likely driven by a combination of factors rather than a single mechanism, including
130 genetic traits, environmental conditions, species interactions, and microbial physiology. This lack
131 of clarity makes it challenging for industry professionals to develop and apply targeted action
132 plans to identify persistent *L. monocytogenes*, manage them accordingly, and eliminate them
133 successfully. There is a need for a unified methodology and standardized criteria for investigating
134 persistence. This standardization would improve the data comparability between studies and

support the development of specialized databases which could be used for advanced modeling to identify the key drivers of persistence and guide future research efforts.

In this study, we applied a unified approach to investigate persistence of *L. monocytogenes* across European food-processing facilities. We conducted a coordinated sampling campaign in food processing facilities across three countries in the European Union using a standardized sampling protocol, and data analysis approach to ensure data comparability. Here, we report the detection of *L. monocytogenes* in the food processing environment, apply a stringent definition for persistence at the SNP-cluster level, and identify genes associated with genomes from persistent SNP clusters found in European food processing facilities.

Materials and Methods

Facility Sampling

A minimum of three repeated samplings were conducted in food processing facilities across three European countries (Austria, Greece, and Spain) over the period of three calendar years (2023-2025). A coordinated sampling protocol was used to isolate *L. monocytogenes* and describe the environmental parameters. Project partners (*Austrian Competence Centre for Feed and Food Quality, Safety, and Innovation, Agricultural University of Athens and University of Burgos)* selected food processing facilities based on accessibility, with the aim of sampling facilities across food sectors.

In each sampling event, up to 50 environmental surface samples were collected using large polyurethane swabs pre-moistened with 10 ml of Hi-Cap™ Neutralizing Broth (Whirl-Pak® PolyProbe™). Approximately half of the samples were taken during production (at the dirty state, pre-cleaning and disinfection), and the other half after production (at the clean state, post-cleaning and disinfection) to also isolate *L. monocytogenes* that survive cleaning and disinfection. The swabbing covered up to 1 m^2 of surface area with suspected *L. monocytogenes* hotspots, including food contact and non-food contact areas (e.g., drains, conveyor belts, processing tables, processing machines). A processing control (swab only) was included. Metadata (surface material, humidity, and temperature) for each sample was recorded whenever possible. Samples were transported to the laboratory at 4°C and processed within 24-48 hours.

Sample Pre-Processing and *L. monocytogenes* Enrichment

In the laboratory, 10 ml of pre-cooled sterile 1× phosphate-buffered saline (PBS) was added to each swab bag. Samples were homogenized with a Stomacher lab blender (BagMixer, Interscience) for 1 min at medium speed. The homogenized material was subjected to a two-step enrichment of *L. monocytogenes* following a modified ISO 11290-1:2017 enrichment protocol (International Organization for Standardization, 2017). For the primary enrichment step, 70 mL of Half-Fraser broth (Biokar Diagnostics, Beauvais, France) was added and incubated for 48 h at 30 °C. After this incubation, 1 ml of the culture was transferred into a 1.5 ml sterile Eppendorf tube and centrifuged at 8,000 × g for 5 min. The supernatant was discarded, and the pellet was resuspended in 50 µl of 1× PBS and streaked on RAPID'L.mono agar (Bio-Rad, Marnes-la-

Coquette, France), which was incubated at 37 °C for 24h. For the secondary enrichment, 1 ml of the Half-Fraser culture was used to inoculate 10 ml of Full-Fraser broth (Biokar Diagnostics, Beauvais, France), which was incubated for 48 h at 37 °C. The secondary enrichments were plated as described above. For each step, negative controls were included to verify sterility throughout the process.

L. monocytogenes Isolation and PCR Confirmation

From the secondary enrichment cultures on RAPID'L.mono agar, one to four presumptive *L. monocytogenes* colonies were selected. Each colony was divided: half was streaked onto fresh Tryptic Soy Agar (TSA; Merck, Darmstadt, Germany) supplemented with 0.6% yeast extract, and the other half was subjected to DNA extraction. The DNA extraction followed a protocol modified from Walsh et al. (1991), in which pure cultures were lysed in 100 µl of 0.01 M Tris/HCl pH=7 (Trizma®base, Sigma-Aldrich, St. Louis, United States) and 400 µl of 2.5% Chelex®100 Resin solution (BioRad, Hercules, United States) at 95°C for 10 min, followed by centrifugation with 15,000 × g for 30 s. A volume of 150 µL of the resulting supernatant was transferred into a fresh sterile tube and used as a template DNA (2 µl) for multiplex PCR (Border et al., 1990). The assay targeted the *hly* gene, encoding listeriolysin O for identification of *L. monocytogenes* (primer sequences: LM1-5'-CCT AAG ACG CCA ATC GAA-3', LM2-5'-AAG CAC TTG CAA CTG CTC-3'), and a fragment of 16S rRNA gene for detection of *Listeria* spp. (primer sequences: U1-5'-CAG CAG CCG CGG TAA TAC-3', L11-5'-CTC CAT AAA GGT GAC CCT-3'). The 25 µl PCR reaction contained 1.25 U/µl Platinum™ Taq DNA-Polymerase (Invitrogen™, Vilnius, Lithuania), 1 × TaqMan PCR buffer, 1.5 mM

193 $MgCl_2$, 1,08 µM of each of the primers (Microsynth, Austria), and 200 μM dNTP Mix (Thermo
194 Scientific™, Vilnius, Lithuania). PCR cycling conditions were: 94 °C for 2 min (*Taq* activation)
195 followed by 30 cycles of 94°C for 30 s (denaturation), 50 °C for 30s (annealing), and 72 °C for 1
196 min (elongation). Each PCR run also included a positive control (*L. monocytogenes* EDGe, DSMZ,
197 Braunschweig, Germany) and a negative control (water for molecular biology, Sigma Aldrich, St.
198 Louis, USA). The amplification products were visualized via agarose gel electrophoresis.

199 Isolates Handling

200 For PCR, confirmed isolates were obtained by preparing overnight cultures (Tryptic Soy Broth +
201 0.6% yeast extract; Merck, Darmstadt, Germany) from the respective TSA agar plates and
202 incubated at 37 °C. From these cultures, glycerol stocks (final concentration of 25 %) were
203 prepared and stored at -80 °C at the respective partner institution. Individual isolates (one colony
204 per swab sample) were streaked onto TSA agar slants and shipped to the respective sequencing
205 facilities for whole genome sequencing. From Greece and Spain 243 isolates were sent to Nestlé
206 Research in Switzerland. From Spain 38 isolates were sequenced at the University of Valladolid,
207 Spain, and 35 isolates from Austria were sequenced at the National Reference Centre/Laboratory
208 for Listeria Austrian Agency for Health and Food Safety (AGES) in Graz, Austria.

209 At Nestlé Research, the isolates were streaked onto ALOA agar (Agar Listeria according to
210 Ottaviani & Agosti, bioMérieux, France) to verify purity. A single colony was picked, transferred to
211 TSA agar, and incubated overnight at 37 °C. These cultures were used next to inoculate Brain Heart
212 Infusion broth (BHI, Oxoid, UK) and incubated at 37 °C for 6 h. One ml of culture was harvested

into sterile 1.5 ml Eppendorf tubes and pelleted at 10,000 × g for 5 min. The supernatant was discarded, and the pellet was stored at -25 °C.

DNA Extraction, Library Preparation and Sequencing

At Nestlé Research, the genomic DNA was extracted using the Promega Maxwell® RSC Cultured Cells DNA Kit was used. Cell pellets were thawed, resuspended 160 µl of P1 buffer and 20 µl of lysozyme (0.2 mg/ µl), vortexed, briefly centrifuged, and incubated at 37 °C for 30 min. After this treatment, DNA was extracted using a Promega Maxwell® RSC Instrument according to the manufacturer's instructions. DNA concentrations were determined using a Quant-iT™ PicoGreen™ dsDNA Assay Kit and normalized to 100 ng using a Myra Liquid Handling System (Bio Molecular Systems). The library preparation was performed using Illumina DNA Prep kits following the Illumina Reference Guide (Illumina, 2021). Before pooling, the libraries were quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit and assessed for fragment size distribution using a fragment analyzer (Advanced Analytical). After determining the correct fragment concentration based on peak integration from the fragment analyzer, the library was pooled using a Myra Liquid Handling System (Bio Molecular Systems) and sequenced with 300 bp paired-end reads on the Illumina NextSeq 2000.

At the hospital in Valladolid, the isolates were grown overnight in BHI broth (BHI, Oxoid, UK) overnight at 37 °C. A 300 µL aliquot of culture was transferred into sterile 1.5 mL Eppendorf tubes and pelleted at 10,000 g for 5 min. The supernatant was discarded, and the pellet was stored at -20 °C. Genomic DNA was extracted using the INDIMAG Pathogen Kit (Indical Bioscience; Ref.

SP947457) on a KingFisher extraction platform. DNA libraries were prepared using the Nextera DNA Prep kit (Illumina, 2016) according to the manufacturer's instructions. Library concentrations were quantified using a Qubit (Thermo Fisher Scientific, Waltham, USA), and libraries were normalized by equimolar pooling based on Qubit-measured concentrations. Sequencing was performed on an Illumina NextSeq platform generating paired-end reads of 35 bp.

At the National Reference Laboratory for Listeria (AGES), genomic DNA was extracted from Brain Heart Infusion broth (BHI, Oxoid, UK) overnight cultures at 37 °C, using the MagAttract HMW DNA Kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA was subsequently used for sequence library preparations with the Nextera XT DNA Library Preparation Kit (Illumina, 2016). Libraries were prepared following the manufacturer's protocol and sequenced on an Illumina MiSeq instrument using 300 bp paired-end reads.

Data Processing and Bioinformatics

Analysis of Metadata from Food Processing Environments

Sampling metadata were analyzed and visualized in RStudio (Version 2024.12.1; R Core Team, 2025) using packages readxl (Version 1.4.5; Wickham and Bryan, 2015), writexl (Version 1.5.3, Ooms, 2017), and tidyverse (Version 2.0.0; Wickham et al., 2019) for data import and filtering. Tables were formatted using knitr (Version 1.50; Xie, 2025), and kableExtra (Version 1.4.0; Zhu, 2017). Finally, data were visualized with multi-panel plots arranged with packages gridExtra (Version 2.3; Auguie, 2010), grid (Version 4.3.2; R Core Team, 2025), ggrepel (Version 0.9.6; Slowikowski, 2016), gtable (Version 0.3.6; Wickham and Pedersen, 2012) and patchwork (Version

1.3.2; Pedersen, 2019) to ensure a clear layout and labelling. Color schemes were generated with viridis (Version 0.6.5; Garnier et al., 2023). All code used for these analyses is provided in Supplementary File S1 (R script).

Whole Genome Sequencing and Quality Control

All sequencing data were generated using sequencing-by-synthesis on Illumina platforms and processed through identical quality filtering and assembly pipelines to ensure comparability across datasets. Additional reads from historical isolates originating from facility D, provided by AGES, were isolated and sequenced as described above, and subsequently subjected to same quality-control and trimming procedures. Samples sequenced at the Hospital in Valladolid were assembled directly following quality assessment due to their shorter read lengths, whereas samples sequenced at Nestlé Research and the National Reference Laboratory for Listeria (AGES) underwent trimming prior to assembly, as described below.

Raw reads were quality-assessed using FastQC (v0.12.1, Andrews, 2010) and summary reports were generated using MultiQC (v1.19, Ewels, 2016). Adapter sequences and short reads shorter than 36 bp were removed using Trimmomatic (v0.39, Bolger et al., 2014) in paired-end mode. Illumina adapters were removed using the ILLUMINACLIP function. Low-quality bases (Phred score < 10) were trimmed from the start and end of reads. Additionally, a sliding window approach (5 bases) was applied to trim regions where the average quality score fell below 20. Reads shorter than 36 bp after trimming were discarded. The resulting reads were re-assessed for quality as described above.

Genome Assembly, Multi-Locus Sequence Typing and Identification of Persistent Strains (SNP Clusters)

De novo genome assembly was performed using SPAdes (v4.1.0; Prjibelski et al., 2020) with default k-mer size and the --careful option enabled to reduce mismatches. Assembly quality was assessed using QUAST (v5.0.2, Gurevich et al., 2013), and sequencing coverage was estimated using BBWrap (BBMap v39.06; Bushnell B, 2024). Whole genome multi-locus sequence typing (MLST typing) was conducted by the Austrian Agency for Health and Food Safety (AGES) using the typing scheme described by Ruppitsch et al. (2015) in Ridom SeqSphere Software (Version 10.5.1; Jünemann et al., 2013). Based on MLST results, sequences were grouped by complex types and facility of origin. In each group, a reference genome was selected based on the assembly metrics (lowest contig number, highest N50 value, genome size, and coverage). The reference genome was then used to run the CFSAN SNP Pipeline (v2.2.1; Davis et al., 2015) with default parameters to calculate the pairwise SNP differences across the isolates in the same group. Strains were classified as putatively persistent if they were isolated at least three different dates from the same facility (based on sampling metadata) and showed ≤10 SNP differences across the whole genome. Additionally, the CFSAN pipeline was run on the complete isolate dataset using a reference genome (selected by the same criteria as described above) to determine high-quality SNP differences. As each facility was sampled for only 7 to 13 months, this study can only describe repeated detection within approximately one year. The resulting SNP alignment was used to infer a maximum-likelihood phylogenetic tree using IQ-Tree (v 3.3.1; Wong et al., 2025) under GTR+ASC substitution model to account for ascertainment bias in SNP alignments. Branch support was

assessed using 1,000 ultrafast bootstrap replicates. The tree was midpoint-rooted and visualized in R using the packages ggtree (Version 4.0.4; Xu et al., 2022) and ggplot (Version 3.5.2; Valero-Mora, 2010). The color schemes were created with viridis (Version 0.6.5; Garnier et al., 2023).

Genome Annotation and Pangenome Analysis

All assembled genomes were annotated using PROKKA (v1.14.6; Seemann, 2014) with default parameters and database for initial gene prediction. Gene presence and absence analysis was performed using Panaroo (v1.5.2; Tonkin-Hill et al., 2020) with strict cleaning mode and core gene alignment. Panaroo groups orthologous genes into gene families, and each gene family was treated as one unit in all downstream analyses. For each gene, its frequency across all genomes was calculated. Gene families were categorized according to their prevalence across the 353 genomes as core (present in 100% of genomes), soft-core (present in ≥95% but <100% of genomes), shell (present in >15% but <95% of genomes), and cloud (present in ≤15% of genomes). Functional annotation (COG categories and PFAM domains) was assigned to each gene family with eggNOG-mapper (v2.1.13 database v5.0.2; Huerta-Cepas et al., 2017) using DIAMOND (v2.1.12; Buchfink et al., 2015). COG categories and PFAM domains are therefore annotations of gene families. The presence of virulence factors was investigated using Abricate (v1.0.1; Seemann T, 2020) with respective VFDB (Virulence Factor Database, nucleotide version, release 14.01.2025; Chen, 2005) and the presence of environmental stress tolerance genes were investigated with BacMET (Version 2.0; Pal et al., 2014)with default parameters (≥80% identity and ≥80% coverage). Partial, truncated or split genes were not distinguished, so “absent” means “not detected”. The

binary heatmap was generated using pheatmap (Version 1.0.13; Kolde, 2010) and color schemes were created with viridis (Version 0.6.5; Garnier et al., 2023).

Genetic Trait Association Analysis

To identify the genes associated with persistence, association testing was performed using Scoary (Version 1.6.16; Brynildsrud et al., 2016) with default parameters. Association testing was performed on Panaroo gene families (one test per gene family). PFAM domains and COG categories were not tested themselves. They were taken from the eggNOG-mapper annotation of each gene family and used only to describe the genes that were significant. Results were filtered for genes with strong statistical support and effect size: Benjamini-Hochberg adjusted p-value <0.05, and an odds ratio ≥ 2 (persistent associated genes) and ≤ 0.5 (for non-persistence-associated genes). Data visualizations were generated using ggplot2 (Version 3.5.2; Valero-Mora, 2010). All color schemes were designed with viridis (Version 0.6.5; Garnier et al., 2023).

Results

Surface Swab Survey Reveals High Positive Rate, Especially in Samples Post-Cleaning

A total of 1,538 swab samples were collected from food processing facilities in three European countries: Spain (n=835), Greece (n=400), and Austria (n=303). Sampling was conducted in three different facility types: meat (n=4), dairy (n=6), and fish (n=1), producing both ready-to-eat and

ready-to-cook products. The highest proportion of samples originated from dairy facilities (n=775), followed by meat (n=598) and fish (n=165). Samples were taken from both food contact surfaces (FCS, n=828) and non-food contact surfaces (NFCs, n=710). Of the samples, 975 were collected during active production, 406 after cleaning and disinfection, and 157 from areas not clearly attributable to either state due to irregular cleaning practices or their distance from production lines (Table 1).

Overall, 22.0% of the swab samples tested PCR positive for *L. monocytogenes* PCR. Meat processing facilities had the highest positive rate at 39.6%, followed by the fish facility 19.4%, and the dairy at 8.9%. Among surface types, FCS showed a positive rate of 20%, while NFCs were higher at 24.2%. Pre-disinfection samples had a positive rate of 22.1%, whereas samples post-cleaning and disinfection were even higher at 26.1%. Samples where the sanitation status was not reported had a positive rate of 10.8%. In total, 338 swab samples were positive for the detection of *L. monocytogenes*, and *L. monocytogenes* could be detected across all facilities, surface types and cleaning and disinfection states (Table 1).

Diverse *L. monocytogenes* Sequence Types Identified Across Facility Types

Of the 338 samples testing PCR-positive for *L. monocytogenes*, 317 isolates were selected for whole genome sequencing. Following trimming and quality control of the raw data, one genome failed at the assembly step due to contamination. In total, 316 genomes underwent subtyping using the Ridom SeqSphere+ software and were further analyzed.

Most isolates belonged to Lineage II (n=270), while a smaller proportion were from Lineage I (n=46) (Table S.1; Figure 1). Both meat and dairy processing facilities harbored isolates from Lineages I and II, with most isolates associated with Lineage II. In contrast, the fish facility only harbored isolates from Lineage II. The isolates could be classified into 18 different STs and 69 different cgMLSTs (Table S.1; Figure 1). Across all facility types, ST8 (n = 70), ST9 (n = 68), and ST121 (n = 46) were the most abundant STs, followed by ST204 (n = 31), ST37 (n = 19), ST7 (n = 17), ST155 (n = 17), ST451 (n = 11), ST6 (n = 10), and ST87 (n = 10). Less frequently detected STs included ST1113 (n = 6), ST4 (n = 3), ST5 (n = 2), and ST54 (n = 2). From ST1, ST21, ST217, and ST504, only a single isolate was detected. Except for ST6 (found in meat and dairy facilities) and ST121 (found in meat and fish facilities), all other sequence types were associated with a single facility type (Figure 1A).

The genomes were further characterized into cgMLSTs to classify them into clusters (Table S1), revealing considerable diversity within some STs. ST9 for example showed the highest diversity with 24 distinct cgMLSTs, followed by ST121 with 9 cgMLSTs, ST204 with 7 cgMLSTs, ST8 with 5 cgMLSTs, ST7 with 4 cgMLSTs, and ST37 with 3 cgMLSTs. In contrast, ST1, ST4, ST5, ST6, ST21, ST54, ST87, ST155, ST217, ST451 and ST504 contained only one to three cgMLSTs. These findings demonstrate the diversity of *L. monocytogenes* isolates found in the European food processing facilities sampled.

Classification of Persistent SNP Clusters Across Facilities Reveals Persistence as a Widespread Phenomenon

Potentially persistent strains (SNP clusters) were defined as groups of isolates belonging to the same ST and cgMLST type that were recovered from the same facility on at least three distinct sampling dates over the two-year sampling period and differed by ≤10 SNPs across the whole genome. Based on these criteria, 177 of the 316 genomes analysed (56.0%) were classified as belonging to potentially persistent strains (SNP clusters) (Table 2). Table 2 additionally lists the 37 historical isolates from facility D (see below).

Most persistent isolates were found in three meat facilities with 136 persistent genomes, followed by 10 in two dairy facilities and 31 in the single fish facility. Persistent isolates were detected in all facility types and countries. Facility A, which exhibited a high overall detection rate, also showed a correspondingly high number of persistent genomes (Table 2). In addition, 62 isolates belonged to cgMLST types detected on only one sampling date within a facility, and 29 isolates to cgMLST types detected on only two sampling dates. A further 48 isolates belonged to cgMLST types detected on three or more sampling dates, but these isolates did not meet the persistence criteria, as they did not form a SNP cluster (≤10 SNPs) across three or more sampling dates.

In total, 17 persistent SNP clusters were identified, representing 10 STs and 15 cgMLST types (Table 2). Two cgMLST types (CT22940 in facility A and CT22966 in facility E) each formed two separate clusters The STs with the highest numbers of persistent isolates were ST8 (n=66), ST121

(n=34), and ST9 (n=33), respectively (Figure 1B). These were followed by ST451, ST204, ST37, ST87, ST155, ST1113, and ST4, which ranged from 10 to 3 persistent isolates (Figure 1B, ST4 counts exclude the historical isolates). Except for ST121, which was found to be persistent in both fish and meat facilities, genomes from persistent clusters in ST8, ST9, ST37, ST87, ST155, ST451, and ST1113 originated exclusively from meat facilities, while persistent genomes in ST4 and ST204, were found only in dairy facilities (Figure 1B). ST9 showed the greatest diversity of cgMLST types (n = 4), followed by ST8 and ST121 (n = 2 each). All other STs containing persistent clusters (ST4, ST37, ST87, ST155, ST204, ST451, and ST1113) were represented by a single cgMLST type (n = 1) (Figure 1B; Table 2).

The broad distribution of persistent isolates across multiple STs and facility types is further illustrated in the phylogenetic tree (Figure S1). An additional 37 historical isolates from facility D (dairy), all belonging to ST4 (CT4605) and obtained outside the present sampling campaign, were included in the downstream analyses, resulting in a total of 353 isolates (Table S2). Including these, the ST4 cluster in facility D comprises 40 isolates (Table 2).

Pangenome-Wide Association Analysis Reveals Genes Associated with Persistence and Non-Persistence

Pangenome-wide association analysis across all 353 isolates revealed genes associated with persistence and non-persistence (Figure 2A, Table S3). In total, 4,569 gene families were identified across the 353 genomes, of which 2,301 were categorized as core, 262 as soft-core, 767 as shell and 1,239 as cloud gene families (Figure S2). Scoary reported 979 gene families with statistics, of

which 831 were significant after BH correction (364 associated with persistence and 467 with non-persistence, adjusted $p < 0.05$), while 148 were not significant after correction (Figure 2A). Applying an additional effect-size threshold (odds ratio ≥ 2 or ≤ 0.5) retained 541 high-confidence gene families, 339 associated with persistence and 202 with non-persistence. These genes were further annotated with eggNOG to obtain the corresponding Clusters of Orthologous Groups (COG) categories and Protein Family (PFAM) domains, which added functional context. After filtering for statistically significant associations with most categories containing domains that were both enriched and depleted (Figure 2B). A large proportion of genes could not be assigned to any COG category. In the unassigned category, 189 genes were enriched and 112 were depleted. Among functionally annotated categories, category S (function unknown) contained 83 enriched and 41 depleted genes, and category L (replication, recombination, and repair) contained 24 enriched and 8 depleted genes, and category K (transcription) we observed 13 to be enriched and ten to be depleted. All other categories had 10 or fewer enriched or depleted genes. Notably, only one gene was enriched in COG category C, only one was depleted in E, and only one gene was depleted in U, GM, and IU. Conversely, only one gene was enriched in KL, MT, N, and O.

In COG category K (transcription, Figure 3A), gene families associated with persistence were annotated with multiple helix-turn-helix (HTH) DNA-binding domains (e.g., HTH_3 and HTH_19) and transcription-associated domains such as AlbA_2 (DNA binding domain), HATPAse_c_4 (ATPase domain for ATP binding), PriCT_11 (primase domain), RrF2 (iron-dependent transcription regulator domain), GntR (transcriptional regulator domains), sigma70_r4_2 (transcription

indicator domain) and ComK (transcription factor domain). Gene families associated with non-persistence were annotated with other associated HTH domains, WYL (DNA damage response), MarR (transcription regulator), and Rep_trans (replication initiation domain).

In COG category L (replication, recombination and repair, Figure 3B), gene families associated with persistence were annotated with DNA maintenance and genome plasticity domains, including helicases (DnaB_2 and Helicase_C), primases and topoisomerases (Topoisom_bac, Toprim, and Toprim_Crpt), recombination proteins (Recombinase, Resolvase, and Zn_ribbon_recom), and DNA methyltransferases (N6_Mtase, N6_N4_Mtase, TypeIII_RM_meth, and Methylase_S). Prominently, many of these enriched domains are often encoded by prophage or mobile genetic elements.

In COG category S (function unknown, Figure 3C), gene families associated with persistence were annotated with uncharacterized domains (DUFs) as well as PFAMs that are involved in DNA packaging, replication, and regulation. Of these PFAMs, 19 were phage-associated. Gene families associated with non-persistence were annotated with other DNA-binding, repair, and transcriptional domains.

PFAM Domains in COG Categories with Uniform Association Direction

In COG categories where all significant gene families showed the same direction of association (K/L, M/T, N, N/U, O and Q for persistence; F, G/M and I/U for non-persistence), the following PFAM domains were detected (Figure 4A). In categories K and L, gene families annotated with Helicase_C (helicase C-terminal domain; involved in DNA unwinding), N6_Mtase (DNA

methyltransferase), ResIII (restriction endonuclease) and SNF2_N (ATPase domain involved in chromatin remodeling and transcriptional regulation) were found only among gene families associated with persistence (Figure 4A). In COG category O, gene families annotated with HTH_3 (helix-turn-helix DNA-binding domain) were found only among gene families associated with persistence, as were gene families annotated with NLPC_P60 (cell wall-associated hydrolase domain) and SH3_3 (bacterial SH3 domain) in categories M and T (Figure 4A). In COG category N, this applied to gene families annotated with Big_2 (bacterial immunoglobulin-like domain). In COG categories N and U, it applied to gene families annotated with LRR_5 (leucine-rich repeat), MucBP (mucin-binding protein domain), WxL (cell wall surface-binding domain) and DUF285 (domain of unknown function). In the same categories, it applied to gene families annotated with T2SSE (type II secretion system protein E) (Figure 4A). Finally, in COG category Q, gene families annotated with Cu-oxidase, Cu-oxidase_2 and Cu-oxidase_3 (multicopper oxidases), Cupredoxin_1 (type I copper centre) and Gram_pos_anchor (Gram-positive cell wall anchor domain) were found only among gene families associated with persistence (Figure 4A).

Five PFAM annotations were found only in gene families associated with non-persistence. These gene families were assigned to COG categories F (nucleotide transport and metabolism), G (carbohydrate transport and metabolism), M (cell wall/membrane/envelope biogenesis), I (lipid transport and metabolism) and U (intracellular trafficking, secretion and vesicular transport) (Figure 4B). In COG category F, gene families annotated with SIR2_2 (SIR2-like domain) and DUF928 (domain of unknown function) were found only among gene families associated with non-persistence, whereas the gene family group_629 had no PFAM annotation. In COG categories

G and M, this applied to NAD_binding_10 (NAD(P)-binding domain), and in categories I and U to Lipase_3 (lipase domain).

Virulence and Environmental Stress Tolerance Gene Profiles are Broadly Conserved Across Persistent and Non-Persistent Isolates

Analysis of virulence genes using the classification based on VFDB (Virulence Factor Database, nucleotide version, release 14.01.2025; Chen, 2005) (Figure 5A) revealed only minor differences between persistent and non-persistent isolates. With a few exceptions, isolates from persistent clusters had nearly the full repertoire of virulence genes that non-persistent genomes harbored. Several persistent isolates, belonging to ST4 (cgMLST 4605), ST8 (cgMLST 22937), ST9 (cgMLST 16305, 22945 and 22953), ST121 (cgMLST 18630 and 8684) and ST1113 (cgMLST 22938) in which *actA* was absent or truncated, which is involved in actin-based motility during host cell invasion. The adhesion-associated gene *ami* was absent or truncated in persistent isolates belonging to ST4 (cgMLST 4605), ST8 (cgMLST 22937 and 22954), ST9 (cgMLST 22945), ST37 (cgMLST 17448), ST87 (cgMLST 19487), ST121 (cgMLST 18630) and ST204 (cgMLST 22966). Virulence genes involved in host receptor interactions, such as *inlF*, were absent or truncated in persistent isolates belonging to ST8 (cgMLST 22937 and 22954), ST9 (cgMLST 22953), ST87 (cgMLST 19487), ST121 (cgMLST 18630) and ST451 (cgMLST 21875). Similarly, *inlJ* was absent or truncated in persistent isolates belonging to ST8 (cgMLST 22937 and 22954), ST9 (cgMLST 22940, 22945 and 22953), ST121 (cgMLST 18630) and ST451 (cgMLST 21875). The invasion-associated gene *aut* was exclusively absent or truncated in persistent isolates of ST4 (cgMLST 4605). In ST8 (cgMLST22937 and

492 ST22954), ST37 (cgMLST17448) and ST204 (cgMLST 22966) the *vip* gene, involved in interaction
493 with host cell receptors, was absent in persistent isolates, but was also absent in non-persistent
494 isolates. The absence or truncation of *actA*, *inlF* and *inlJ* in ST121 (all persistent and non-persistent
495 isolates) and of *vip* in ST8, ST37 and ST204 (also in non-persistent isolates) was shared by nearly
496 all isolates of the ST and therefore reflects clonal background rather than persistence. Absences
497 in ST4 cannot be separated from persistence, as no non-persistent ST4 isolates were available.
498 Most persistent isolates did not harbor Listeriolysin S-toxin associated genes (*llsA, llsB, llsD, llsG,*
499 *llsH, llsP, llsX,* and *llsY)*. The isolates from persistent clusters that harbored all or at least one of
500 these genes all belonged to ST4 (cgMLST 4605). Overall, virulence gene profiles were highly
501 conserved across isolates, and gene absences were largely ST-specific.

502 Environmental resistance genes showed a similar pattern of presence and absence compared to
503 the virulence genes (Figure 5B). Many environmental resistance genes were widely conserved
504 across all isolates, regardless of phenotype. Among the arsenic resistance genes, persistent
505 isolates of ST9 (cgMLST 16305, 22940, 22945, and 22953) carried *arsA, arsC* and *arsR*. For arsenic
506 resistance gene *arsD* was only present in one persistent ST8 (cgMLST 22954) isolate. Persistent
507 isolates from ST155 (cgMLST 1234) harbored *arsC,* ST204 (cgMLST 22966) isolates carried the
508 *arsA*, *arsD*, and *arsR*, and ST1113 (cgMLST 22938) carried *arsA*, *arsC*, and *arsR*. For cadmium
509 resistance genes, *cadC* was present in persistent isolates from ST8 (cgMLST 22937, and 22954),
510 ST9 (cgMLST 16305 and 22940), ST121 (cgMLST 8684 and 18630), ST155 (cgMLST1234) and
511 ST1113 (cgMLST22938), whereas copper resistance gene *copB* was harbored by ST8 (cgMLST
512 22937 and 22954), ST9 (cgMLST 16305 and 22940) and ST1113 (cgMLST 22938). Withing the iron

uptake operon *fetAB* (encoding *fetA/yybI* and *fetB/ybbM*), the gene *fetA/yybL* was harbored by persistent belonging to ST4 (cgMLST 4605), ST8 (cgMLST 22937 and 22954), ST9 (cgMLST 16305, 22945, and 22953), ST87 (cgMLST 19487), ST204 (cgMLST 22966) and ST1113 (cgMLST 22938). Additionally, *fetB/ybbM* was encoded in all ST8 (cgMLST 22954), ST1113 (cgMLST 22938), and some ST9 (cgMLST22940) isolates.

In multidrug efflux genes *evgA* was found in persistent isolates from ST4 (cgMLST 4605), ST8 (cgMLST22937 and 22954), ST9 (cgMLST 22940, 22945 and 22953), ST37 (cgMLST 17448), ST87 (cgMLST 19487), ST121 (cgMLST 18630) and ST451(cgMLST 21875). The efflux pump gene *smrA* was present in a subset of one isolate of ST4 (cgMLST 4605), ST8 (cgMLST 22937), ST121 (cgMLST 18630) and ST451 (cgMLST 21875). The gene *amvA* was detected in only a single ST8 (cgMLST 22937) isolate. Looking at stress response and zinc resistance genes, cueR/ybbI was detected in persistent isolates from ST4 (cgMLST 4605), zntR/yhdM was present in ST121 (cgMLST 18630), and *ziaA* together with *ziaR* were found in persistent isolates from ST204 (cgMLST 22966). Overall, the distribution of environmental resistance genes seemed to be primarily ST-specific (notably ST4, ST8, ST9, ST37, ST87, ST121, ST204, ST451, and ST1113), rather than associated with persistence.

Discussion

In this study, we applied a unified approach to investigate the persistence of *Listeria monocytogenes* across European food-processing facilities. A coordinated sampling campaign

was carried out in facilities from three partner countries within the European Union over the course of three calendar years (2023 -2025) using a standardized sampling protocol and data-analysis pipeline to maximize comparability of the resulting data.

Our study reveals a high number of *L. monocytogenes* positive environmental swab samples for *L. monocytogenes* (22.0%) across the surveyed European food processing facilities. This result is higher when compared with previous studies, including Muhterem-Uyar et al. (2015) which reported an overall occurrence of 12.6% across 12 European food processing facilities, and Bolten et al. (2024) which observed a positive rate of 13% in eight dairy facilities in the United States. In our study, meat facilities exhibited a higher positive detection rate overall, largely due to one facility (facility A) with an exceptionally high number of positive samples, indicating potential localized hygiene issues or structural challenges specific to this facility. Meat facilities typically harbor *L. monocytogenes* more frequently than dairy facilities, as is consistent with Muhterem-Uyar et al. (2015) who reported a positive rate of 32% in European meat facilities and 8.8% in European dairy facilities. Reported positive rates in fish processing facilities range from 10% (Zakrzewski et al., 2024) to 22% (Hoffman et al., 2003), indicating that our results are within a expected range. Most of the studies above also note that there are differences in environmental swab detection rates between facilities, which is also what we observe in our dataset. This heterogeneity in *L. monocytogenes* detection is likely a reflection of differences in cleaning practices and efficiencies, facility design, and microbial ecology of specific *L. monocytogenes* subtypes.

552 The substantial number of positive post-sanitation samples highlights the importance of sampling
553 both during production and after cleaning and disinfection, providing a more accurate assessment
554 of *L. monocytogenes* presence and potential persistence. Since no contextual data on the initial
555 hygienic status of the facilities are available, we can only hypothesize regarding the underlying
556 reasons for the high detection rate in facilities following cleaning and disinfection. However, these
557 findings might reflect insufficient cleaning and sanitation practices in the individual facilities.
558 Alternatively, the high rate of detection, particularly of persistent strains, may result from
559 disruption of the resident microbial community, including other *Listeria* species, potentially
560 facilitating the colonization and persistence of *L. monocytogenes* (Ottesen et al., 2016; Zwirzitz et
561 al., 2021). Recent studies have highlighted the role of co-occurring microbiota in the promotion
562 of *L. monocytogenes* persistence in food processing environments (Burnett et al., 2025). Microbial
563 community data were not analyzed in this study, so we cannot assess whether co-occurring
564 *Listeria* species or other microbiota influenced persistence.

565 Analysis of the genetic lineages revealed that isolates from Lineage II were more abundant in food
566 processing environments, consistent with prior observations (Orsi et al., 2011). Most persistent
567 clusters belonged to Lineage II, with only three STs belonging to Lineage I (Fagerlund et al., 2022;
568 Palaiodimou et al., 2021). In addition to the frequently described STs containing persistent
569 clusters (STs 4, 8, 9, and 121), we also identified less frequently described STs (STs 37, 87, 155,
570 204, 451, and 1113) as persistent (Guidi et al., 2021; Knudsen et al., 2017; Møretrø et al., 2017;
571 Muhterem-Uyar et al., 2018; Pasquali et al., 2018; Wang et al., 2021). Overall, persistent isolates
572 were detected across all countries and facility types, highlighting the persistence of *L.*

573 *monocytogenes* as a widespread and multifaceted issue in the food production environment.
574 However, the accurate definition of persistence and the SNP cutoff used may vary depending on
575 the environmental context. As persistence might differ across different contexts, the precise
576 definition and SNP cutoff used potentially needs to be adjusted to individual facilities and
577 hotspots. Defining persistence therefore remains challenging, as both overly strict or overly
578 permissive SNP cutoffs may lead to strain misclassification.

579 Our goal was to identify highly similar persistent SNP clusters while still allowing for minor genetic
580 changes, such as phage integration or the gain and loss of plasmids, which can influence overall
581 SNP differences. Notably, applying a more stringent SNP threshold (≤10 SNP differences across
582 the whole genome) did not reveal a clear genetic pattern associated with persistence. This
583 suggests that persistence cannot be fully captured by SNP-based definitions alone and must be
584 interpreted in the context of each facility. Moreover, SNP-based thresholds consider only the total
585 number of nucleotide differences, without accounting for their genomic location or functional
586 impact. While SNP counts serve as a practical measure of genomic relatedness in epidemiological
587 analyses, the location of individual SNPs may be relevant in the investigation of genetic
588 mechanisms potentially associated with persistence.

589 The hygienic state of the facility should also be incorporated into the assessments of persistence.
590 Although the hygienic conditions of the facilities were beyond the scope of this study, they likely
591 influence the overall picture, as limited sanitation may allow certain strains to remain in the
592 facility over extended periods without necessarily reflecting persistence. Our pangenome-wide
593 association analysis across 353 *L. monocytogenes* isolates revealed genomic patterns associated

with persistence in food processing environments. We observed a similar number of genes significantly associated with persistence and non-persistence (Figure 2A). This pattern may be the result of the underlying genetic diversity across the *L. monocytogenes* STs included in the analysis. This finding may also indicate a more complex interplay between genes rather than the influence of a single gene or biomarker on the persistence phenomenon.

Our association analysis identified gene families that may be of interest for future studies of persistence in *L. monocytogenes*. Gene families associated with persistence were frequently annotated with PFAM domains linked to DNA replication, recombination and repair (Helicase_C, DnaB_2, N6_Mtase, ResIII, Topoisom_bac, Toprim, Toprim_Crpt, Recombinase, Resolvase, Zn_ribbon_recom) and transcription regulation (HTH_3, HTH_19, AlbA_2, PriCT_11, RrF2, GntR, sigma70_r4_2, ComK). Whether these functions contribute to long-term survival in food processing environments remains to be tested. Gene families associated with persistence were also annotated with PFAMs linked to prophages and mobile genetic elements (DUF2800, DUF285, Terminase_1, Phage_capsid, among others) (Bateman et al., 2021), many in COG category S (Bateman et al., 2021). This is consistent with prior work showing that prophages have been associated with stress tolerance, DNA packaging, secretion, and surface interactions (Gutiérrez et al., 2025) and have been proposed to contribute to environmental persistence(Cherifi et al., 2018; Palma et al., 2020). However, persistent genomes were dominated by a few clones sampled repeatedly in single facilities, and prophage content was not analyzed, so these associations may reflect clonal composition. Gene families associated with persistence were also annotated with PFAMs linked to cell wall remodeling, surface interaction, transport, motility, secondary

615 metabolite biosynthesis and uncharacterized functions (NLPC_P60, Big_2, LRR_5, MucBP, WxL,
616 Gram_pos_anchor, Cu-oxidase, Cu-oxidase_2, Cu-oxidase_3, Cupredoxin_1). This suggests that
617 persistent clusters may require stress response and surface adaptation functions, which
618 potentially support biofilm formation, adhesion to surfaces, and general environmental resilience
619 (Janež et al., 2021; Omelchenko et al., 2026; Xayarath and Freitag, 2012). Whether these
620 functions are relevant for biofilm formation, surface adhesion or environmental resilience cannot
621 be concluded from gene presence or absence and needs functional testing.

622 Conversely, gene families associated with non-persistence were annotated with PFAMs linked to
623 nucleotide, carbohydrate and lipid metabolism, cell envelope biogenesis and intracellular
624 trafficking (SIR2_2, DUF928, NAD_binding_10, Lipase_3). This pattern might reflect a trade-off
625 between resources allocated to metabolic versatility and basic survival necessities, such as
626 genome maintenance, stress resilience, and surface adaptation. Indicating a potential advantage
627 for persistent strains of shifting to a reduced metabolic state or dormancy under stressful
628 conditions in the food processing environment (Arvaniti et al., 2021; Arvaniti and Skandamis,
629 2022). Importantly, many genes in the *L. monocytogenes* genome remain poorly annotated and
630 lack functional information, highlighting the need for additional studies to characterize these
631 genes and their functions. In our analysis, annotating each gene family with its PFAM domains
632 provided additional functional context for interpreting the associated gene families. Overall, the
633 accessory genome of 2,268 gene families, together with mobile genetic elements such as phage
634 and plasmids (Castro et al., 2021; Palma et al., 2020; Schmitz-Esser et al., 2021), indicate a large
635 genetic repertoire and genomic flexibility, which may be crucial in the support of persistence.

In our dataset, virulence gene profiles differed mainly between STs and not between persistent and non-persistent isolates (Figure 5A).

These findings highlight the importance of further investigating persistence mechanisms, since persistent *L. monocytogenes* still have the potential to cause listeriosis outbreaks. Overall, our findings support the hypothesis that *L. monocytogenes* are generalists that can become well adapted to food processing environments, due to their genetic repertoire and genomic flexibility. However, persistence is likely a result from a complex interplay between genetic factors, environmental pressures and interactions with the co-occurring microbiomes (Carpentier & Cerf, 2011; Hingston *et al.*, 2017).

Conclusion

Persistence of *L. monocytogenes* remains a challenge in food production environments and a threat to public health via contamination of food products. The identification of key factors and mechanisms underlying persistence, however, is still lacking. This highlights the need for identifying an appropriate level of resolution for defining and investigating persistence, which accounts for both facility-specific contamination dynamics and the adaptive potential of persistent *L. monocytogenes.*

Our study illustrates the application of a unified sampling and analysis approach for studying of *L. monocytogenes* persistence. We revealed that persistence of *L. monocytogenes* is a widespread phenomenon across the facilities and countries sampled. The combination of environmental

prevalence, genetic diversity and the size of the accessory genome is consistent with the ability of *L. monocytogenes* to survive repeated disturbances or difficult environments in food processing environments. Gene families associated with persistence were annotated with functions in DNA replication, recombination and repair, transcription regulation, and included gene families linked to mobile genetic elements and prophages. These associations may partly reflect the clonal structure of the dataset and need confirmation by prophage and plasmid analysis and functional studies. Future studies of *L. monocytogenes* persistence could benefit from integrating environmental metadata and whole genome sequencing of isolates with predictive modelling tools, which may provide a practical approach to identify key drivers of persistence. Such unified approaches with standardized sampling, data collection, and analysis in combination with predictive tools will support the development of targeted management practices to control persistent *L. monocytogenes* in food processing environments.

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Declaration of Competing Interests

The authors declare that they have no known competing financial or personal interests that could have influenced the work reported in this paper.

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Figures and Tables

Table 1: Detection of *Listeria monocytogenes* in 1,538 environmental swabs from European food facilities, summarized by country, facility type, surface type, and disinfection state.

	Group	Total Samples	% of Total Samples	Positive	Negative	% Positive	% Negative
Overall							
	All samples	1538	100	338	1200	22.0	78.0
Facility Country							
	Austria	303	19.7	37	266	12.2	87.8
	Greece	400	26	197	203	49.3	50.7
	Spain	835	54.3	104	731	12.5	87.5
Facility Type							
	Dairy	775	50.4	69	706	8.9	91.1
	Fish	165	10.7	32	133	19.4	80.6
	Meat	598	38.9	237	361	39.6	60.4
Surface Type							
	FC	828	53.8	166	662	20	80
	NFC	710	46.2	172	538	24.2	75.8
Disinfection State							
	pre	975	63.4	215	760	22.1	77.9
	post	406	26.4	106	300	26.1	73.9
	NA	157	10.2	17	140	10.8	89.2

Table 2. Characteristics of persistent *Listeria monocytogenes* SNP clusters identified across sampled facilities. For each persistent SNP cluster, the table presents the facility, sequence type (ST), cgMLST type (CT), total number of isolates, number and dates of distinct sampling events, time span between first and last detection (days), and maximum pairwise SNP distance within the cluster. Clusters were classified as persistent when isolates were detected on at least three distinct sampling dates. In Facility D, 37 historical isolates were included in the SNP cluster identified as ST4/CT4605, in addition to the isolates collected during prospective sampling.

Facility	ST	cgMLST type (CT)	No. of isolates	No. of sampling dates	Sampling dates	First–last detection (days)	Maximum pairwise SNP distance
A	1113	22938	5	3	28 Jun 2023; 21 Nov 2023; 02 Jul 2024	370	6
A	155	1234	6	3	28 Jun 2023; 27 Mar 2024; 02 Jul 2024	370	7
A	451	21875	10	3	21 Nov 2023; 27 Mar 2024; 02 Jul 2024	224	4
A	8	22937	60	4	28 Jun 2023; 21 Nov 2023; 27 Mar 2024; 02 Jul 2024	370	6
A	8	22954	6	3	21 Nov 2023; 27 Mar 2024; 02 Jul 2024	224	5
A	9	16305	3	3	21 Nov 2023; 27 Mar 2024; 02 Jul 2024	224	8
A	9	22940	5	3	28 Jun 2023; 21 Nov 2023; 02 Jul 2024	370	7
A	9	22940	3	3	21 Nov 2023; 27 Mar 2024; 02 Jul 2024	224	4
A	9	22945	12	4	28 Jun 2023; 21 Nov 2023; 27 Mar 2024; 02 Jul 2024	370	5
A	9	22953	10	3	21 Nov 2023; 27 Mar 2024; 02 Jul 2024	224	5
C	121	18630	31	5	13 Jun 2023; 07 Nov 2023; 08 Nov 2023; 15 Jan 2024; 16 Jan 2024	217	4
D	4	4605	40	3	26 Jun 2023; 27 Jun 2023; 02 Aug 2023	37	8
E	204	22966	4	3	11 Dec 2023; 21 May 2024; 16 Jan 2025	402	8
E	204	22966	3	3	11 Dec 2023; 21 May 2024; 16 Jan 2025	402	6
G	37	17448	7	3	17 Jan 2024; 12 Jun 2024; 10 Feb 2025	390	9
H	121	8684	3	3	17 Jan 2024; 31 May 2024; 27 Jan 2025	376	4
H	87	19487	6	3	17 Jan 2024; 31 May 2024; 27 Jan 2025	376	7

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Table S.1: Typing information of *L. monocytogenes* isolates, showing Facility Type, Lineage, ST, and cgMLST.

Table S.2: Typing information of *L. monocytogenes* historical isolates (facility D), showing Facility Type, Lineage, ST and cgMLST.

Table S.3: Gene-trait associations identified by Scoary, showing the 979 gene families with presence/absence in persistent and non-persistent genomes, odds ratios, p-values and eggNOG annotations (COG categories, PFAM domains).

Figure 1: Number of isolates from persistent clusters per sequence type. A. Number of isolates per sequence type (ST) by Lineage and facility type. B. Number of isolates from persistent clusters per ST by Lineage and facility type.

Figure 2: Gene Enrichment and Depletion in Genomes from Persistent SNP Clusters. A. Volcano plot of genes ($\log_2$ odds ratio vs $-\log_{10}$ BH-adjusted p value). B. Mean $\log_2$ odds ratio of the significant gene families in each COG category, calculated separately for gene families associated with persistence and non-persistence (point size = number of gene families).

Figure 3: Gene families associated with persistence or non-persistence in *L. monocytogenes* by COG categories K, L and S. (A–C) Gene families significantly associated with persistent or non-persistent SNP clusters in COG categories K (Transcription), L (Replication, recombination and repair) and S (Function unknown). Each point is one gene family, labelled with the PFAM annotation(s) assigned to it; "-" marks gene families without a PFAM annotation, and numeric suffixes (.1, .2, …) distinguish gene families with identical labels. Plots show the $\log_2$ odds ratio

and 95% confidence interval. Positive values indicate association with persistent SNP clusters, negative values with non-persistent isolates.

Figure 4: COG categories in which all significantly associated gene families were associated with persistence (A) or with non-persistence (B) in *L. monocytogenes*. Each point is one gene family, labelled with the PFAM annotation(s) assigned to it; "-" marks a gene family without a PFAM annotation. Plots show he $\log_2$ odds ratio and 95% confidence interval. Positive values indicate association with persistent SNP clusters, negative values with non-persistent isolates.

Figure 5: Heatmap of virulence and environmental stress tolerance genes in *L. monocytogenes* isolates. A. Presence and absence of virulence genes determined using the VFDB (Virulence Factor Database, nucleotide version, release 14.01.2025), ordered by persistence status (persistent vs. non-persistent) and sequence type (ST). Virulence genes are grouped by functional categories. B. Presence and absence of environmental stress tolerance genes, determined using the BacMet database (version 2.0), ordered by phenotype and ST, with genes grouped by functional categories.

Figure S1: Maximum-likelihood phylogenetic tree of *L. monocytogenes* isolates. Isolates sequenced at the Hospital in Valladolid were excluded due to insufficient read length. The tree is midpoint-rooted and annotated by sequence type (ST), persistence status, and facility type.

Figure S2: Number of genes in each pangenome category. Core (2,301), soft-core (262), shell (767), and cloud (1,239) genes among the 4,569 analyzed.

Supplementary File S1: R script used to perform all analyses and generate figures presented in this study. The script includes data import, cleaning, filtering, statistical analysis, and figure generation.

Data availability

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB110404 (https://www.ebi.ac.uk/ena/browser/view/PRJEB110404).

Figures

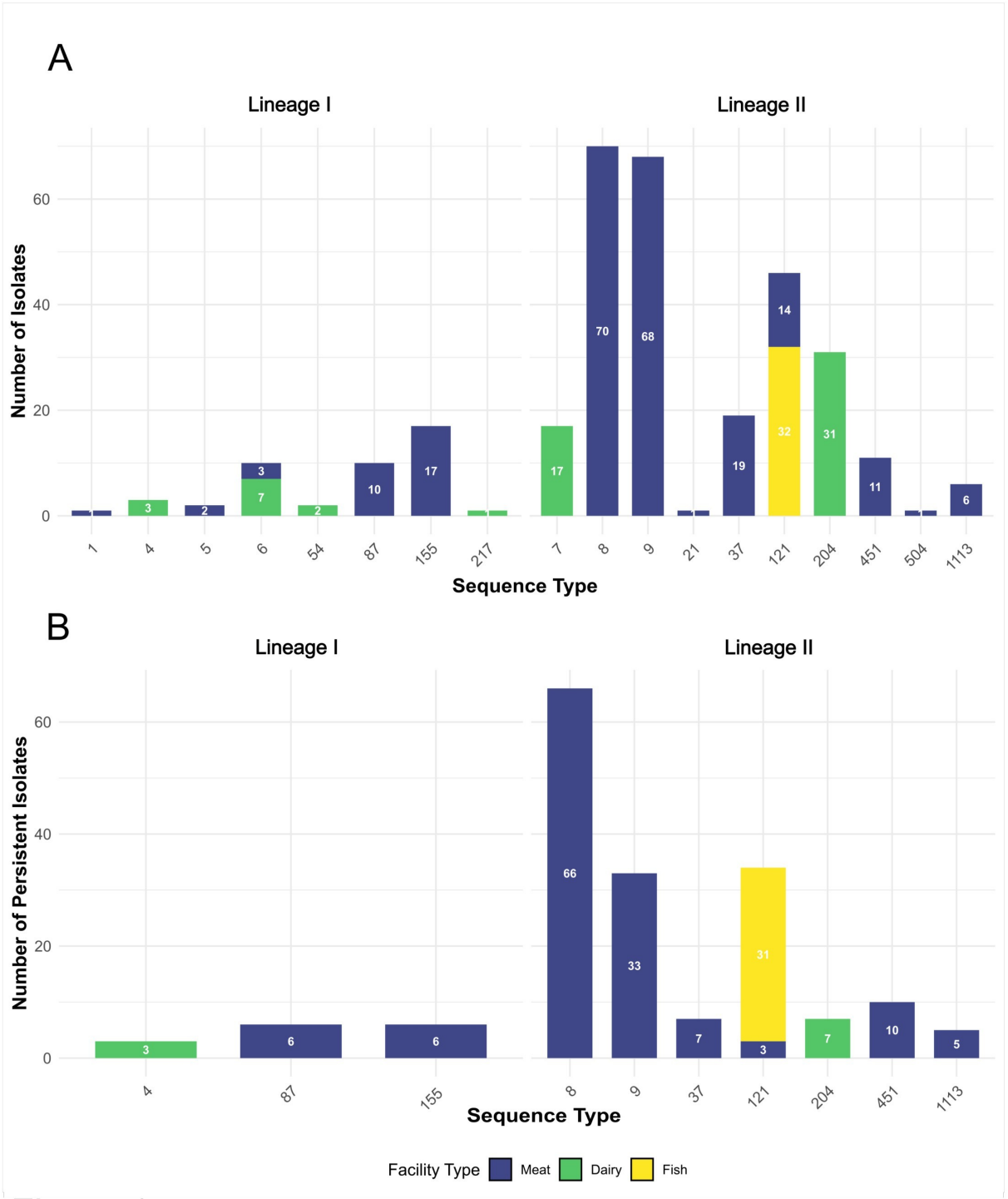


Figure 1

Figure 1

Number of isolates from persistent clusters per sequence type. A. Number of isolates per sequence type (ST) by Lineage and facility type. B. Number of isolates from persistent clusters per ST by Lineage and facility type.

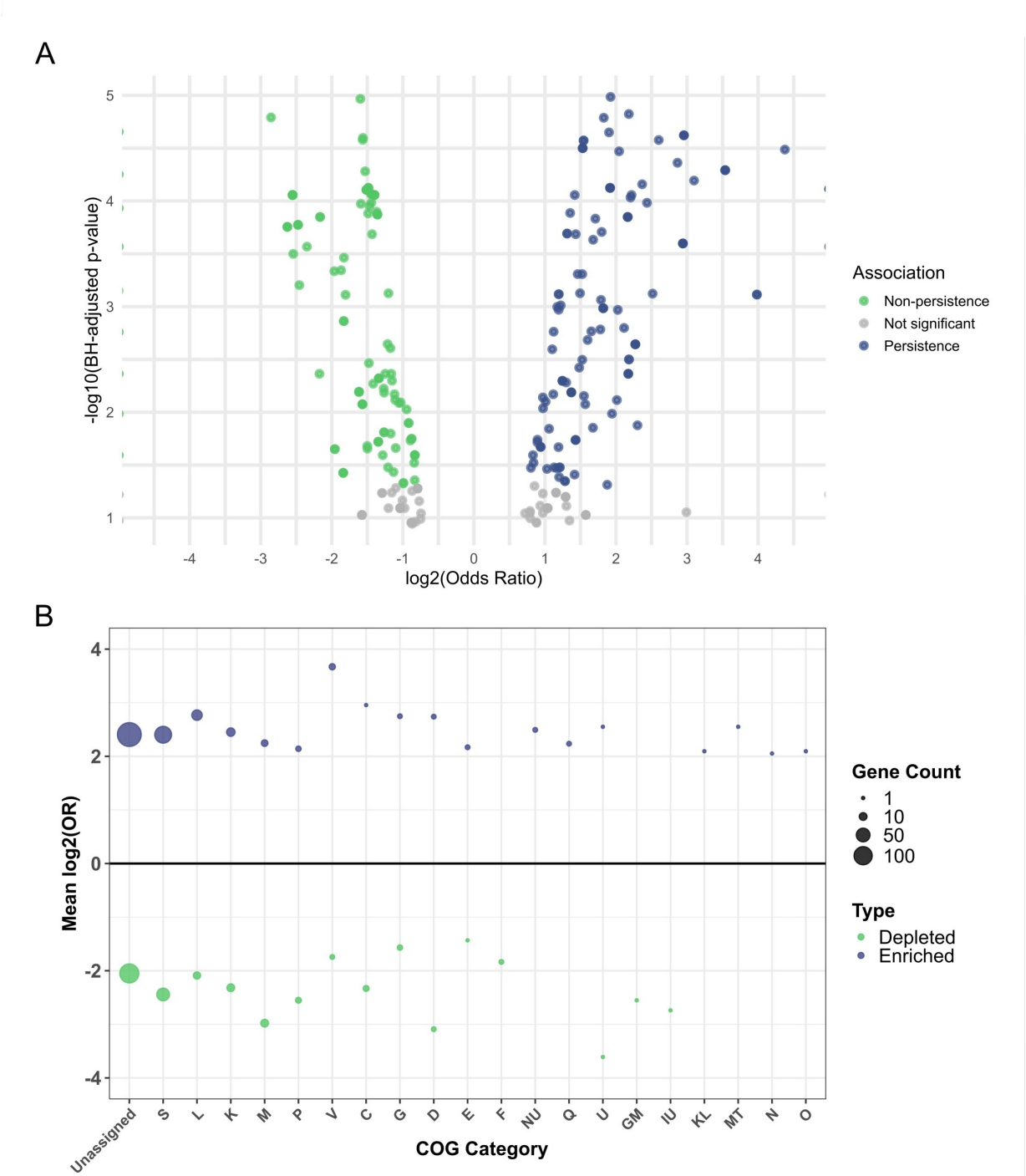


Figure 2

Figure 2

Gene Enrichment and Depletion in Genomes from Persistent SNP Clusters. A. Volcano plot of genes (log_2 odds ratio vs $-log_{10}$ BH-adjusted p value). B. Mean log_2 odds ratio of the significant gene families in each COG category, calculated separately for gene families associated with persistence and non-persistence (point size = number of gene families).

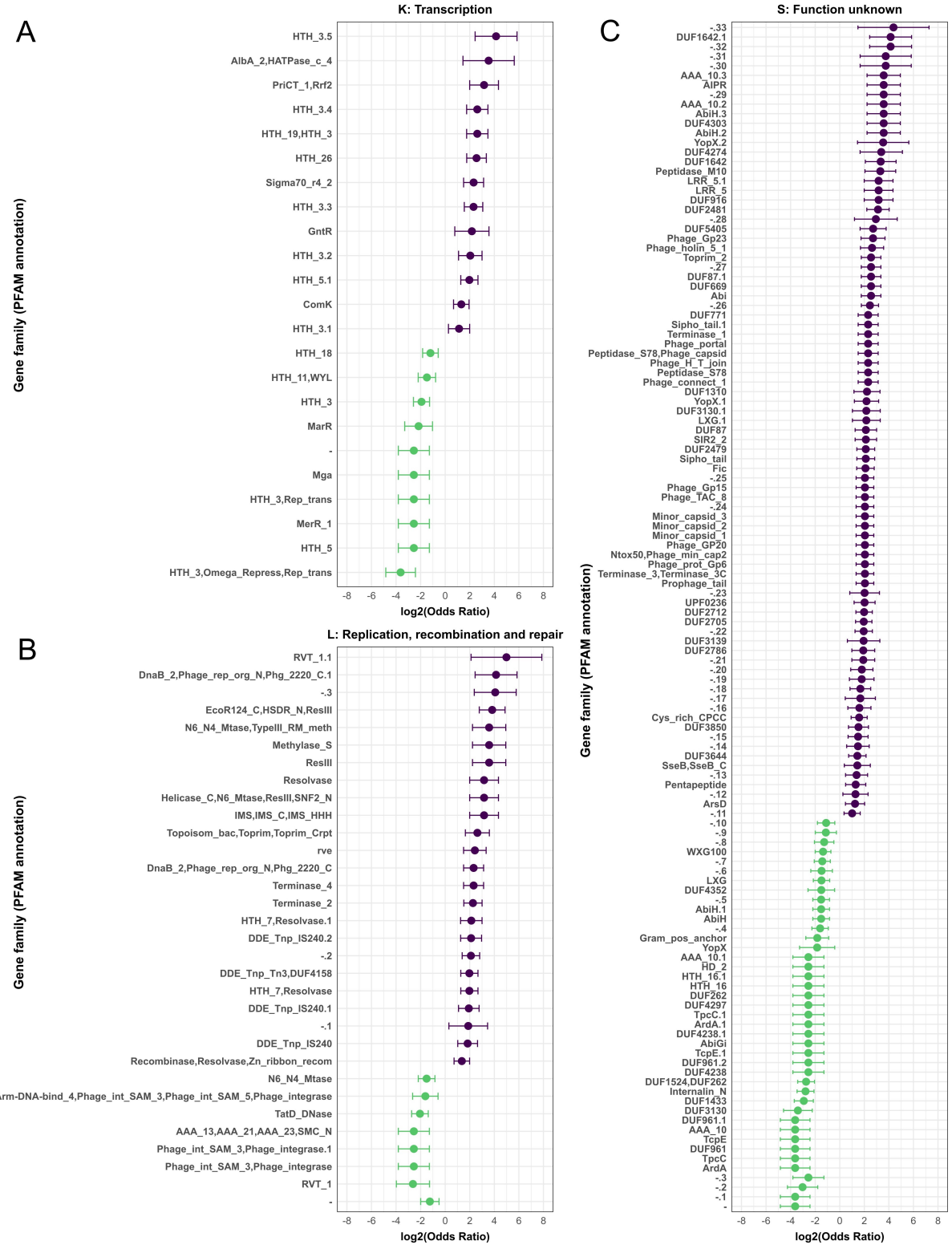


Figure 3

Gene families associated with persistence or non-persistence in L. monocytogenes by COG categories K, L and S. (A–C) Gene families significantly associated with persistent or non persistent SNP clusters in COG categories K (Transcription), L (Replication, recombination and repair) and S (Function unknown). Each point is one gene family, labelled with the PFAM annotation(s) assigned to it; "-" marks gene families without a PFAM annotation, and numeric suffixes (.1, .2, …) distinguish gene families with

identical labels. Plots show the $\log_2$ odds ratio 61 and 95% confidence interval. Positive values indicate association with persistent SNP clusters, negative values with non-persistent isolates.

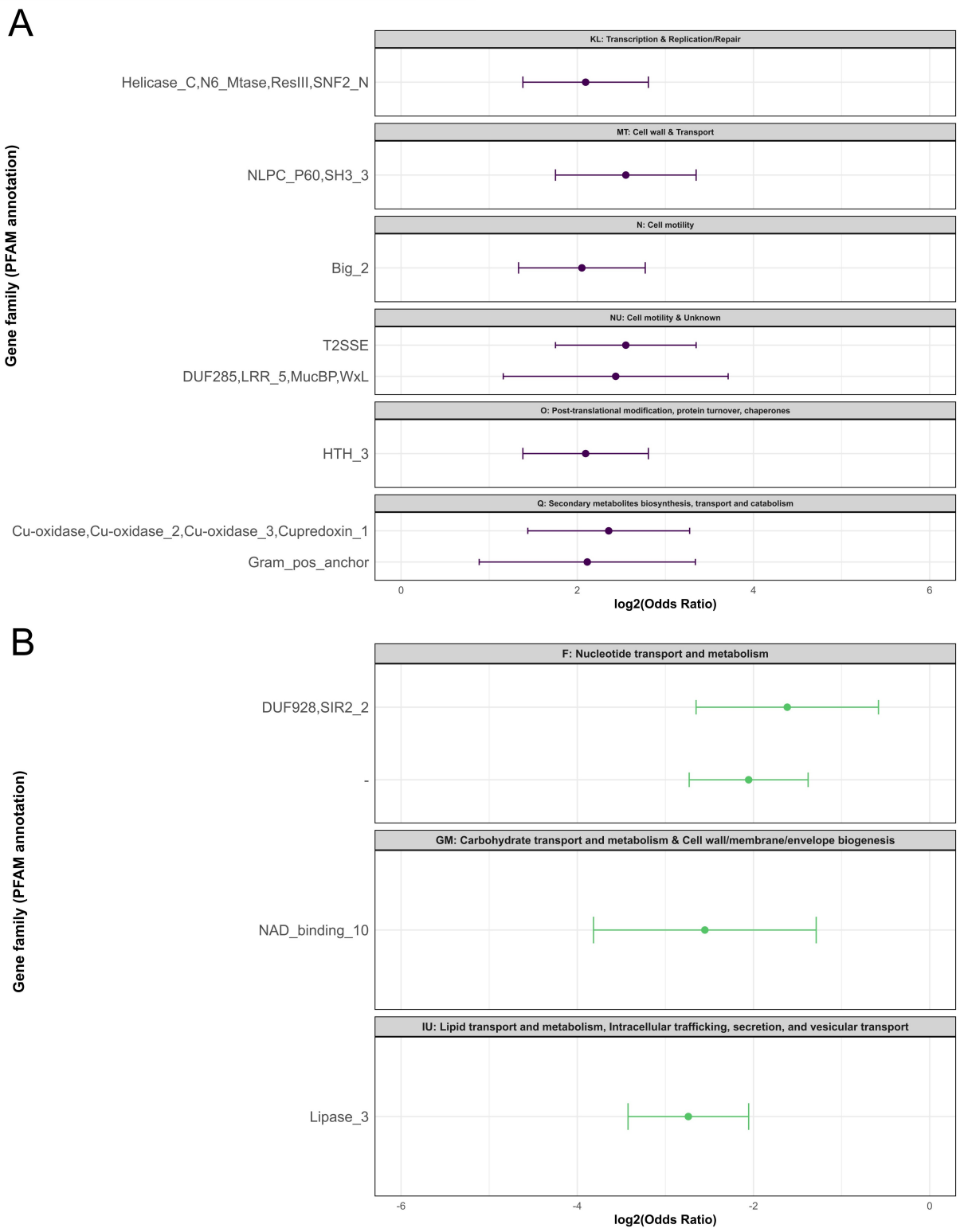


Figure 4

COG categories in which all significantly associated gene families were associated with persistence (A) or with non-persistence (B) in L. monocytogenes. Each point is one gene family, labelled with the PFAM

annotation(s) assigned to it; "-" marks a gene family without a PFAM annotation. Plots show he $\log_2$ odds ratio and 95% confidence interval. Positive values indicate association with persistent SNP clusters, negative values with non-persistent isolates.

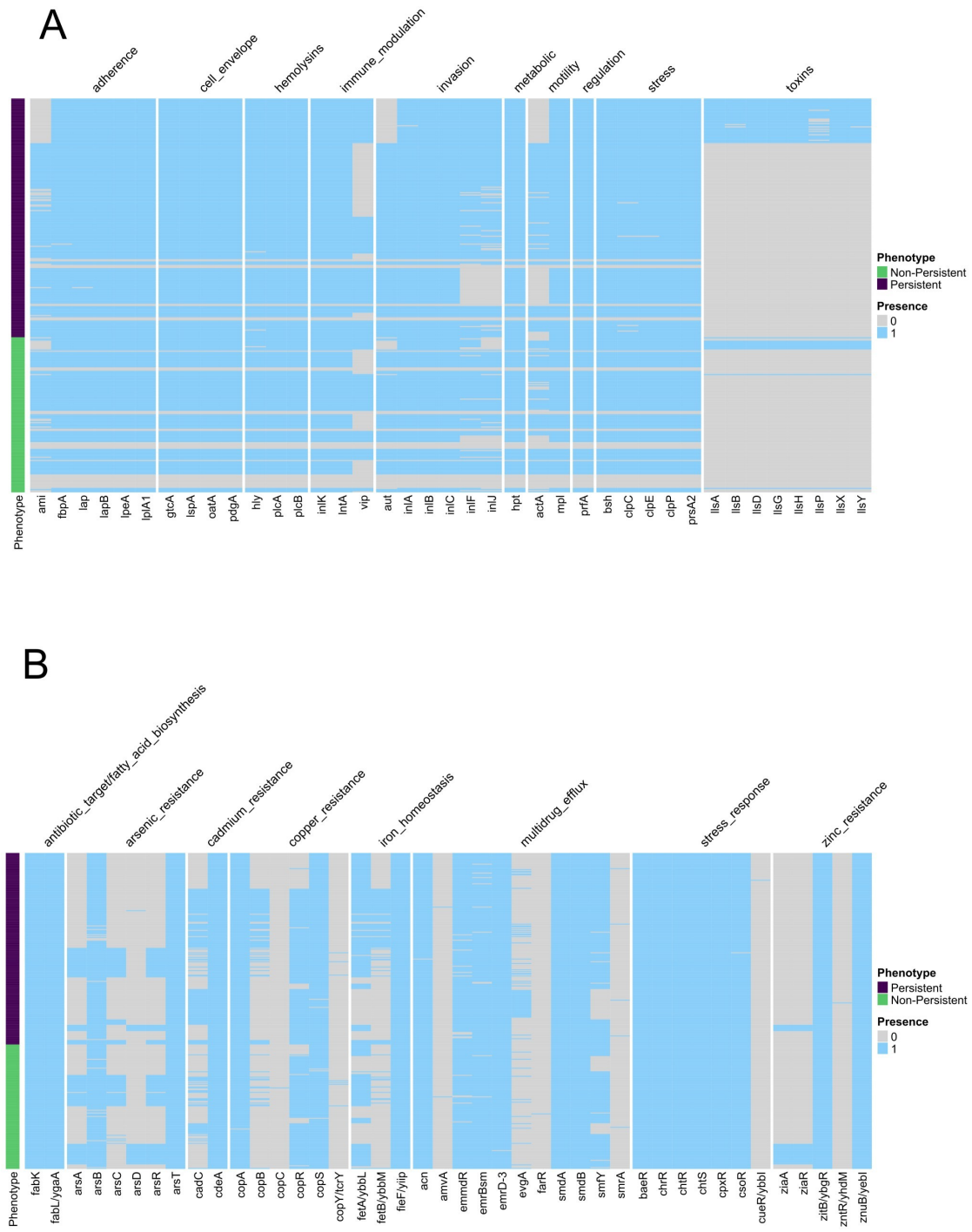


Figure 5

Figure 5

Heatmap of virulence and environmental stress tolerance genes in *L. monocytogenes* isolates. A. Presence and absence of virulence genes determined using the VFDB (Virulence Factor Database, nucleotide version, release 14.01.2025), ordered by isolate phenotype (persistent vs. non-persistent) and sequence type (ST). Virulence genes are grouped by functional categories. B. Presence and absence of environmental stress tolerance genes, determined using the BacMet database (version 2.0), ordered by phenotype and ST, with genes grouped by functional categories.

Supplementary Files

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- FigureS2.jpg
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- TableS2.csv
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- SupplementaryFileS1RCode.r