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Listeria monocytogenes: do we know enough about this pathogen?

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Listeria monocytogenes: do we know enough about this pathogen?

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Editorial: *Listeria monocytogenes*: do we know enough about this pathogen?

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KEYWORDS

whole genome sequencing (WGS), transcriptome sequencing, genetic diversity, biofilm, food safety

Editorial on the Research Topic
Listeria monocytogenes: do we know enough about this pathogen?

Listeria (*L.*) *monocytogenes* is a gram positive foodborne pathogen responsible for listeriosis, a rare, but severe infection disease. *L. monocytogenes* was discovered by E.G.D. Murray in 1926 investigating an outbreak affecting rabbits and guinea pigs in animal care houses in England (Murray et al., 1926). First human cases were reported in 1929, considering listeriosis as a zoonosis. More than 40 years later, a human listeriosis outbreak was finally directly linked to the consumption of *L. monocytogenes* contaminated food (Schlech et al., 1983). Now, *L. monocytogenes* is unambiguously recognized as a food borne pathogen, that can infect humans and animals. In healthy individuals, listeriosis is presented as a non-invasive, self-limiting gastroenteritis, but *L. monocytogenes* can also be silently present in the gastro-intestinal tract. In contrast, in immunocompromised and elderly individuals, newborns and pregnant women, a severe and systemic infection can occur, resulting in meningoencephalitis, septicaemia or abortion (Vazquez-Boland et al., 2001).

Our knowledge on the occurrence, genetic diversity, pathogenicity and behavior of *L. monocytogenes* has largely increased in the last decades using whole genome sequencing and analysis, transcriptomics and *in vitro* and *in vivo* virulence models. Moreover, *Listeria* is widely used as a model microorganism studying the interplay between a pathogenic microbe, host tissues and microbiota *in vivo*.

But there are still many open questions. We still do not understand the behavior of *Listeria* on food and within the food producing environment particularly in relation to microbial interactions and biofilm formation. Moreover, our knowledge on the genetic diversity of *L. monocytogenes* from "non-classical- sources" like soil or unexplored countries is still limited.

This Research Topic, which is composed of nine articles, addresses these aspects including different environments like soil of carrot farming, leafy vegetables, beef, drains in a meat processing plant and a frozen vegetable producing environment.

Nowak et al. analyzed the prevalence of *L. monocytogenes* in soil samples from organic carrot crops in Poland. They found *L. monocytogenes* in 10.8% of the samples. The isolated strains were characterized including antibiotic resistance, disinfectant tolerance, biofilm formation and virulence.

Virulence of *L. monocytogens* was also the focus of the review by Sousa et al.. They in depth discussed the different methods available to evaluate virulence among clonal complexes.

Prieto et al. focused on the genetic diversity of *L. monocytogenes* in the food chain in Montenegro using whole genome sequencing and analysis. The 160 isolates belonged to 21 clonal complexes, among them ST8, ST9, ST121 and ST155 were the most prevalent. This was the first study of *L. monocytogenes* from Montenegro.

The study of Zhang et al. also studied the genetic diversity of *L. monocytogenes*, but they focused on one food source namely beef. They conducted gene profiling of virulence and stress resistant genes and pangenomic analysis including international 344 strains.

The study of Fagerlund et al. investigated the temporal variation and population dynamics of *L. monocytogenes* in drains in a meat processing plant in Norway, focusing on the diversity of *L. monocytogenes* and the impact of the resident microbiota. *L. monocytogenes* was detected in the majority of samples and four different CCs were identified with up to three CCs in the same sample. Analysis of the microbiota in drains and enrichment cultures by 16S rRNA gene amplicon sequencing and metagenomic or quasimetagenomic sequencing, revealed that the drain microbiota remained relatively stable over time, with *Pseudomonas*, *Acinetobacter, Janthinobacterium, Chryseobacterium, Staphylococcus,* and *Sphingomonas* as the most commonly identified genera. There were no apparent differences in the microbial genera present in *L. monocytogenes* positive and negative drains or samples.

Pracser et al. analyzed the occurrence of *Listeria* in biofilms in a European frozen vegetable processing facility. Biofilms were present on 12.7% sites. In two cases, *L. innocua* was detected in a biofilm, which was the first study confirming the presence of *Listeria* within a biofilm in a real environment. Furthermore, the resident microbial and the co-occurrence of bacterial taxa with *Listeria* were investigated by 16S rRNA gene sequencing. *Pseudomonas*, *Acinetobacter*, and *Exiguobacterium* dominated the microbial community of the processing environment. Using differential abundance analysis, Enterobacterales and *Carnobacterium* were found to be significantly higher abundant in *Listeria*-positive samples.

Culliney and Schmalenberger aimed to analyse the bacterial community of leafy vegetables like spinach and their effect on *L. monocytogenes* growth post-harvest. They further tested the effect of the different cultivation conditions like polytunnel on the microbiota and revealed that cultivation conditions determine bacterial phyllosphere community structure, which consequently influenced *L. monocytogenes* growth.

As exopolysaccharides enhance the ability of *L. monocytogenes* to colonize and persist on surface of fresh fruit and vegetables, Elbakush et al. investigated the effect of maple compounds on biofilm formation. They discovered that maple lignans inhibit not only biofilm formation, but enhanced biofilm dispersal via sortase A inhibition. The role of sortase A is to anchors surface proteins to the cell wall, including EPS.

How exogenous fatty acids (FA) influence the growth of *L. monocytogenes* at low temperature was the research question of Quilleré et al. using transcriptomic analysis. They demonstrated that *Listeria* regulates the synthesis of saturated FA in its membrane. Moreover, they detected that unsaturated FA upregulated genes involved in flagellar assembly, resulting in numerous and long-looped flagella.

Summarizing, this Research Topic enlarges our knowledge on the interaction of *L. monocytogenes* with bacteria in food and food producing environment. Furthermore, it unravels genetic diversity of *L. monocytogenes* strains from novel sources.

Author contributions

KR: Writing – review & editing, Writing – original draft. NW-K: Writing – review & editing. KS: Writing – review & editing.

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Exploring the occurrence of *Listeria* in biofilms and deciphering the bacterial community in a frozen vegetable producing environment

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The establishment of *Listeria (L.) monocytogenes* within food processing environments constitutes a significant public health concern. This versatile bacterium demonstrates an exceptional capacity to endure challenging environmental conditions in the food processing environment, where contamination of food products regularly occurs. The diverse repertoire of stress resistance genes, the potential to colonize biofilms, and the support of a co-existing microbiota have been proposed as root causes for the survival of *L. monocytogenes* in food processing environments. In this study, 71 sites were sampled after cleaning and disinfection in a European frozen vegetable processing facility, where *L. monocytogenes* in-house clones persisted for years. *L. monocytogenes* and *L. innocua* were detected by a culture-dependent method at 14 sampling sites, primarily on conveyor belts and associated parts. The presence of biofilms, as determined by the quantification of bacterial load and the analysis of extracellular matrix components (carbohydrates, proteins, extracellular DNA) was confirmed at nine sites (12.7%). In two cases, *L. innocua* was detected in a biofilm. Furthermore, we explored the resident microbial community in the processing environment and on biofilm-positive sites, as well as the co-occurrence of bacterial taxa with *Listeria* by 16S rRNA gene sequencing. *Pseudomonas*, *Acinetobacter*, and *Exiguobacterium* dominated the microbial community of the processing environment. Using differential abundance analysis, amplicon sequence variants (ASVs) assigned to Enterobacterales (*Enterobacter*, *Serratia*, unclassified *Enterobacteriaceae*) and *Carnobacterium* were found to be significantly higher abundant in *Listeria*-positive samples. Several *Pseudomonas* ASVs were less abundant in *Listeria*-positive compared to *Listeria*-negative samples. *Acinetobacter, Pseudomonas*, *Janthinobacterium*, *Brevundimonas*, and *Exiguobacterium* were key players in the microbial community in biofilms, and *Exiguobacterium* and *Janthinobacterium* were more relatively abundant in biofilms. Further, the microbial composition varied between the different areas and the surface materials.

KEYWORDS

microbiota, food processing, colonization, 16S rRNA, microbial interaction

1 Introduction

Listeria (L.) monocytogenes is of particular concern due to its versatility and ability to adapt to harsh environmental conditions such as low temperatures (Schmid et al., 2009; Muchaamba et al., 2022; Osek et al., 2022). *L. monocytogenes* is further able to persist for years in diverse food processing environments despite hygiene measures (Guidi et al., 2021; Chen et al., 2022; Sullivan et al., 2022; Chowdhury and Anand, 2023; Schiavano et al., 2023) elevating the risk of food contamination. Long-term survival of *L. monocytogenes* has been previously linked to gene content involved in stress resistance, which provides tolerance against disinfectants, a wide range of temperatures, acidic and alkaline conditions, and high salinity (Bucur et al., 2018; Lakicevic et al., 2022). However, there are indications that the genomic content is not the only factor explaining the persistence of *L. monocytogenes*. Our previous study confirmed the presence of in-house clones in a European frozen vegetable-producing environment. However, no differences in the stress resistance gene pattern were observed between in-house and non-in-house clones (Pracser et al., 2024). Other factors that could support the persistence of *L. monocytogenes* in food processing environments include biofilms and interactions with co-occurring microbiota (Møretrø and Langsrud, 2017; Tan et al., 2019; Finn et al., 2023).

Biofilms are composed of a multitude of bacterial cells embedded in a self-produced extracellular matrix consisting of carbohydrates, proteins and extracellular DNA (eDNA). The biofilm architecture allows for the exchange of genetic material, provides nutrients and protects cells from stressors such as disinfectants or desiccation (Esbelin et al., 2018; Li et al., 2021; Flemming et al., 2023). Various studies have investigated the biofilm-forming potential of *L. monocytogenes* and other bacterial species commonly found in food processing environments in single- and multispecies biofilms in the lab. Indeed, there are extensive variations in the biofilm-forming ability of different bacterial species and strains (e.g., Wagner et al., 2021; Di Ciccio et al., 2022). Multispecies biofilm models showed that *L. monocytogenes* is able to colonize a monospecies *Pseudomonas* biofilm (Rodríguez-López et al., 2022; Sterniša et al., 2023) or even a multi-taxa biofilm (Rolon et al., 2024b). These studies showed that the bacterial interactions in multi-species biofilms are complex and that *L. monocytogenes* within biofilms is protected from environmental stressors, e.g., from antimicrobials. For example, the biofilm matrix reduces the diffusion of antimicrobials or extracellular enzymes in the matrix degrade antimicrobials, resulting in lower concentration of antimicrobials (Maillard and Centeleghe, 2023). A shortcoming of these studies is that the behavior of *Listeria* within a biofilm has only been studied in biofilm models.

The composition of the resident microbiota of a food producing environment is influenced by food products and diverse environmental factors like nutrient avaiablility, humidity, sanitation methods or temperature, and also by the workers. Therefore, the microbiota of food processing environments is often composed of versatile microorganisms, which can adapt to various environmental stressors (Maes et al., 2019). *Pseudomonas*, *Acinetobacter*, *Staphylococcus*, *Psychrobacter*, *Stenotrophomonas*, *Serratia*, and *Microbacterium* are prevalent in food processing environments. In addition, the environmental conditions may promote the growth and increase the relative abundance of certain bacterial taxa. The resident microbiota may interact competitive or cooperative with other bacteria including food pathogens (De Filippis et al., 2021; Fagerlund et al., 2021; Tadielo et al., 2023; Xu et al., 2023). Diverse bacterial taxa isolated from food processing environments were shown to influence the survival and growth of *Listeria* under laboratory conditions (Sinclair et al., 2022; Lake et al., 2023; Rolon et al., 2024a,b). Positive and negative associations of members of microbial communities with *L. monocytogenes* and other *Listeria* spp. were already investigated in meat processing facilities (Zwirzitz et al., 2021; Belk et al., 2022; Cherifi et al., 2022), tree fruit packing facilities (Rolon et al., 2023), the fish, meat and dairy industry (Rodríguez-López et al., 2019), and distribution centers for fresh produce (Townsend et al., 2023). However, there is still limited knowledge on the extent to which *Listeria* spp. are present in biofilms in the food-producing environment and which bacterial taxa are present in biofilms.

In this study, we examined the presence of *Listeria* spp., the presence of biofilms, and the composition of the microbial community in a European frozen vegetable processing facility. Sampling was performed at 71 environmental sites, including food-contact surfaces and non-food-contact surfaces in three different subareas. To our knowledge, this is the first study describing the presence of *Listeria* in biofilms and the specific residing microbiota in a frozen vegetable processing environment.

2 Materials and methods

2.1 Sampling

Sampling of environmental surfaces was performed on two sampling visits in April and May 2022 in a European frozen vegetable processing facility. On the first visit, samples were taken in the subarea "Production" where processing lines for blanching and deep-freezing of fresh vegetables were located. The second sampling visit comprised two subareas. In "Packaging Room A," processing lines for mixing vegetables and packaging of frozen vegetables were sampled. In "Packaging Room B," sampling was conducted on processing lines for frozen fried vegetable products and the packaging of frozen vegetable products. All samples were taken after cleaning and disinfection and included food-contact, indirect food-contact (surfaces touching, e.g., parts of equipment, which are in direct food contact) and non-food-contact surfaces. In total, 71 regular samples, seven supplementary samples and 19 negative controls were taken (Supplementary Tables S1, S2).

Each site was sampled using two sets of sampling devices: First, for biofilm detection a scraper (Cell Scraper; length: 225 mm, blade width: 20 mm; Carl Roth) and Nylon® flocked swabs (552C, FLOQSwabs, COPAN) (Maes et al., 2017; Wagner et al., 2020) were used. The sampling surface areas were horizontally and vertically wiped with the scraper and flocked swabs. Second, for *Listeria* detection and analysis of the microbial community, a larger area of the same sampling site was sampled with a hydrated polyurethane sponge (PU) swab in foil bags (HiCap Neutralizing swabs, Nasco Whirlpak). The size of surface areas of sites sampled for biofilm detection ranged from 5 to 300 cm^2 and for *Listeria* detection and microbial community description from 20 to 1,000 cm^2. Surface areas that were not accessible with both sets of devices (e.g., too small for PU swabs such as the inner wall of a water hose outlet), were only sampled with scraper and flocked swabs. Scraper and flocked swabs

were transported in 10 mL of ¼ Ringer solution (B. Braun Austria GmbH). All samples were transported under cool conditions and were processed within 24 h.

2.2 Culture-depended detection of *Listeria*

Listeria spp. were recovered from surfaces wiped with PU-swabs. To each foil bag containing a PU-swab, 10 mL of 1X Phosphate Buffered Saline (PBS) (Thermo Fisher Scientific) were added. The samples were homogenized using a Stomacher blender at middle speed for 1 min. From the homogenized suspension, 8 mL were removed from the foil bag and transferred to a separate tube for total DNA recovery (see Section 2.3). Isolation of *Listeria* was performed according to ISO 11290-1:2017 with modifications. 50 mL of half-Fraser broth (Noack) were added to each foil bag containing a PU-swab. The samples were incubated at 30°C for 36 h for primary enrichment. For the secondary enrichment, 100 µL of the primary enrichment were transferred to 9 mL of Fraser broth (Noack) and incubated at 37°C for 48 h. The primary and secondary enrichment were plated out on ALOA (Merck Millipore) and PALCAM (Biokar Diagnostics) agar plates. The agar plates incubated at 37°C for 48 h.

2.3 DNA extraction

Total DNA was extracted from putative *Listeria* spp. colonies on the selective agar plates using a Chelex-based (Chelex 100, Sigma-Aldrich) extraction procedure (Walsh et al., 1991). A Chelex 100 stock solution was prepared with 2.5 g Chelex 100, 2.5 mL 0.01 M Tris–HCl (pH 7.0) and 95 mL autoclaved distilled water. Putative *Listeria* isolates were transferred from selective agar plates to 1.5 mL tubes containing 100 µL 0.01 M Tris–HCl (pH 7.0) and 400 µL of the Chelex stock solution, respectively. The suspensions were vortexed and incubated at 95°C for 10 min shaking (500 rpm). After centrifugation at 15,000 × g for 30 s, 150 µL of the supernatant containing extracted DNA of each *Listeria* isolate was transferred to a new 1.5 mL tube.

In addition, bacterial cells were recovered from samples wiped with PU-swabs and scraper and flocked swabs, respectively, for subsequent DNA extraction. The 8 mL bacterial cell suspension recovered from sampling with PU-swabs (see Section 2.2) was centrifuged at 3,220 × g for 10 min at 20°C. The resulting pellet was resuspended in 500 µL PBS (Thermo Fisher Scientific) and stored at −80°C until DNA extraction.

The sample processing of scraper and flocked swabs was done according to a previous study (Wagner et al., 2020). Briefly, we added 2 g of hydrated cation exchange resin (CER, Amberlite® HPR110, 20–50 mesh, Sigma-Aldrich) to each sample collected with scraper and flocked swabs, followed by 15 min of shaking at 500 rpm. Then, the suspension was centrifuged at 3,220 × g at 20°C for 15 min. The supernatant was sterile filtered through a 0.22 µm filter membrane (Filtropur S0.2, Sarstedt AG & Co KG) and stored at −20°C until analysis of extracellular matrix components (see Section 2.5). The cell pellet including CER was stored at −20°C until DNA extraction. Bacterial cell pellets including CER were thawed to prepare for DNA extraction at room temperature and 5 mL 1X PBS (Thermo Fisher Scientific) were added to each sample. Mixing of the samples was achieved via vortex agitation. Subsequently, CER was allowed to settle for 2 min resulting in a separation of the supernatant and CER. The supernatant was transferred to a new centrifugation tube. The washing step with PBS was repeated twice. Then, the collected supernatant was centrifuged for 5 min at 3,220 × g. The supernatant was discarded resulting in the recovery of a bacterial cell pellet without CER.

Total DNA was extracted from bacterial cell pellets using the DNeasy®PowerSoil®ProKit (Qiagen) according to manufacturer's instructions with small modifications. For bacterial cell disruption, bead beating (4.5 m/s, 45 s, 5 repetitions) with Matrix Lysis A (MP Biomedicals) was performed on a FastPrep-24™ 5G (MP Biomedicals). The elution step was performed twice with 25 µL 70°C sterile water (Sigma-Aldrich). DNA concentration was determined using the Qubit fluorometer (Invitrogen) with the Qubit high sensitivity dsDNA kit (Invitrogen) and 2 µL of DNA. DNA was stored at −80°C until further analysis.

In addition, *L. monocytogenes* isolates were sent to the Austrian National Reference Laboratory for *Listeria monocytogenes* (AGES) for total DNA extraction using the MagAttract high-molecular-weight (HMW) DNA kit (Qiagen). Total DNA of *L. innocua* isolates was extracted with the GeneJet Genomic DNA Purification kit (Thermo Fisher Scientific) following the manufacturer's instructions for purification of genomic DNA from gram-positive bacteria with minor modifications. The final elution step was carried out with 50 µL 37°C sterile water (Sigma-Aldrich).

2.4 Molecular characterization

For differentiation of *Listeria* spp., two PCRs were performed (Border et al., 1990; Bubert et al., 1999). One PCR targeted the *hly* gene, which encodes for listeriolysin O (primer sequences: 5′-CCT AAG ACG CCA ATC GAA-3′, 5′-AAG CAC TTG CAA CTG CTC-3′) for identification of *L. monocytogenes*, and the fragment of 16S rRNA for detection of *Listeria* spp. (primer sequences: 5′-CAG CAG CCG CGG TAA TAC-3′, 5′-CTC CAT AAA GGT GAC CCT-3′). The reaction volume of the PCR was 25 µL and contained 1.5 µL of each primer (stock concentration 18 µM, final concentration: 1.08 µM), 8.5 µL of sterile water (Sigma-Aldrich), 2.5 µL of 10X PCR Buffer (-$MgCl_2$) (Invitrogen), 0.75 µL 50 mM $MgCl_2$, 5 µL of 4 mM deoxynucleotide triphosphate mix (dNTPs, Thermo Fisher Scientific), 0.25 µL of 5 U/µl Platinum™ Taq DNA Polymerase (Invitrogen), and 2 µL of DNA template. PCR was carried out with following settings: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min.

The second multiplex PCR approach targeted the *iap* gene for differentiation of *Listeria* spp.. Primers specific for *L. monocytogenes* (5′-CAA ACT GCT AAC ACA GCT ACT-3′), *L. innocua* (5′-ACT AGC ACT CCA GTT AAA C-3′), *L. grayi* (5′-CCA GCA GTT TCT AAA CCT GCT-3′), the *L. ivanovii/L. seeligeri/L. welshimeri* group (5′-TAA CTG AGG TAG CGA GCG AA-3′), were used in pair with the primer sequence 5′-TTA TAC GCG ACC GAA GCC AAC-3′. The reaction volume of the PCR was 25 µL and contained 2 µL of each primer (stock concentration 1.6 µM, final concentration: 128 nM), 4.45 µL of sterile water (Sigma-Aldrich), 2.5 µL of 10X PCR Buffer (-$MgCl_2$) (Invitrogen), 0.75 µL 50 mM $MgCl_2$, 5 µL of 4 mM deoxynucleotide triphosphate mix (dNTPs, Thermo Fisher Scientific), 0.3 µL of 5 U/µl Platinum™ Taq DNA Polymerase (Invitrogen), and

2 µL of DNA template. PCR was carried out with following settings: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, followed by a final extension step at 72°C for 5 min. PCR products were visualized by gel electrophoresis.

A quantitative real-time PCR (qPCR) targeting the 16S rRNA gene (primer sequences: 5′-CCT ACG GGA GGC AGC AG-3′, and 5′-ATT ACC GCG GCT GCT GG-3′) was applied to calculate total bacterial cell equivalents (BCE) in all samples as previously described (Dixon et al., 2019; Wagner et al., 2020). Briefly, the volume of a single qPCR reaction was 20 µL containing 1 µL template DNA, 7 µL sterile water (Sigma-Aldrich), 1 µL of each primer (final concentration 250 nM), and 10 µL Brilliant III Ultra-Fast SYBR® Green qPCR master mix with low ROX (Agilent). Amplification was performed with one cycle at 95°C for 3 min, 40 cycles at 95°C for 5 s and at 60°C for 20 s, followed by generation of a melting curve at 95°C for 1 min, 60°C for 30 s, and 95°C for 30 s. Copy numbers of background controls of the DNA extraction kit, which determined the bacterial contamination of reagents, were subtracted from copy numbers of the samples. An average of 5.3 16S rRNA gene copy numbers was estimated using rrnDB for calculation of total BCE (Větrovský and Baldrian, 2013; Stoddard et al., 2015).

Whole genome sequencing of *L. monocytogenes* isolates was carried out by AGES. Briefly, whole genome sequencing libraries were prepared using the Nextera XT kit (Illumina) and paired-end sequencing (2 × 300 bp) was performed on a MiSeq platform (Illumina). Whole genome sequencing of *L. innocua* isolates was performed by Microsynth (Balgach, Switzerland). Briefly, Illumina Nextera two-step PCR libraries were prepared and paired-end sequencing on an Illumina MiSeq platform (2 × 250 bp) was done.

DNA from samples taken with PU-swabs was sent to Microsynth (Balgach, Switzerland) for sequencing of the V3-V4 region of the 16S rRNA gene. Preparation of Illumina Nextera two-step PCR libraries (Illumina) and paired-end sequencing on an Illumina MiSeq platform (2 × 250 bp) was performed by Microsynth.

2.5 Biochemical characterization of biofilm matrix components

The amounts of carbohydrates, eDNA and proteins were determined in the supernatant of the processed biofilm samples.

2.5.1 Presence of carbohydrates in the biofilm matrix

Concentration of carbohydrates in each sample was attained via evaporation for 1 h at 90°C shaking at 300 rpm. A phenol-sulfuric acid method was applied to determine the carbohydrate content in each sample (Masuko et al., 2005). Glucose was used for creating a standard curve that was used for calculation of glucose equivalents in each sample. The limit of quantification (LOQ) was 12.6 mg/L glucose equivalents.

2.5.2 Presence of eDNA in the biofilm matrix

Ethanol precipitation according to Zetzmann et al. (2015) was done in order to precipitate eDNA. To each sample, 0.1 × (of sample volume) 3 M Na-acetate, 0.1 × 0.1 M $MgCl_2$ and 2.5 × ice-cold ethanol absolute were added, and samples incubated at −20°C for 24 h. Precipitated DNA was recovered by centrifugation at 20,817 × g for 15 min. The supernatant was discarded, and the pellet was washed with 1 × (of sample volume) 70% ethanol. The centrifugation step was repeated, and the resulting pellet was resuspended in 30 µL of water. Samples were measured for eDNA content on a DeNovix DS-11 FX+ spectrophotometer. The limit of blank (LOB), as assessed from negative precipitation controls, was 2 ng/µl.

2.5.3 Presence of proteins in the biofilm matrix

Precipitation of proteins was done with 0.1 × (of sample volume) TCA/Acetone (1 g/mL) and 0.01 × 2% sodium deoxycholate at 4°C over-night (Rychli et al., 2016). Precipitated proteins were recovered by centrifugation at 20,817 × g at 4°C for 30 min. The pellet was washed with ice-cold acetone and the centrifugation step was repeated. The air-dried pellet was dissolved in 20 µL of 0.05 M Tris–HCl, and the dissolved samples incubated for 3 h at room-temperature. Presence of proteins was determined using the Quant-iT protein assay kit (Invitrogen) according to manufacturer's instructions. The Quant-iT protein buffer was spiked with bovine serum albumin (final concentration: 1.7 ng/µl BSA), since the sensitivity of the protein assay kit was not high enough for the low biomass samples of the current study. Fluorescence of the spike solution was measured and subtracted from other samples. A limit of blank (LOB) (305 ng) was calculated from the spiked Quant-iT protein buffer solution.

2.6 Bioinformatics

2.6.1 Analysis of *Listeria* genome data

Quality of demultiplexed raw reads of all *Listeria* spp. isolates was assessed with fastqc v0.11.9 (Andrews, 2010) and MultiQC v1.0 (Ewels et al., 2016). Read trimming and adapter removal was done with trimmomatic v0.39 (Bolger et al., 2014). Subsequently, adapter-removed and trimmed reads were assembled using SPAdes v3.15.4 (Bankevich et al., 2012) with default settings. Quality of genomes was assessed with QUAST v5.0.2 (Gurevich et al., 2013) and reads were mapped back to the genome assemblies using BBmap v39.01 (Bushnell, 2014) to determine coverage. Subtyping of *L. monocytogenes* isolates was performed using SeqSphere+ v9.0.3 (Ridom GmbH) by the identification of the multi locus sequence typing-sequence type (MLST-ST) and the core genome multi locus sequence typing-complex type (cgMLST-CT) according to the subtyping scheme from Ruppitsch et al. (2015). The MLST-ST of *L. innocua* isolates was determined using the BIGSdb-Pasteur database (Institut Pasteur, France; https://bigsdb.pasteur.fr/). Further, a whole genome SNP analysis of *L. innocua* and *L. monocytogenes* isolates was performed using the CFSAN SNP pipeline v2.2.1 (Davis et al., 2015) with default settings. As reference genome, an internal draft genome was selected based on total length, low number of contigs and coverage. A phylogenetic tree was constructed with the filtered SNP matrix with IQ-TREE v2.0.3 (Minh et al., 2020) using the GTR + ASC substitution model with 1,000 ultrafast bootstrap replicates (Hoang et al., 2017). Visualization of phylogenetic trees was performed with iTOL (Interactive Tree of Life) (Letunic and Bork, 2021).

2.6.2 Analysis of 16S rRNA sequencing data

Analysis of the microbial community with sequencing results from the V3-V4 region of the 16S rRNA gene was carried out as follows: Demultiplexed raw reads were quality checked with fastqc

v0.11.9 (Andrews, 2010) and MultiQC v1.0 (Ewels et al., 2016). Removal of residual adapter sequences was done using trimmomatic v0.39 (Bolger et al., 2014). Subsequently, reads were imported into QIIME-2 v2023.2.0 (Bolyen et al., 2019). Reads were trimmed and quality-filtered using dada2 implemented in QIIME-2 and the resulting amplicon sequence variants (ASVs) were taxonomically classified using the SILVA database SSU 138 (Quast et al., 2013). Potential contaminant ASVs were filtered from the ASV table by using the "isNotContaminant" function (threshold 0.5) in the *decontam* v1.20.0 R package for low biomass samples, which applies a prevalence-based method to identify potential contaminants (Davis et al., 2018). Initial dataset exploration was done in R v4.3.1 (R Core Team, 2023) using the *phyloseq* package v1.44.0 (McMurdie and Holmes, 2013). Samples with less than 1,000 total reads were removed from the dataset ($n = 19$). ASVs detected in less than 10% of samples were removed for differential abundance analysis ($n = 1{,}026$), which was subsequently conducted with *DESeq2* v1.40.2 (Love et al., 2014). For exploring alpha- and beta-diversity, the dataset was rarefied to the lowest read depth (1,280 reads). Alpha diversity was investigated using *vegan* v2.6–4 (Oksanen et al., 2022) with indices Observed, Chao1, Shannon, Simpson, InvSimpson, ACE, and Fisher's alpha. Beta-diversity was explored using the Bray-Curtis index with a t-distributed stochastic neighbor embedding method [tSNE; *tsnemicrobiota* package v0.1.0 (Lindstrom, 2023)]. Figures were created with *ggplot2* v3.4.4 (Wickham, 2016).

2.7 Statistical analyses

The Wilcoxon rank sum test with Benjamini-Hochberg adjustment for *p*-value was used for pairwise comparisons of the alpha-diversity indices Observed, Chao1, Shannon, Simpson, InvSimpson, ACE, and Fisher's alpha. Differences in the bacterial community compositions (beta diversity) were assessed by calculating a permutational analysis of variance (PERMANOVA) with 5,000 permutations using the "adonis2" function in the *vegan* v2.6–4 package with R v4.3.1. A p-value < 0.05 was considered as significant for statistical calculations.

3 Results

3.1 Bacterial load in a frozen vegetable processing facility

Three different rooms ("Production", "Packaging Room A", "Packaging Room B") were sampled with two sets of sampling devices (scraper and flocked swabs for biofilm detection; PU-swabs for *Listeria* detection and analysis of the bacterial community). The different sampling devices were necessary as the material of the PU sponge swab interferes with biofilm matrix analyses. Scraper and flocked swabs failed to recover *Listeria* spp. and to recover enough biomass for the microbiota analysis. Therefore, sampling sites were additionally wiped with PU sponge swabs.

Quantification of the 16S rRNA gene revealed the presence of bacteria in all 71 samples collected with PU-swabs (Figure 1; Supplementary Table S1). The highest bacterial load was detected on "blancher 1—conveyor belt—blanching zone" in the "Production" room (site 3) with 7.6 log BCE/cm^2, on a conveyor belt guide roller in "Packaging Room A" (site 43) with 6.9 log BCE/cm^2, and in the drain next to blancher in the "Production" room (site 11) with 6.8 log BCE/cm^2. The lowest BCE was observed on a screw conveyor (site 56) with 0.3 log BCE/cm^2.

We additionally analyzed the bacterial load in samples collected with scraper and flocked swabs, which are suitable for biofilm analysis. This was essential as the presence of bacteria is a prerequisite for the formation of bacterial biofilms. We detected bacteria in 70 samples ranging from 0.006 to 5.8 log BCE/cm^2. By trend, the bacterial load was overall higher in samples collected with PU-swabs than in samples collected with scraper and flocked swabs.

3.2 Presence of biofilms

For the detection of biofilms, the presence of matrix components such as carbohydrates, proteins, and eDNA was determined. Carbohydrates were detected in 12 samples, proteins in 7 samples, and eDNA in 12 samples (Figure 2). The carbohydrate content ranged from 0.70–13.86 µg/cm^2, the protein amount ranged from 31.67 to 296.47 ng/cm^2, and the eDNA concentration ranged from 14.31 ng/cm^2 to 100.99 ng/cm^2 (Supplementary Figure S1).

In total, nine biofilm sites (12.68% of 71 total samples) were identified based on the criteria of the presence of at least two matrix components and a positive bacterial cell equivalent (BCE) count (Figure 2). In the "Production" room, biofilms were present on a metal tub (site 21), a conveyor belt (site 36) and an air separator (site 39). In "Packaging Room A", biofilms were identified on a conveyor belt (site 45), a conveyor screw (site 50), a conveyor belt guide roller (site 51), a filling funnel (site 54), and a metal funnel (site 57). In "Packaging Room B", a biofilm was detected on a conveyor belt guide roller (site 63).

3.3 Presence and characterization of *Listeria* spp.

The presence of *Listeria* spp. was assessed in samples collected with PU-swabs. In total, 14 samples were positive for *Listeria* spp. (Figure 2; Table 1; Supplementary Table S3). In the "Production" room, *L. innocua* (ST1481) was present in a drain (site 32) and on two conveyor belts after the freezer exit (site 34 and 35). In "Packaging Room A," *L. innocua* (ST2347) was detected on a conveyor belt (site 42) and on conveyor belt guide rollers (site 43, 46, and 51). Additionally, *L. monocytogenes* (ST8-CT8851) was recovered from a conveyor belt guide roller (site 43). In "Packaging Room B," *L. innocua* (ST2347) was isolated from drains (site 61 and 66) and a conveyor belt guide roller (site 63), *L. innocua* (ST1489) was isolated from a drain (site 71), and *L. monocytogenes* (ST224-CT5623) was detected in a drain (site 64), and on two conveyor belts (site 65 and 68). Of note, the *L. innocua* positive sites 51 and 63, both conveyor belt guide rollers in "Packaging Room A and B", were also positive for biofilms. In addition, a ST224-CT4656 (*L. monocytogenes*) isolate and a ST1481 (*L. innocua*) were recovered from employees' shoe soles (Supplementary Tables S2, S3).

By applying whole genome SNP analysis, 1–3 SNPs could be identified among ST224-CT5623 isolates. Additionally,

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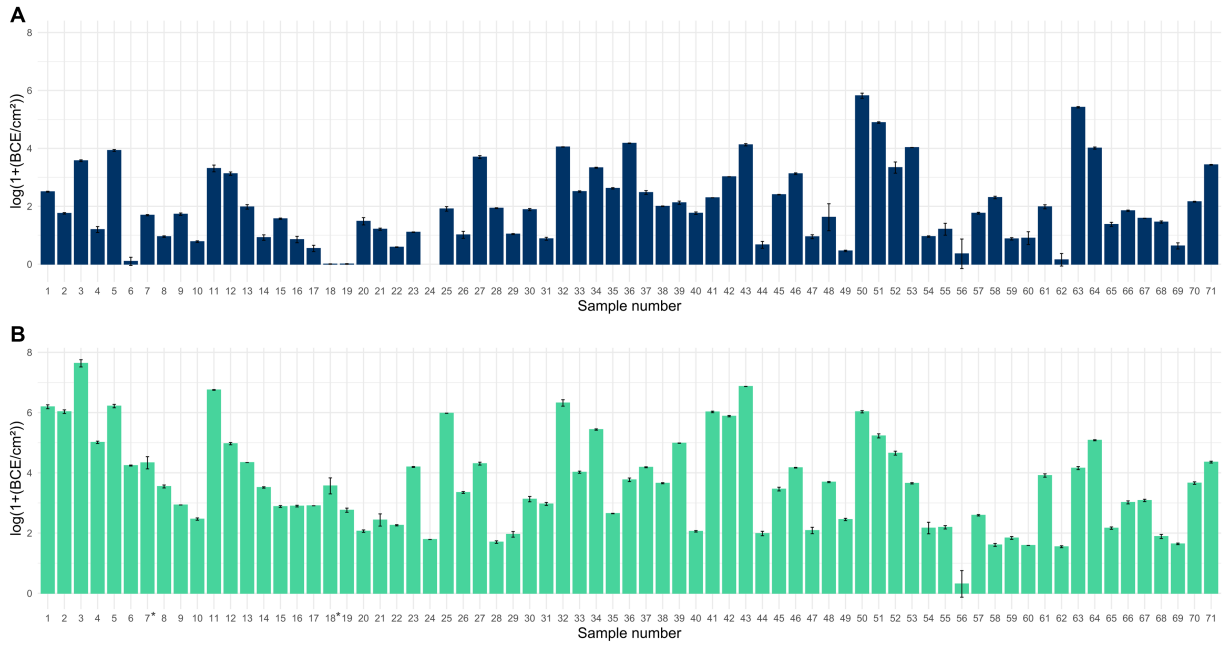

FIGURE 1
Bar chart displaying the mean bacterial load in log(1+ BCE/cm^2) ± SD for samples collected with scraper and flocked swabs to detect biofilms **(A)** and for samples collected with PU-swabs **(B)** for microbiota analysis (the addition of 1 to raw BCE/cm^2 values before log transformation was applied, to only obtain positive values after log transformation, since BCE/cm^2 values <1 would result in negative values). *: sites 7 and 18 were sampled using two swabs each. Mean values from two different swabs taken at sites 7 and 18, respectively.

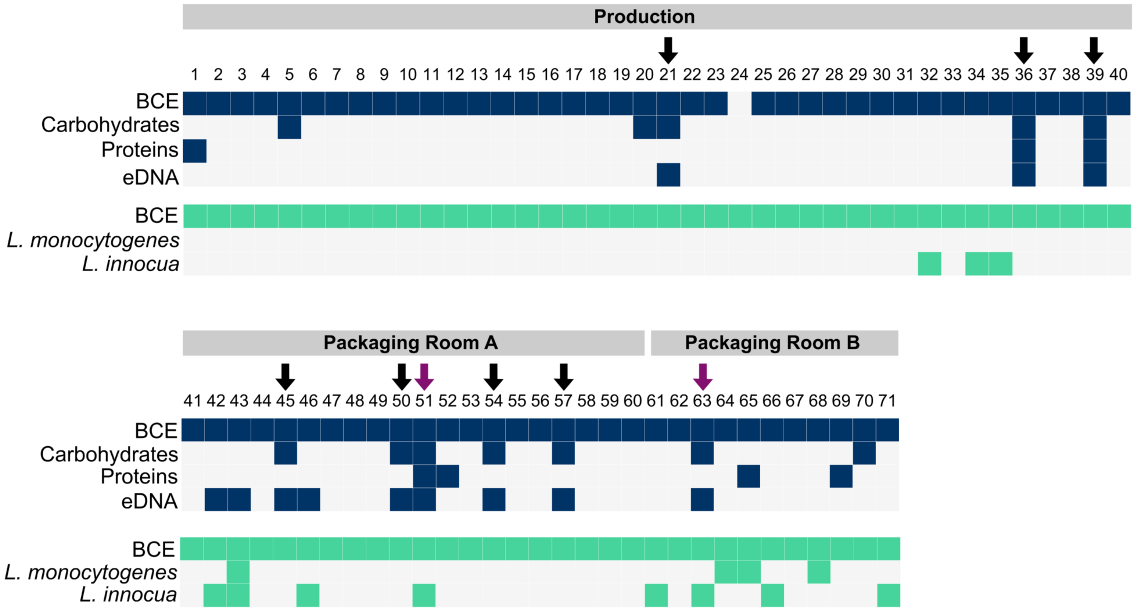

FIGURE 2
The presence of bacteria (BCE), carbohydrates, proteins and eDNA in samples collected with scraper and flocked swabs for biofilm detection (dark blue), and the presence of bacteria (BCE), *L. monocytogenes*, and *L. innocua* in samples collected with PU-swabs (green) in the production and packaging rooms A and B. Arrows indicate the presence of biofilms. Purple arrows indicate the presence of *Listeria* spp. in biofilms.

ST224-CT5623 clustered together in a phylogenetic tree (Supplementary Figure S3; Supplementary Table S5). Moreover, ST224-CT5623 isolates were identified in a previous study as in-house clones surviving for years in the frozen vegetable facility, indicating their re-occurrence in processing lines of "Packaging Room B" over time (Pracser et al., 2024).

L. innocua isolates within MLST-ST groups were closely related and clustered together in a phylogenetic tree (Supplementary Figure S2), with

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TABLE 1 *Listeria* spp. isolated from the frozen vegetable processing environment.

Isolate	Species	Isolation source	MLST-ST	cgMLST-CT*
Li-121	*L. innocua*	Site 42—conveyor belt	2,347	NA
Li-122	*L. innocua*	Site 43—conveyor belt guide rollers	2,347	NA
Li-125	*L. innocua*	Site 46—conveyor belt guide rollers	2,347	NA
Li-130	*L. innocua*	Site 51—conveyor belt guide rollers	2,347	NA
Li-142	*L. innocua*	Site 61—drain	2,347	NA
Li-144	*L. innocua*	Site 63—conveyor belt guide rollers	2,347	NA
Li-147	*L. innocua*	Site 66—drain	2,347	NA
Li-153	*L. innocua*	Site 71—drain	1,489	NA
Li-154	*L. innocua*	Site 88.S—employee's shoe soles	1,481	NA
Li-264	*L. innocua*	Site 32—drain	1,481	NA
Li-272	*L. innocua*	Site 34—conveyor belt	1,481	NA
Li-273	*L. innocua*	Site 35—conveyor belt	1,481	NA
MRL-22-01015	*L. monocytogenes*	Site 43—conveyor belt guide rollers	8	8,851
MRL-22-01016	*L. monocytogenes*	Site 64—drain	224	5,623
MRL-22-01017	*L. monocytogenes*	Site 65—conveyor belt	224	5,623
MRL-22-01018	*L. monocytogenes*	Site 68—conveyor belt	224	5,623
MRL-22-01019	*L. monocytogenes*	Site 88.S—employees' shoe soles	224	4,656

NA, not applicable; *: subtyping scheme according to Ruppitsch et al. (2015).

0 SNPs identified among isolates of ST1481 and 0–2 SNPs among isolates of ST2347 through whole genome SNP analysis (Supplementary Table S4).

3.4 Bacterial communities in the frozen vegetable processing environment

Microbial diversity within samples was estimated using alpha diversity indices (Observed, Chao1, ACE, Shannon, Simpson, Inversed Simpson, and Fisher) for the different sample groups. No significant differences between alpha diversity indices were observed within *Listeria*-positive and -negative sites (Wilcoxon test, Observed: $p = 0.306$, Chao1: $p = 0.306$, ACE: $p = 0.326$, Shannon: $p = 0.219$, Simpson/InvSimpson: $p = 0.189$, Fisher: $p = 0.306$), within biofilm and non-biofilm harboring sites (Wilcoxon test, Observed: $p = 0.723$, Chao1: $p = 0.627$, ACE: $p = 0.537$, Shannon: $p = 0.969$, Simpson/InvSimpson: $p = 1$, Fisher: $p = 0.723$), as well as within samples from different rooms (Kruskal-Wallis test, Observed: $p = 0.085$, Chao1: $p = 0.119$, ACE: $p = 0.095$, Shannon: $p = 0.143$, Simpson/InvSimpson: $p = 0.333$, Fisher: $p = 0.085$) (Supplementary Figures S4A, S5A, S6A). In terms of trends, higher species richness (Chao1) was observed in the microbial community within samples from the "Production" and "Packaging Room B" compared to "Packaging Room A", as well as in the microbiota within *Listeria* positive samples compared to *Listeria* negative samples. The alpha indices Observed ($p = 0.02$), Chao1 ($p = 0.018$), ACE ($p = 0.025$) and Fisher ($p = 0.02$) were significantly higher within samples from steel surfaces compared to plastic surfaces indicating higher species richness on steel surfaces (Supplementary Figure S7A).

A PERMANOVA analysis was conducted to statistically assess beta diversity among the different sampling groups. Microbial communities were significantly different ($p = 0.034$) between *Listeria*-positive and *Listeria*-negative samples (Supplementary Figure S4B). In addition, the different room types ("Production," "Packaging Room A," "Packaging Room B"; $p = 0.0001$) and the surface material ("steel," plastic"; $p = 0.021$) were significant factors influencing the microbial community structure (Supplementary Figures S6B, S7B). No significant differences between the microbiota in the biofilm-positive and -negative sites were detected (Supplementary Figure S5B, $p = 0.262$).

Further, we examined the taxonomic composition of the microbial community in the frozen vegetable processing environment. The 10 most abundant genera in microbiota of the processing facility across all samples were *Pseudomonas* (median relative abundance: 15.7%), *Acinetobacter* (median relative abundance: 5.43%), *Exiguobacterium* (median relative abundance: 1.39%), *Massilia* (median relative abundance: 1.35%), *Brevundimonas* (median relative abundance: 0.56%), *Paracoccus* (median relative abundance: 0.39%), unclassified *Comamonadaceae* (median relative abundance: 0.28%), *Flavobacterium* (median relative abundance: 0.27%), *Sphingomonas* (median relative abundance: 0.22%) and *Rhodococcus* (median relative abundance: 0.21%) (Figure 3).

The five most abundant genera in the biofilm-positive sample group were *Acinetobacter* (median relative abundance: 5.17%), *Pseudomonas* (median relative abundance: 4.02%), *Janthinobacterium* (median relative abundance: 1.61%), *Brevundimonas* (median relative abundance: 1.20%), and *Exiguobacterium* (median relative abundance: 1.13%) (Figure 4).

3.4.1 Differential abundance of ASVs in *Listeria*-positive versus *Listeria*-negative samples

In total, 30 ASVs were significantly differentially abundant between *Listeria*-positive and -negative samples (Figure 5A). Of these, six ASVs classified as *Carnobacterium*, *Enterobacter*, *Serratia* and unclassified *Enterobacteriaceae* were more abundant when *Listeria* was present. The other 24 differentially abundant ASVs, assigned to 15 different genera, were significantly more abundant in

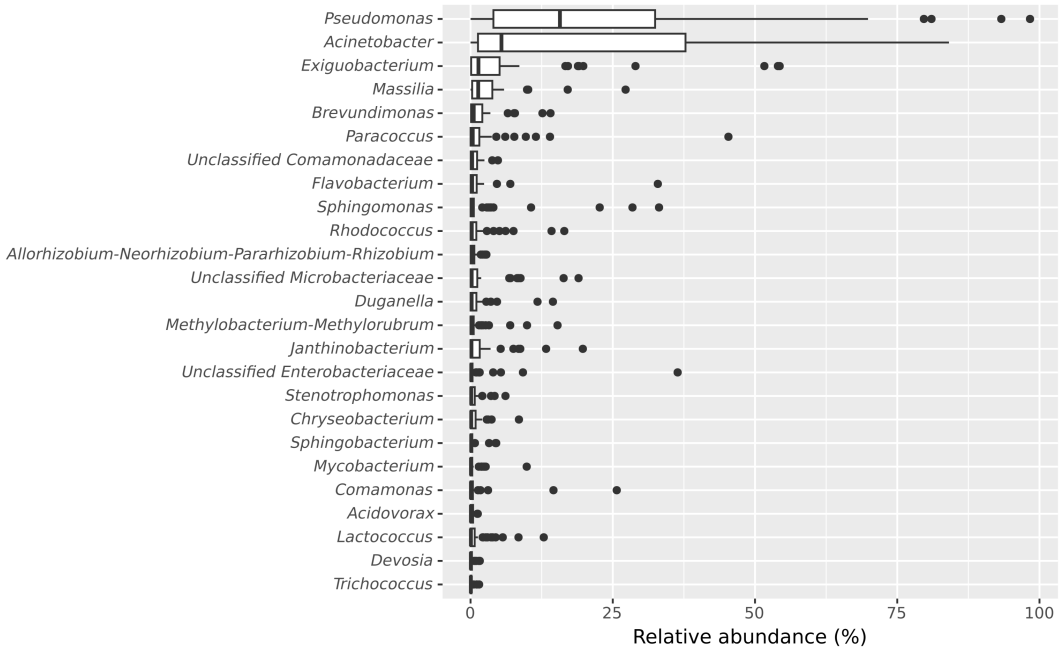

FIGURE 3
Boxplot showing the 25 most abundant genera within all samples from the frozen vegetable processing environment. Genera are decreasingly ordered by their relative median abundance.

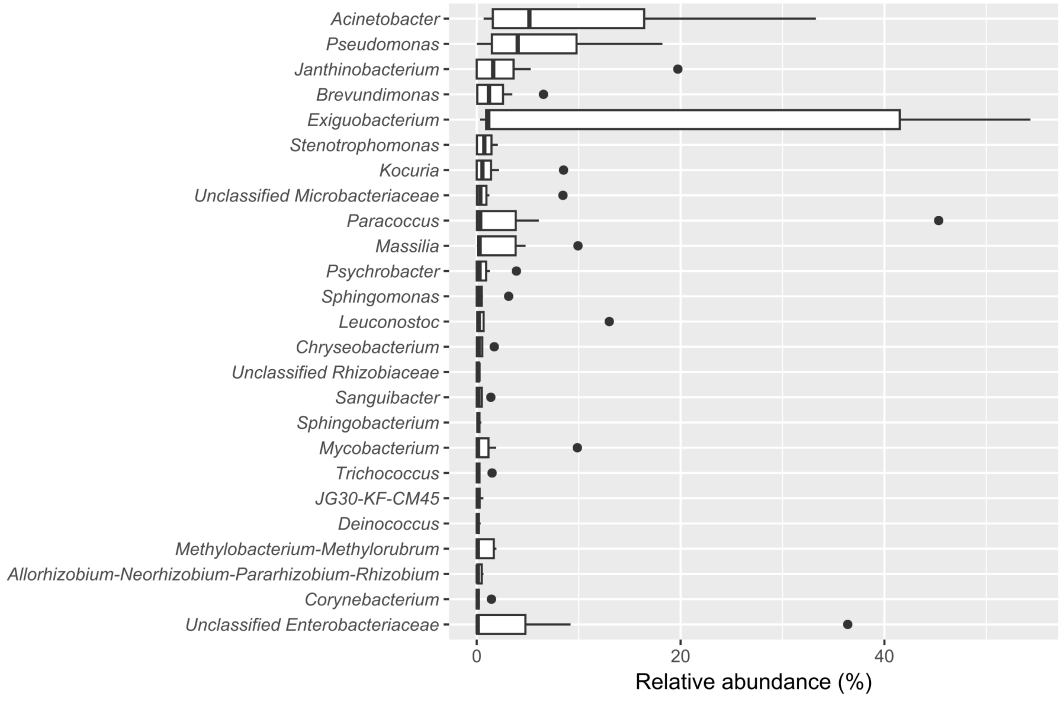

FIGURE 4
Boxplot showing the 25 most abundant genera within samples from the biofilm-positive sample group. Genera are decreasingly ordered by their relative median abundance.

Listeria-negative samples (*Aeromonas*, *Bacillus*, *Corynebacterium*, *Delftia*, *Duganella*, *Flavobacterium*, *Kocuria*, *Lactococcus*, *Leuconostoc*, *Massilia*, *Methylophilus*, *Mycobacterium*, *Pseudomonas*, *Sanguibacter*, *Streptomyces*).

3.4.2 Differential abundance of ASVs in samples from biofilm harboring sites

When comparing biofilm-positive and -negative sites, 31 ASVs were significantly differentially abundant (Figure 5B). Three ASVs

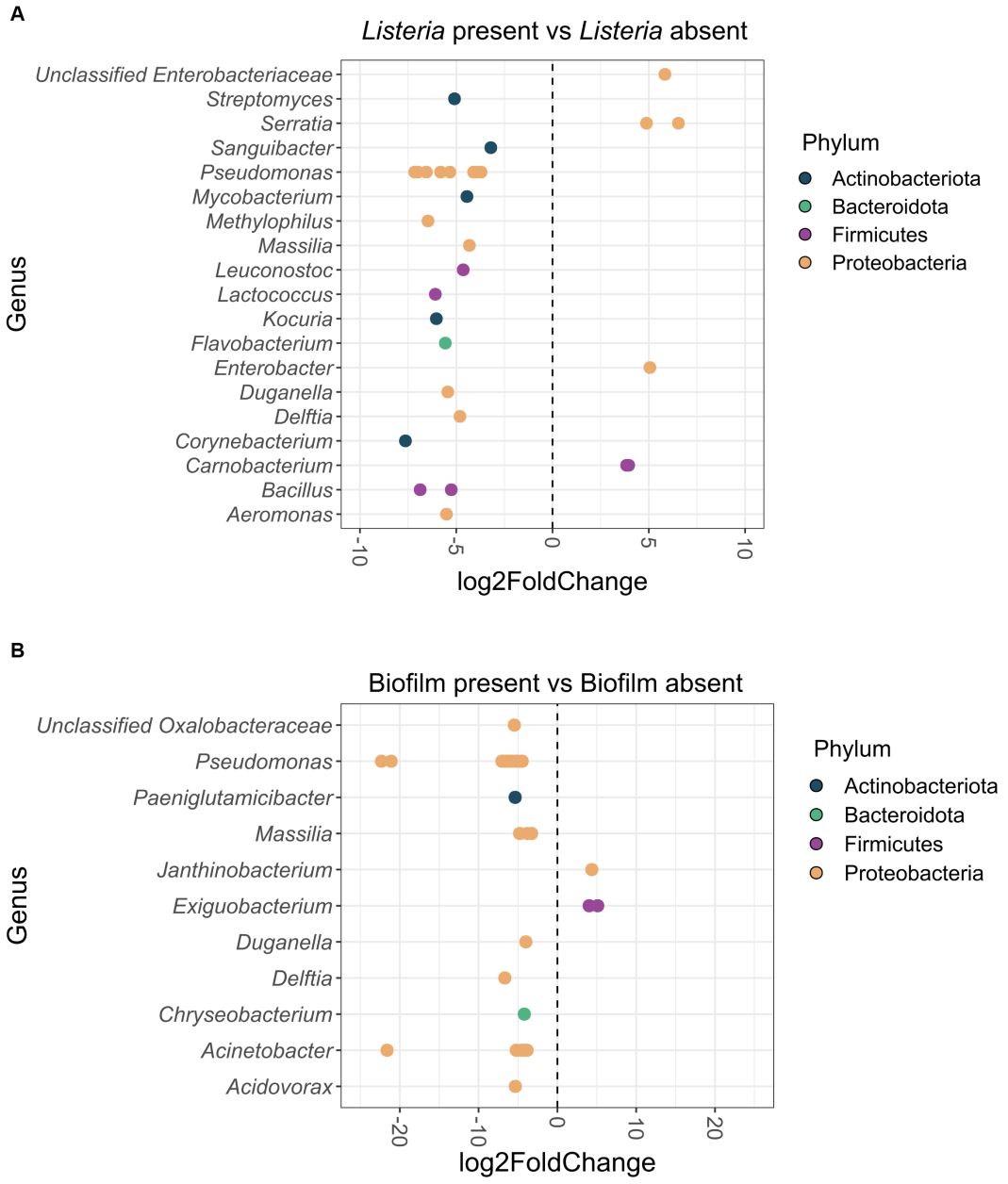

FIGURE 5
(A) Differentially abundant ($p < 0.05$) ASVs at genus-level in the *Listeria*-positive versus *Listeria*-negative sample group. Positive $\log_2$Fold Change values indicate a higher abundance of ASVs in the *Listeria*-positive sample group and negative $\log_2$Fold Change values indicate a lower abundance of ASVs in the *Listeria*-positive sample group. ASVs are displayed as dots for each separate genus. ASVs are additionally color-coded by phylum. **(B)** Differentially abundant ($p < 0.05$) ASVs at genus-level from biofilm-positive versus biofilm-negative sites. Positive $\log_2$Fold Change values indicate a higher abundance of ASVs in the biofilm-positive sample group and negative $\log_2$Fold Change values indicate a lower abundance of ASVs in the biofilm-positive sample group. ASVs are displayed as dots for each separate genus. ASVs are additionally color-coded by phylum.

classified as *Exiguobacterium* and *Janthinobacterium* were more abundant in biofilms, whereas 28 ASVs classified as *Acidovorax*, *Acinetobacter*, *Chryseobacterium*, *Delftia*, *Duganella*, *Massilia*, *Paeniglutamicibacter*, *Pseudomonas*, and unclassified *Oxalobacteraceae* were significantly more abundant on biofilm-negative sites.

3.4.3 Differential abundance of ASVs in different rooms of the frozen vegetable processing facility

Differential abundance analysis identified 126 ASVs from 60 different genera that were significantly differentially abundant between "Packaging Room A" and the "Production" room (Supplementary Figure S8), and 79 ASVs from 43 genera between "Packaging Room B" and the "Production" room (Supplementary Figure S9). Only 34 ASVs of 12 genera were differentially abundant between "Packaging Room A" and "Packaging Room B" (Supplementary Figure S10).

In "Packaging Room A", 23 ASVs, among them prevalent genera such as *Exiguobacterium*, *Pseudomonas*, *Acinetobacter*, and *Psychrobacter*, were significantly more abundant than in the "Production" room. Also, 123 ASVs, such as those assigned to *Pseudomonas*, *Exiguobacterium*, *Acinetobacter*, or *Massilia*, were

significantly more abundant in the "Production" room compared to "Packaging Room A.

In "Packaging Room B", 14 ASVs were significantly more abundant compared to the "Production" room, among them genera such as *Pseudomonas*, *Exiguobacterium* or unclassified *Enterobacteriaceae*. Other ASVs of prevalent genera such as *Acinetobacter*, *Exiguobacterium*, *Massilia*, *Paracoccus* and *Pseudomonas* were significantly more abundant in the "Production" room than in "Packaging Room B." The majority of differentially abundant ASVs (65 ASVs) were significantly more abundant in "Production" when compared to "Packaging Room B".

In "Packaging Room A", 22 ASVs were significantly more abundant than in "Packaging Room B," among them ASVs assigned to *Acinetobacter*, *Corynebacterium*, *Exiguobacterium*, *Massilia*, *Pseudomonas*, *Psychrobacter*, and *Sphingobacterium*, and 12 ASVs (*Brevundimonas*, *Enterobacter*, *Exiguobacterium*, *Kocuria*, *Pseudomonas*, *Sphingomonas*, and unclassified *Enterobacteriaceae*) were significantly lower abundant.

3.4.4 Differential abundance of ASVs between steel and plastic surfaces

Differential abundance of ASVs between samples from steel and plastic surfaces was additionally assessed. 51.61% of samples from the packaging rooms and 67.5% from the "Production" room collected from steel surfaces. We identified 104 ASVs assigned to 52 genera that were significantly differentially abundant between samples from steel versus plastic surfaces (Supplementary Figure S11). In samples from steel surfaces, 86 ASVs were identified as significantly more abundant, among them were 27 ASVs of prevalent genera such as *Acinetobacter*, *Enterococcus*, *Exiguobacterium*, *Flavobacterium*, *Massilia*, *Paracoccus*, *Pseudomonas*, and *Sphingomonas*. Of note, a similar number of differentially abundant ASVs were identified when both packaging rooms were compared to the "Production" room.

4 Discussion

This study aimed to investigate the presence of *Listeria* in biofilms and characterize the co-existing microbiota in the processing environment of a European frozen vegetable processing facility, where *L. monocytogenes* in-house clones persisted for years (Pracser et al., 2024). We collected all samples after cleaning and disinfection to assess the hygiene status of the processing environment while avoiding the presence of food residues, which would interfere with the analysis of extracellular matrix components of biofilms.

The microbiota in the processing facility, where bacteria encounter temperatures ranging from −20°C to +70°C and significant moisture, was dominated by *Pseudomonas*, *Acinetobacter* and *Exiguobacterium*. While *Exiguobacterium* is often found in extreme environments (Vishnivetskaya et al., 2009), *Pseudomonas* and *Acinetobacter* are commonly found in diverse food processing environments (Zwirzitz et al., 2021; Rolon et al., 2023; Serrano Heredia et al., 2023; Tadielo et al., 2023; Valentino et al., 2023; Xu et al., 2023). In addition, members of *Pseudomonas* (Zhang et al., 2019; Chauhan et al., 2023), *Acinetobacter* (Kim et al., 2018; Herrera et al., 2021) and *Exiguobacterium* (Vishnivetskaya et al., 2007; Rodrigues et al., 2008) are capable of adapting to low temperatures.

In this study, biofilms were detected on 12.7% (9/71) of the sampled sites, which is in line with other studies using similar methods for biofilm detection. Previous research reported a biofilm prevalence of 9.3% in a meat processing environment (Wagner et al., 2020) and 17% across multiple types of food processing environments, including those producing oven foods, dairy products, meat products, baker's yeast, sauces and egg products (Maes et al., 2017). In our study, biofilms were mainly located on direct and indirect food contact surfaces such as on conveyor belts and conveyor belt guide rollers (44% of biofilm positive sites), a screw conveyor, a metal tub, an air separator, and two steel funnels. Moreover, the highest bacterial loads were found in biofilms located on a screw conveyor (5.8 log BCE/cm^2) and two conveyor belt guide rollers (5.4 log BCE/cm^2, 4.9 log BCE/cm^2). It is well known that biofilms have high cell densities (Flemming et al., 2016). On sites with attached bacterial cells and established biofilms, chemical cleaning together with mechanical cleaning strategies such as scraping or enzymatic disruption of biofilms would be essential for biofilm removal (Chmielewski and Frank, 2003; Kumar et al., 2021; Mazaheri et al., 2022). The top five most abundant genera within the biofilm-positive sample group were *Acinetobacter*, *Pseudomonas*, *Janthinobacterium*, *Brevundimonas*, and *Exiguobacterium*. Different studies have demonstrated the biofilm-forming ability of all five of these genera (Pantanella et al., 2007; Douterelo et al., 2014; Chauhan et al., 2018; Wagner et al., 2021; Chernogor et al., 2022; Gricajeva et al., 2022; Huang et al., 2022; Liu et al., 2023; Maifreni et al., 2023). In addition, *Acinetobacter*, *Pseudomonas*, *Janthinobacterium*, and *Brevundimonas* were previously found in biofilms in food processing environments (Dzieciol et al., 2016; Wagner et al., 2020). In biofilms, *Janthinobacterium* and *Exiguobacterium* were more abundant than in the other samples. Some *Exiguobacterium* species have been described as having the potential to degrade plastic materials such as polystyrene or polypropylene. The biodegrading activity of bacteria can lead to alterations in surface material properties, such as changes in hydrophobicity or damage to the material (Chauhan et al., 2018; Sun et al., 2023). Surface defects or increased roughness make surfaces challenging to clean, which is favorable for biofilm formation, as previously discussed (Dass and Wang, 2022; Sharan et al., 2022). In this study, 38.4% of sample surfaces were built from plastics. Although *Pseudomonas* was present in biofilm samples, we detected 13 significantly lower abundant *Pseudomonas* ASVs in biofilms compared to biofilm-negative sites. This was unexpected, as *Pseudomonas* is a frequent biofilm-former and *Pseudomonas* biofilms were described to provide other bacterial species, e.g., *L. monocytogenes*, protection from environmental stressors such as cleaning and disinfection (Sterniša et al., 2023; Thomassen et al., 2023; Zarei et al., 2023). Since *Pseudomonas* is widespread in the food processing environment, we assume that species or strain-level differences are responsible for the findings of our study. This hypothesis is supported by a study which demonstrated inter- and intra-species differences in the biofilm-forming ability of *Pseudomonas* isolates from meat and dairy processing environments (Fernández-Gómez et al., 2021). Microbial interactions may pose another factor influencing the microbiota at biofilm-positive sites, since Singh et al. (2019) reported an anti-biofilm activity of *Exiguobacterium indicum* against *Pseudomonas aeruginosa* by quorum sensing inhibition. Nevertheless, further research with additional data, such as metagenomes or from the characterization of isolates would be required to explore functional correlations at the species and strain levels.

Different studies hypothesized that (multispecies) biofilms support *Listeria* persistence in food processing environments, as the biofilm matrix protects *Listeria* from disinfectants (Van der Veen and Abee, 2011; Oxaran et al., 2018; Rolon et al., 2024b). In this study, we detected *L. innocua* on two biofilm harboring sites, located at conveyor belt guide rollers with *Exiguobacterium* present in abundance. These results demonstrate for the first time that *Listeria* spp. are able to colonize biofilms in the processing environment. Due to the low sample number, further research would be necessary to test if *Exiguobacterium* supports *Listeria* survival and colonization (on plastic surfaces) under conditions found in food processing environments. Yet, no biofilm was detected on the majority of *Listeria*-positive sites (12/14 sites), indicating that biofilms did not play a significant role in *Listeria* survival and persistence. Both, *L. innocua* and *L. monocytogenes* were detected on conveyor belts and the associated conveyor belt guide rollers, and in drains. In previous studies, conveyor belts, especially modular-built conveyor belts with multiple joints, were described as hard-to-clean niches, which contribute to the survival of *Listeria* and the establishment of biofilms (Veluz et al., 2012; Langsrud et al., 2016; Fagerlund et al., 2017; Belias et al., 2022). The proportion of modular conveyor belts was higher in the packaging rooms than in the production room, which could explain the higher prevalence of biofilms and *Listeria* in the packaging rooms. In addition, differences in cleaning and disinfection efficiency (Burnett et al., 2022; Maillard and Centeleghe, 2023) or surface defects (Hua and Zhu, 2024) can also influence biofilm establishment and the survival of *Listeria*. It is frequently reported that drains serve as reservoirs for *Listeria* (Rückerl et al., 2014; Kaszoni-Rückerl et al., 2020; Stessl et al., 2020; Bardsley et al., 2024) and biofilms (Dzieciol et al., 2016; Wagner et al., 2020). In our study, we detected *Listeria* in five drains, but none of the total seven drains harbored a biofilm. Clearly, conveyor belts and drains provide a potential reservoir for *Listeria*. The difficulty to clean the type of modular conveyor belts used in the processing facility promotes the survival of *Listeria*. In floor drains, accumulation of organic matter, moisture and the potential presence of biofilms may provide beneficial conditions for *Listeria*.

Characterization of *Listeria* spp. revealed the presence of ST8 and ST224 *L. monocytogenes* isolates in the processing environment. Both MLST-STs were prevalent in the same frozen vegetable processing environment in our previous study. ST224-CT5623 was identified as an in-house clone in the packaging area, specifically in "Packaging Room B" (Pracser et al., 2024). Our findings, combined with the results of our previous research, clearly show that ST224-CT5623 is still present after multiple cleaning and disinfection cycles. Furthermore, whole genome SNP analysis of *L. innocua* isolates in the ST1481 and ST2347 groups detected 0 and 0–2 SNPs, respectively, and isolates within a MLST-ST group clustered together with 100% bootstrap support in a phylogenetic tree, may indicating a close genetic relatedness in relation to the metadata (Jagadeesan et al., 2019).

We additionally investigated the microbiota co-occurring with *Listeria* in the frozen vegetable processing environment. In different food producing environments, different genera have been found to correlate with the presence of *Listeria*. For example, *Pseudomonas*, *Acinetobacter*, or *Janthinobacterium* were reported to be indicative for *Listeria* presence in a meat processing environment (Zwirzitz et al., 2021). Also, *Pseudomonas*, *Stenotrophomonas*, and *Microbacterium* were associated with *Listeria* presence in tree fruit packing facilities (Rolon et al., 2024b), and *Carnobacterium*, *Psychrobacter*, and *Pseudomonas* were more abundant in samples positive for *Listeria* in distribution centers for fresh produce (Townsend et al., 2023). We also detected significant differences in the community structure between *Listeria*-positive and *Listeria*-negative samples. *Carnobacterium*, *Enterobacter*, *Serratia* and ASVs from unclassified *Enterobacteriaceae* were found to be higher abundant in *Listeria*-positive samples. Interestingly, several *Pseudomonas* ASVs were less abundant in *Listeria*-positive compared to *Listeria*-negative samples. This is in line with another study conducted in a meat processing environment, which reported a higher relative abundance of *Pseudomonas* (not significant) in *Listeria*-negative samples (Belk et al., 2022), but is inconsistent with the results from Zwirzitz et al. (2021), where the presence of *Listeria* was associated with the occurrence of *Pseudomonas*. To further analyse this discrepancy, we compared the significantly lower abundant *Pseudomonas* ASVs (from partial 16S rRNA gene sequencing) in *Listeria*-positive samples of this study, to the significantly higher abundant *Pseudomonas* ASVs (nearly full length 16S rRNA gene sequencing) in *Listeria*-positive samples of the study from Zwirzitz et al. (2021). Our analysis detected 100% nucleotide identity (compared to the sequences from Zwirzitz et al., 2021) for only one *Pseudomonas* ASV, whereas all other ASVs shared between 95.79 and 99.07% nucleotide identity. This finding indicates that species- and/or strain-level differences are among the driving forces for shaping the microbial community. Therefore, further research using whole metagenome sequencing rather than 16S rRNA gene sequencing is necessary to investigate the microbial community structure on species and strain level.

Similar to the findings of Belk et al. (2022) and other studies (Dzieciol et al., 2016; Cobo-Díaz et al., 2021), we found distinct specific communities in various areas of food processing environments. Major differences were observed between the "Production" room and both packaging rooms, as in the "Production" room 123 ASVs were significantly more abundant than in "Packaging Room A" and 65 ASVs compared to "Packaging Room B". The variance in the composition of the microbiota may be explained by environmental conditions such as temperature. In the packaging rooms, the temperature ranged from cold to ambient conditions, whereas in the "Production" room mostly ambient to hot conditions were predominant during operation. Furthermore, we observed significant variations in alpha-diversity measures (Observed, Chao1, ACE, Fisher) and in beta-diversity when we compared the microbiota of samples from steel and plastic surfaces. Surprisingly, in samples from steel surfaces, we found higher richness within the microbiota. Differential abundance analysis revealed 86 ASVs were higher in abundance in samples from steel surfaces than in samples from plastic surfaces, mainly built from polyacetal. The materials have different surface properties, as for example steel is generally more hydrophilic, whereas polyacetal is rather hydrophobic. There is evidence that bacterial attachment to surfaces is influenced by individual strain abilities, roughness, and hydrophobicity of the surface material (Ashok et al., 2023; Cai et al., 2023; Finn et al., 2023), which may explain the variations in the microbiota of steel and plastic surfaces. For instance, significantly more biomass of *Acinetobacter baumannii* accumulated on polycarbonate compared to other surface materials such as glass, rubber, porcelain and polypropylene in a study (Greene et al., 2016). An increased albeit not significant biofilm formation of *Pseudomonas fluorescens* was observed on polystyrene compared to glass and stainless steel (Gagné et al., 2022). However, the factors influencing attachment and biofilm formation on different

surface materials are complex. A study reported a significantly increased biofilm formation of *Pseudomonas fluorescens* on stainless steel at 25°C compared to 4°C. This temperature-dependent effect on biofilm formation was reversed but not significant on polystyrene and not visible on polytetrafluoroethylene (Maifreni et al., 2023). Moreover, the number of hard-to-clean niches in the two sample groups and/or the sanitation efficacy on the plastic and steel surface materials may affect the composition of the microbiota, as a study reported differences in cleaning and disinfection efficacy of smooth plastic surfaces built from high-density polyethylene surfaces compared to stainless-steel (Ohman et al., 2023). The frozen vegetable producer applies multiple cleaning and disinfection agents including chlorine-based agents, foaming surfactants and peracetic acid. There were no differences in the cleaning and disinfection regime between the different rooms. There is still a gap in knowledge about which factors are the (main) drivers for shaping the microbiota. It is unclear if the differences in the microbiota were driven by the surface material or by environmental conditions in the rooms. The fact that different *Pseudomonas* ASVs were either higher or lower abundant in samples from steel surfaces further supports potential differences at the species or strain level and their colonization of niches with distinct properties in food processing environments.

In summary, we detected *Listeria* spp. on 14 sites after cleaning and disinfection in the frozen vegetable processing environment. Of note is that the quality control team of the frozen vegetable processing facility regularly monitors the microbial safety of food products, which strongly reduces the risk for customers. *Listeria* spp. were present in two out of nine biofilms, demonstrating (to our knowledge) for the first time that *Listeria* spp. are a member of the microbial community in "real" biofilms in a food processing environment. Biofilms were detected by determining the presence of bacteria by qPCR and the presence of at least two extracellular matrix components of the biofilm (carbohydrates, proteins, eDNA). The finding that the majority of *Listeria* were found on non-biofilm sites indicates that biofilms are only one factor for *Listeria* survival in the processing environment. Biofilms and *Listeria* were frequently found on modular conveyor belts and/or conveyor belt guide rollers, hard-to-clean niches which showed the highest bacterial load. Therefore, we recommend substituting these conveyors to enhance hygiene in the frozen vegetable processing facility. ASVs assigned to *Carnobacterium*, *Serratia*, *Enterobacter* and unclassified *Enterobacteriaceae* were associated with *Listeria* presence. *Janthinobacterium* and *Exiguobacterium*, both known biofilm formers, were significantly more abundant in biofilms. In addition, there were indications that the different room types and their specific environmental factors, such as temperature or surface materials, play a role in shaping the existing microbiota. Our findings demonstrate the complex nature of microbial interactions in the food-producing environment, including biofilm and other *Listeria* niches, and suggest that further research is needed to identify the main drivers for modulating the microbiota and the presence of *Listeria* in food-processing environments.

Data availability statement

The datasets presented in this study can be found in online repositories. The datasets analyzed for this study can be found in the NCBI repository under the BioProject number PRJNA1075930 https://www.ncbi.nlm.nih.gov/.

Author contributions

NP: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. EV: Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing. ST: Data curation, Investigation, Methodology, Writing – review & editing. AP: Data curation, Methodology, Writing – review & editing, Investigation. ES: Conceptualization, Formal analysis, Supervision, Writing – review & editing. MW: Funding acquisition, Resources, Supervision, Writing – review & editing, Project administration. KR: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1404002/full#supplementary-material

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

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

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

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

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

KEYWORDS

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

1 Introduction

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

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

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

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

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

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

2 Materials and methods

2.1 Origin and sample collection

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

2.2 Cultural detection of *Listeria monocytogenes*

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

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

2.3 DNA extraction and whole genome sequencing

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

2.4 Sequence data analysis

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

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

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

NCBI AMRFinder+ v3.11.2. was used with the EXACT method at 100% setting together with the BLAST alignment at >90% of protein sequences against the AMRFinder+ database to detect antimicrobial resistance genes (ARG) (Michael et al., 2019). The Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Listeria; accessed on 6th June 2023) was used to detect VGs (Liu et al., 2022). Thresholds were set for the target scanning procedure to ≥85% identity with the reference sequence and ≥ 99% with the aligned reference sequence. Pathogenicity islands LIPI-3 and LIPI-4 were extracted from genome data. The CGE Mobile Element Finder v1.0.5. was used with the <90% identity and > 95% alignment method to detect Mobile Genetic Elements (MGE) (Johansson et al., 2021). Chromosome & Plasmid Finder v1.0. through MOB-suite v3.1.4. available in SeqSphere+ v8.5.1. was used with the Mash Neighbor Distance >0.06 to detect plasmids (Robertson and Nash, 2018). Isolates of the most prevalent STs of *L. monocytogenes* of our study (ST8, ST9, ST121, ST155, ST204) were compared with those from pubMLST database[1] and the Austrian Reference Laboratory for Listeria, which currently includes more than 20,000 genomes.

3 Results

3.1 Detection of *Listeria monocytogenes*

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

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

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

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

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

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

Industry (No. of isolates)	Years	Sequence type	Prevalence per industry (%)
Meat industry 1 (23)	2015 2016 2017 2018 2019 2021 2022	ST9 ST9, ST121 ST8, ST9 ST4, ST9 ST7, ST8 ST8, ST9, ST121 ST9	ST4 (4%) ST7 (4%) ST8 (13%) ST9 (48%) ST121 (31%)
Meat industry 2 (34)	2016 2019 2020 2021 2022	ST8, ST9, ST37, ST121 ST31, ST101, ST580 ST8, ST121, ST155, ST451, ST580 ST8 ST8	ST8 (50%) ST9 (10%) ST31 (6%) ST37 (3%) ST101 (6%) ST121 (6%) ST155 (6%) ST451 (3%) ST580 (10%)
Meat industry 3 (9)	2017 2020 2021	ST9, ST736 ST8 ST736	ST8 (22%) ST9 (22%) ST736 (56%)
Meat industry 4 (1)	2021	ST1	ST1 (100%)
Meat industry 5 (6)	2019 2020	ST9, ST121, ST321 ST9	ST9 (50%) ST121 (33%) ST321 (17%)
Meat industry 6 (12)	2015 2016 2017 2019 2020	ST9 ST8, ST9, ST451 ST121 ST9 ST8, ST37, ST489	ST8 (18%) ST9 (37%) ST37 (9%) ST121 (9%) ST451 (9%) ST489 (17%)
Meat industry 7 (1)	2019	ST155	ST155 (100%)
Meat industry 8 (4)	2015 2019	ST7, ST9 ST8	ST7 (25%) ST9 (50%) ST9 (25%)
Meat industry 9 (10)	2016 2019	ST1, ST321 ST8, ST121, ST321	ST1 (10%) ST8 (30%) ST121 (40%) ST321 (20%)
Meat industry 10 (2)	2018 2019	ST121 ST37	ST37 (50%) ST121 (50%)
Meat industry 12 (1)	2016	ST3	ST3 (100%)
Meat industry 13 (2)	2016 2017	ST8 ST121	ST8 (50%) ST121 (50%)
Meat industry 15 (3)	2016	ST8, ST37, ST451	ST8 (34%) ST37 (33%) ST451 (33%)
Meat industry 16 (1)	2016	ST8	ST8 (100%)
Meat industry 17 (3)	2015	ST2	ST2 (100%)
Milk industry 1 (2)	2020	ST101	ST101 (100%)
Milk industry 2 (9)	2016 2017 2018	ST8, ST204 ST204 ST37, ST204	ST8 (11%) ST37 (11%) ST204 (78%)
Milk industry 3 (7)	2016 2017 2018	ST155 ST155 ST155	ST155 (100%)
Milk industry 4 (1)	2017	ST7	ST7 (100%)
Small farms (5)	2017 2018 2019	ST1 ST7, ST121, ST204 ST26	ST1 (20%) ST7 (20%) ST26 (20%) ST121(20%) ST204 (20%)
Unknown industries (11)	2014 2015	ST9, ST101, ST517, ST736 ST1, ST2, ST124, ST451	ST1 (9%) ST2 (9%) ST9 (37%) ST101 (9%) ST124 (9%) ST451 (9%) ST517 (9%) ST736 (9%)
Imported (13)			
IMP 1 IMP 2 IMP 3 and IMP 4 IMP 5	2017 Bosnia 2020 France 2018–2019 Serbia 2022 Italy	ST7 ST37 ST14, ST155 ST155	ST7 (8%) ST14 (8%) ST37 (8%) ST155 (77%)

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

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

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

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

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

10.3389/fmicb.2024.1418333

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

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

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

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

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

3.2.1 *Listeria monocytogenes* ST8 (*n* = 29)

Twenty-nine *L. monocytogenes* ST8 isolates (18% of the total) were obtained from ten industries in Montenegro during the period 2016–2022. ST8 isolates were the most prevalent in Meat IND 2 (*n* = 16, 55,2% of ST8), Meat IND 13 (*n* = 1, 50%) and Meat IND 16 (*n* = 1, 100%). *L. monocytogenes* ST8 isolates were distributed to cluster 1 (*n* = 18), cluster 7 (*n* = 7) and four singletons. Cluster 1 comprised one isolate from Meat IND 1 (3.4% of the ST8), fifteen isolates from Meat IND 2 (48%), one isolate from Meat IND 6 (3.4%) and one isolate from Meat IND 13 (3.4%) with a maximum of eight allelic differences within the cluster (Figure 1). Cluster 7 comprised seven isolates differing by a maximum of three alleles. One isolate was obtained from Meat IND 2 (3.4%), two isolates from Meat IND 3

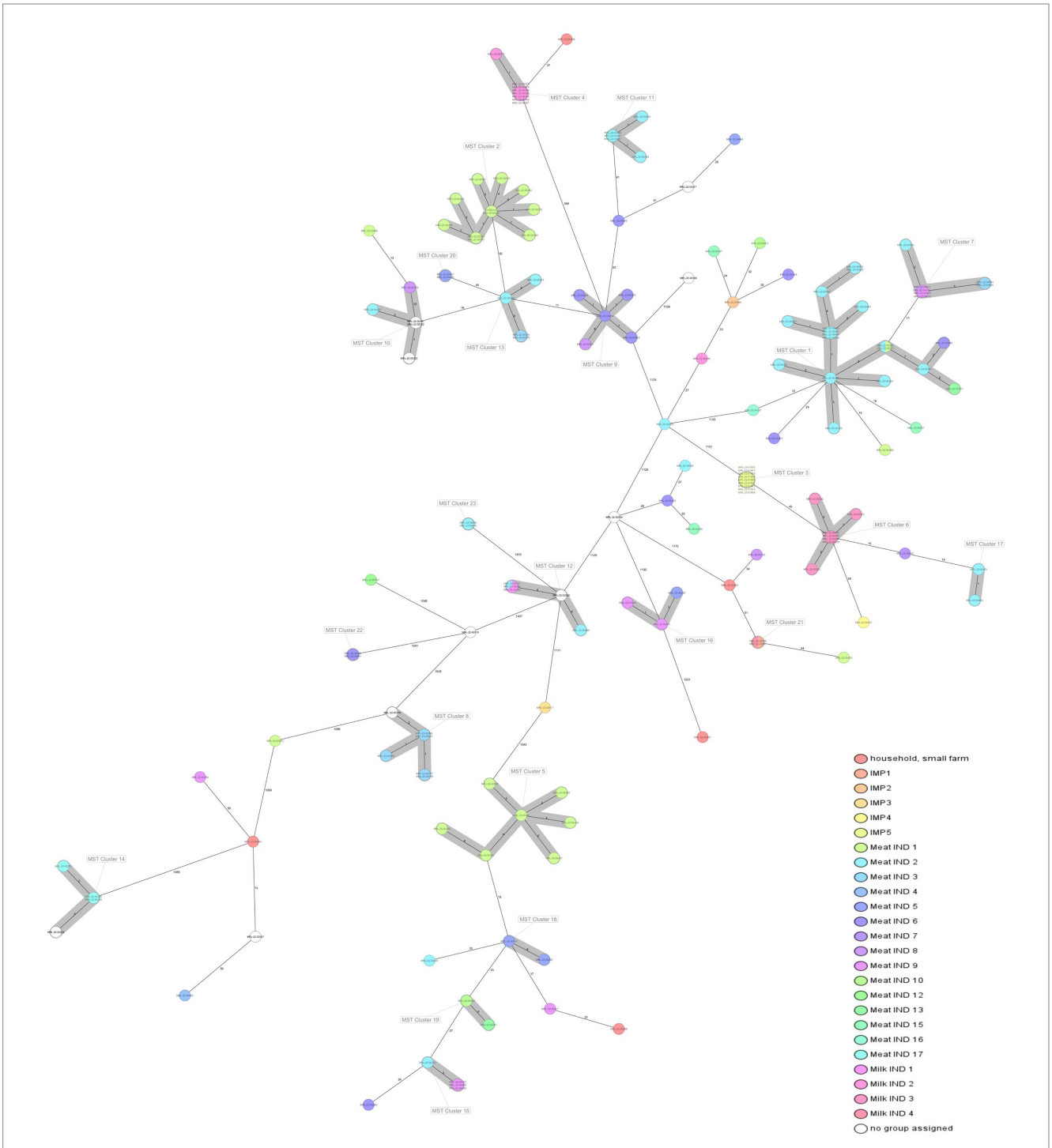

FIGURE 1
Minimum spanning tree (MST) of 160 *L. monocytogenes* isolates included in the study based on the cgMLST defined by Ruppitsch et al. (2015). Cluster definition was set in 10 allelic differences. Isolates are coloured by company/industry. Information in every isolate: ST, alias, year of isolation.

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

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

3.2.2 *Listeria monocytogenes* ST9 ($n = 31$)

Thirty-one *L. monocytogenes* ST9 isolates (19% of the total) were obtained from ten different industries in Montenegro from the period 2014 to 2022. ST9 isolates were the most prevalent type in Meat IND 1 ($n = 12$, representing the 38% of ST9), Meat IND 5 ($n = 3$, 10%), Meat IND 6 ($n = 5$, 16%), and Meat IND 8 ($n = 2$, 6%) (Table 1; Figure 2). *L. monocytogenes* ST9 isolates were distributed to five clusters (cluster 2, 9, 10, 13 and 20) and four isolates were singletons. Cluster 2 isolates ($n = 11$, 35.48% of ST9) were obtained from beef meat from Meat IND 1 from 2015 to 2022, whereas isolates from the other clusters were derived from pork meat. Cluster 9 isolates ($n = 5$, 16.12% of ST9) were obtained from Meat IND 6 and Meat IND 8, with a maximum of eight allelic difference. Isolates within Cluster 10 ($n = 5$, 16.12% of ST9) were obtained from Meat IND2 ($n = 1$, 3.22%), Meat IND 8 ($n = 1$, 3.22%) and from and unknown industries ($n = 3$, 9.67%), with a maximum of ten allelic differences within the cluster. Cluster 13 isolates ($n = 4$; 12.90% of ST9) were obtained from Meat IND 2 and Meat IND 3 with a maximum difference of nine alleles. Isolates in Cluster 20 ($n = 2$, 6.45% of ST9) derived from Meat IND 5 showed no allelic difference in their core genome.

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

3.2.3 *Listeria monocytogenes* ST121 ($n = 19$)

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

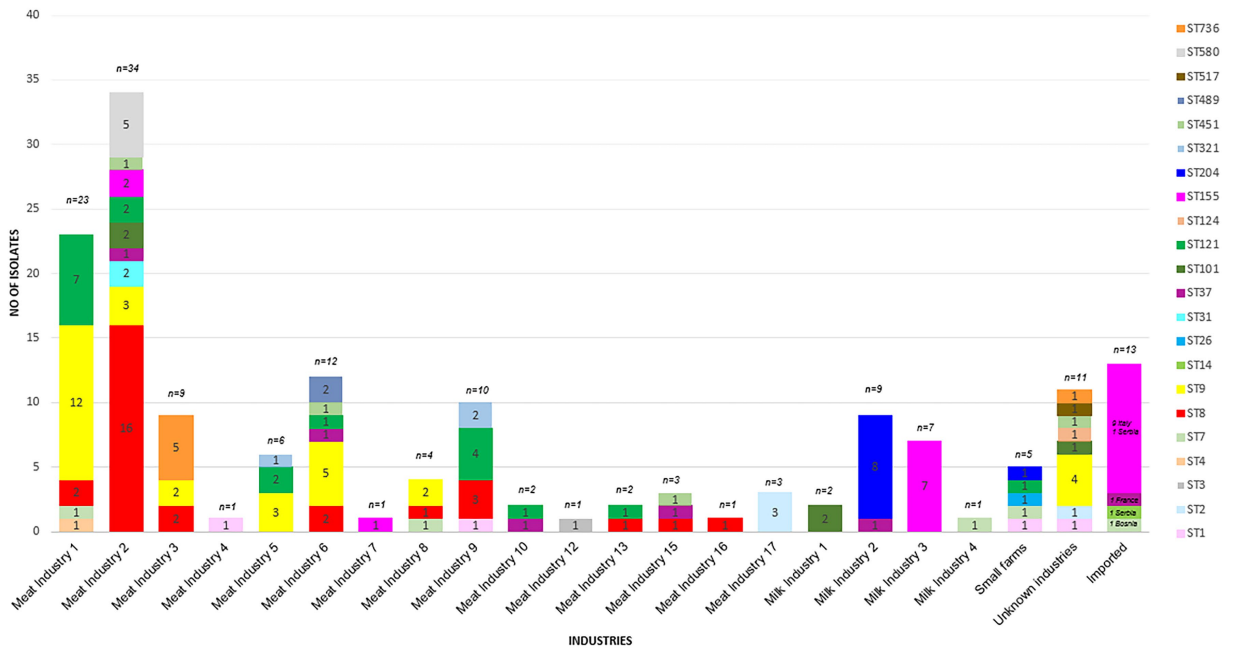

FIGURE 2
Prevalence of each ST sorted by industry.

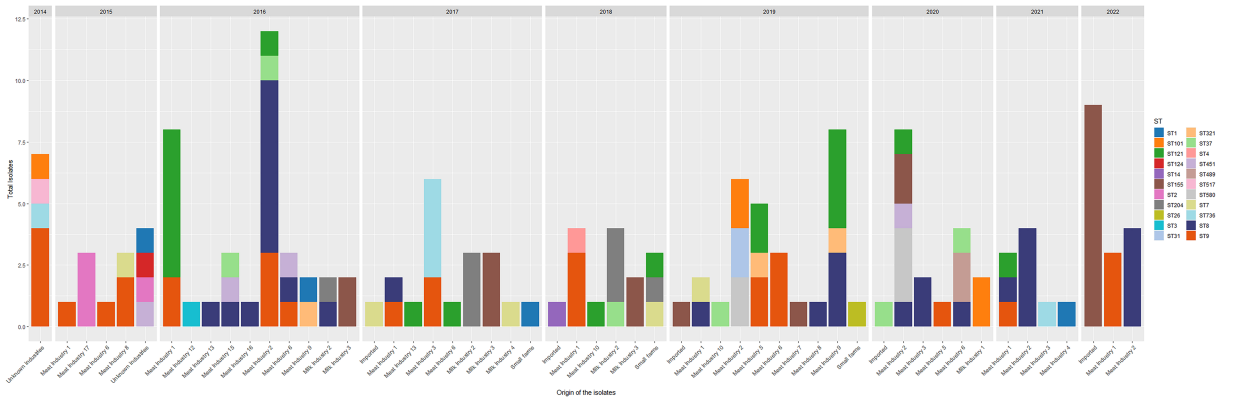

FIGURE 3
ST sorted by industry and year.

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

3.2.4 *Listeria monocytogenes* ST155 (*n* = 20)

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

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

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

3.2.5 *Listeria monocytogenes* ST204 (*n* = 9)

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

3.2.6 Other *Listeria monocytogenes* STs

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

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

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

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

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

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

3.3 Mobile genetic elements and plasmids

One hundred eleven out of 160 isolates carried at least one MGE. Nine isolates (5.6% of the total) carried over five MGEs. The most prevalent MGE was the unit transposon *Tn5422*, which was found in eighty-four isolates (52.5% of the total) followed by the insertion sequences *ISLmo1* and *ISLmo9* found in forty-three (26.8% of the total) and forty-seven (29.3% of the total) isolates, respectively (Supplementary Table S1). All *L. monocytogenes* ST8, ST9 and ST121 isolates carried MGE *Tn5422*. *ISLmo1*, *ISLmo7*, *ISLmo8*, and *ISLmo9* were present in 32, 39, 39, and 55% of *L. monocytogenes* ST9, respectively. All ST204 and two ST155 isolates carried *ISLmo1*. The remaining eighteen ST155 isolates and ST1, ST2, ST4, ST7, ST451, ST489, ST517, ST736 isolates carried no MGEs. ST3, ST9, ST31, and ST580 carried 5–7 MGEs (Supplementary Table S1).

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

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

3.4 Antimicrobial genes, pathogenicity islands, and virulence genes

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

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

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

4 Discussion

4.1 Genetic diversity

Controlling *L. monocytogenes* in the food chain is an ongoing and important challenge due to its resilience in different environments. Over a period of 8 years, samples from food products and food production environments of different origin and composition were examined, showing a prevalence rate of 0.7% among all samples analysed. In a similar study conducted in the USA from the period 2005–2017, the prevalence of *L. monocytogenes* in 150,000 RTE meat samples was 0.4% (Mamber et al., 2020), which is similar to the prevalence in RTE meat samples in our study (0.6%). The incidence of *L. monocytogenes* in RTE pork in our study (69%) is significantly higher compared to similar studies (Gamboa-Marin et al., 2012; Wang et al., 2018), where percentages ranged from 13.5–33.9%. The presence of *L. monocytogenes* in RTE milk products was 12%, which is consistent with findings from other studies (Chen et al., 2020). The occurrence in RTE fish was 0.5%, which is lower than reported in recent studies (Korsak et al., 2012; European Food Safety Authority, and European Centre for Disease Prevention and Control, 2017).

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

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

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

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

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

In our study ST101 strains were detected in products from two different industries (Milk IND 1 and Meat IND 2), located within the same industrial yard and being part of the same production brand. While the production procedures of these two industries are separated, our findings suggest that cross-contamination of products with this ST101 strain consistently present in the environment is likely occurring. Recognizing the environment as a key source of *L. monocytogenes*, Linke et al. (2014), underscored the significance of the environment as a primary source of *L. monocytogenes*, as shown in their two-year study of soil and water samples, which revealed ST101 as one of the dominant strains (Linke et al., 2014). In the Listeria SEQuencing (LiSEQ) project ST101 isolates were linked with milk/milk products (Painset et al., 2019). The detection of two identical ST7/CT16794 isolates in 2017, one from fresh cow's milk from Montenegro and the other from sour cream from Bosnia might be explained by the import of milk contaminated with listeria from Bosnia and Serbia (personal communication). Along with the identification of ST7 strains in clinical and food samples, it has been linked to listeriosis cases in cattle and sheep (Annette et al., 2020; Zhao et al., 2021). Among the 20 different STs isolated from meat products ST7 has been reported, along with others like ST1, ST2, ST3 and ST155 to persist in industrial plants for extended periods. The consistent isolation of isolates with no allelic differences from spatially distant industries suggests a possible contamination originating from the raw material. Notably, the only isolate (ST37) detected in frozen vegetables during the entire testing period was detected in a product imported from France in 2020. This same ST was also found in isolates coming from pork from various meat industries and in cow milk cheese. In addition to a frequent isolation from clinical samples, ST37 has been isolated from meat, fish as well as dairy products (Alvarez-Molina et al., 2021; Kubicová et al., 2021; Maćkiw et al., 2021). ST37 has also been isolated from mallard, pheasant and teal feces, moose carcass and wild boar (Fredriksson-Ahoma et al., 2022). In Austria in 2017, ST37 was the fifth most frequent ST in the category food including meat, dairy products, and vegetables (Cabal et al., 2019).

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

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

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

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

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

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

4.3 Antimicrobial resistance genes, plasmids, and mobile genetic elements

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

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

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

5 Conclusion

Monitoring the presence of *L. monocytogenes* over a longer period is a reliable indicator of this pathogen's opportunities in the food chain. Our results reveal a high genetic diversity and variability of *L. monocytogenes* in the Montenegrin food chain, underscoring the importance of ongoing surveillance. This data is crucial for improving and ameliorating the public health and food safety sector. Whole-genome sequencing (WGS) allows researchers the tracking of the trajectory and persistence of *L. monocytogenes* among other pathogens, and the identification of genetic markers of resistance and virulence. The combination of WGS and seeking the epidemiological link provides a comprehensive view of the pathogen's spread and impact. Furthermore, the Austrian reference database for *L. monocytogenes*, alongside other extensive and continuously updated pathogen databases, has allowed us to verify that many of our isolates share a close genetic relationship with isolates of the same sequence type (ST) widely distributed across different European countries. These findings reinforce the relevance of our study in a broader European context, not only at the level of Montenegro, establishing relevant connections in the field of food safety and public health.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

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

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Conflict of interest

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

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Supplementary material

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

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Deciphering the impact of exogenous fatty acids on *Listeria monocytogenes* at low temperature by transcriptome analysis

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Listeria monocytogenes is a ubiquitous and psychrotrophic foodborne pathogen commonly found in raw materials, ready-to-eat products, and food environments. We previously demonstrated that *L. monocytogenes* can grow faster at low temperature when unsaturated fatty acids (UFA) are present in its environment. This could question the maintenance of food safety for refrigerated foods, especially those reformulated with a higher ratio of UFA versus saturated fatty acids (SFA) to fit with nutritional recommendations. In this study, we used transcriptomics to understand the impact of UFA on the behavior of *L. monocytogenes* at low temperature. We first demonstrated that *fabK*, a key gene in SFA synthesis, is up-regulated in the presence of UFA but not SFA at low temperature. *L. monocytogenes* can thus regulate the synthesis of SFA in its membrane according to the type of FA available in its environment. Interestingly, we also observed up-regulation of genes involved in chemotaxis and flagellar assembly (especially *cheY* and *flaA*) in the presence of UFA but not SFA at low temperature. TEM observations confirmed that *L. monocytogenes* acquired a remarkable phenotype with numerous and long-looped flagella only in the presence of UFA at 5°C but not at 37°C. As flagella are well known to be involved in biofilm formation, this new finding raises questions about the structure and persistence of biofilms settled in refrigerated environments using unsaturated lipid-rich products.

KEYWORDS

foodborne pathogen, refrigeration, exogenous fatty acids, fatty acid membrane composition, transcriptomics, RT-qPCR, flagella, *cheY* gene

1 Introduction

Listeriosis, caused by the foodborne pathogen *Listeria monocytogenes*, is the fifth most reported zoonosis and has a high fatality rate (13.7% in Europe, in 2021) (European Food Safety Authority and European Centre for Disease Prevention and Control, 2022). *L. monocytogenes* is ubiquitous and can be found in a wide variety of foodstuffs, widely present in food-associated environments (raw materials, food chain, and retail environments) leading

to a high prevalence of this bacterium in foodstuffs. Ready-to-eat (RTE) products are consumed without cooking or other processing that could remove or reduce pathogens to acceptable levels. For this food category, which can support the growth of *L. monocytogenes*, EC Regulation No. 2073/2005 established that no more than 100 CFU/g should be found in 5 samples at the end of shelf-life. During shelf-life, *L. monocytogenes* growth is mainly controlled by refrigeration temperature.

The cold adaptation response is a common mechanism, well-described in psychrotrophic bacteria such as *Bacillus cereus* and *L. monocytogenes* (Alvarez-Ordóñez et al., 2015). Low temperature directly impacts cell integrity and basal cell functions including membrane fluidity, nutrient uptakes, protein folding and assembly of macromolecules (Zhu et al., 2005; Tasara and Stephan, 2006). The cold-related loss of membrane fluidity is due to a membrane phase transition from liquid-crystalline to gel state (Denich et al., 2003). The first transient adaptive response to low temperature is the regulation of the fatty-acid synthesis type II (FASII) for increasing the proportion of FA with lower melting points into the phospholipids and restoring optimal membrane fluidity (Annous et al., 1997).

The FA composition of *L. monocytogenes* mainly consists of *iso*- or *anteiso*-branched fatty acids (*i*-BFA and *a*-BFA) (84% of the membrane FA at 37°C in TSB) (Touche et al., 2023). BFA are produced from valine (*i*-C14, *i*-C15), leucine (*i*-C15, *i*-C17), and isoleucine (*a*-C15, *a*-C17) by transamination and decarboxylation to form acyl-CoA derivatives that enter then in the elongation module (Zheng et al., 2005). The FA profile of *L. monocytogenes* also contains saturated (SFA) and unsaturated (UFA) fatty acids at smaller proportions (13 and 3%, respectively at 37°C in TSB) (Touche et al., 2023). SFA are produced from acetate through the initiation and elongation modules. Although the presence of UFA in its membrane was previously described, as was the increase of their proportion when temperature decreases, the pathway of UFA synthesis has not been yet identified (Hingston et al., 2017; Touche et al., 2023). Two pathways for UFA synthesis are described in other bacterial species: (1) *de novo* synthesis through the FabM or FabN pathway; or (2) desaturation of an acyl chain through desaturase activity. As far as we know, none of them have been described in *L. monocytogenes*. The FabM or FabN pathway, described respectively in *Streptococcus* spp. and *Enterococcus faecalis*, converts *trans*-2-enoyl-ACP into acyl-ACP with a double bond leading to UFA synthesis (Fujita et al., 2007; Dong and Cronan, 2022). This FabM/N pathway enters in competition with the FabK pathway leading to SFA synthesis. The regulation between these two pathways modulates the SFA/UFA ratio and thus membrane fluidity. Therefore, if this pathway exists in *L. monocytogenes*, the bacteria could sustain optimum membrane fluidity by modulating the ratio UFA/SFA, besides the classical modulations of the membrane fluidity in Gram-positive bacteria (ratios of BFA/SFA, *a*-BFA/*i*-BFA, length of the FA acyl chain (Annous et al., 1997; Tasara and Stephan, 2006)).

Moreover, *L. monocytogenes* was recently shown to incorporate exogenous FA (#FA) into its membrane (Flegler et al., 2022; Touche et al., 2023). Direct incorporation of #FA could save the energy cost of *de novo* FA synthesis. Touche et al. (2023) showed that the incorporation of #FA into the membrane of *L. monocytogenes* is temperature-dependent but non-selective. All #FA are highly incorporated into the membrane at low temperature, but not at 37°C, and the effect on bacterial growth depends on the type of FA: #UFA promote and #SFA inhibit bacterial growth. This phenomenon is of major importance in terms of food safety. Indeed, today's nutritional and health challenges are to reduce the ratio of SFA/UFA in the consumers' diet. Consequently, food operators tend to follow these recommendations and reformulate their products in this way in the last 20 years. However, the increase of UFA in refrigerated foods can favor the growth of *L. monocytogenes* and can compromise food safety before the end of shelf-life (Touche et al., 2023).

The mechanisms of FA incorporation and its effects on general metabolism are still unknown. Gram-positive bacteria, such as *Staphylococcus aureus* and *Streptococcus* spp., can incorporate #FA through a FakAB system working as a two-component system (TCS). FakA, a FA kinase, phosphorylates FakB, the FA-binding protein, which in turn transfers the phosphate to the FA. The resulting acyl-phosphate is then transferred to acyl-carrier protein (ACP) by PlsX, the acyltransferase (Parsons et al., 2014). Different FakB chains with differences in selectivity have been described: FakB1 exclusively binds SFA, FakB2 binds SFA and mono-UFA, and FakB3 binds poly-UFA (Gullett et al., 2019). As far as we know, this mechanism is still not described in *L. monocytogenes*.

In this study, we first analyzed the global transcriptome of *L. monocytogenes* at 5°C and 37°C with or without oleic acid to identify the genes associated with the observed impact of UFA on *L. monocytogenes* grown at low temperature (RNA-seq). Targeted transcriptomic analysis (RT-qPCR) and phenotypic characterizations (FA membrane profiles and TEM observations) were then conducted with different types of #FA and at different growth phase points to further decipher the impact of #FA on the metabolism and behavior of *L. monocytogenes* at low temperature.

2 Materials and methods

2.1 Bacterial strain and culture conditions

Listeria monocytogenes CNL895805, shortly named Lm208, used in this study was isolated from sheep brain in France (serotype 1/2a, CC7, BioSample SAMN39851069) (Tabouret et al., 1992; Van Langendonck et al., 1998; Touche et al., 2023). This strain was chosen among nine *L. monocytogenes* strains of different origins because of its highest overgrowth capabilities in the presence of #UFA at low temperature (Touche et al., 2023). It was stored in Tryptone Soy Broth (TSB, bioMérieux, Marcy l'Etoile, France) supplemented with 20% (v/v) glycerol at −80°C (Invitrogen™, Thermo Fisher Scientific, Waltham, United States). The strain was inoculated at 1% v/v in TSB (≈10^6 CFU/mL) with a standardized inoculum obtained after two successive subcultures at 30°C without shaking. When indicated, the medium was supplemented with different #FA, namely myristic acid (#C14), oleic acid (#C18:1), and linoleic acid (#C18:2) (Larodan Fine Chemicals, Malmö, Sweden). These #FA solutions were prepared as previously described (Touche et al., 2023) and the final FA concentration in the culture medium was 0.045 mM FA in 0.05% bovine serum albumin (w/v) (BSA, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). For RNA sequencing, *L. monocytogenes* was grown with #C18:1 or without and incubated at 5°C or 37°C. For RT-qPCR, membrane FA analysis, and TEM observations, *L. monocytogenes* was grown with #C14, #C18:1, #C18:2 or without at 5°C.

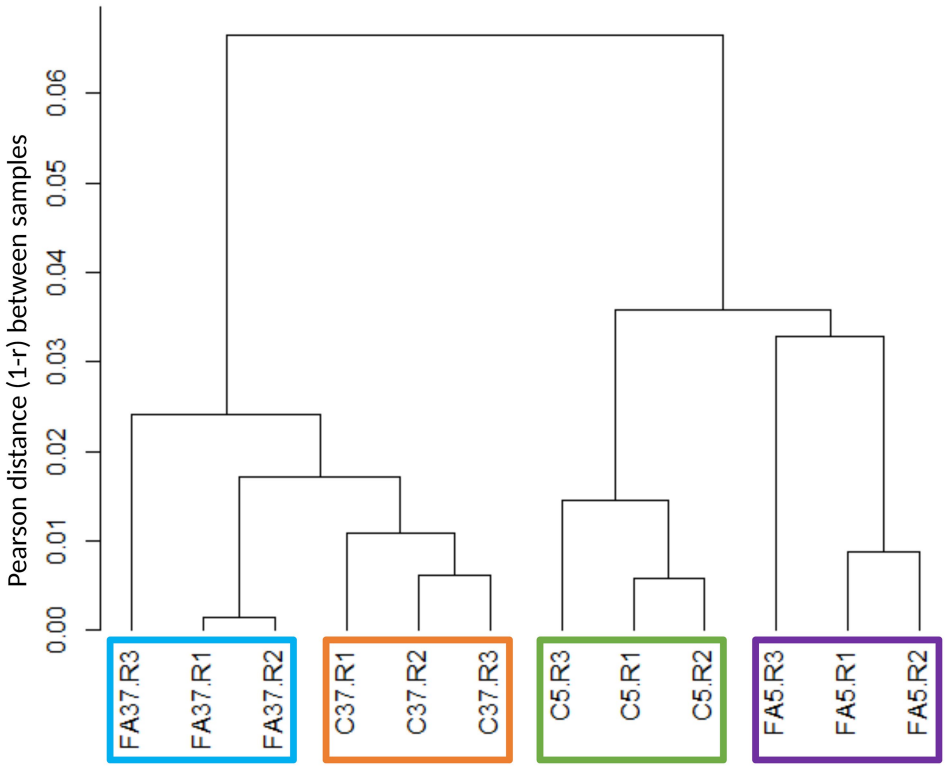

FIGURE 1
Hierarchical clustering tree of RNA sequencing biological samples obtained by calculating the Pearson coefficients per pair (*r*) and represented through the Pearson (1 – *r*). C indicates control cultures, FA indicates cultures grown with #C18:1, 5 and 37 refer to the culture temperature in °C. R1, R2 and R3 are the three replicates of each condition.

2.2 Cell harvest at different time points

Bacterial growth was followed by measuring optical density (OD) at 600 nm (Genesis 30 spectrophotometer, Thermo Fisher Scientific, Villebon-sur-Yvette, France) and cells were harvested at different time points: the mid-exponential phase at $OD_{600} = 0.1 \pm 0.05$ (T1), the end of the exponential phase at $OD_{600} = 0.4 \pm 0.05$ (T2) and the beginning of the stationary phase at $OD_{600} = 0.7 \pm 0.05$ (T3). Cells were harvested at T1 for RNA sequencing and at T1, T2, and T3 for RT-qPCR and TEM observations. The harvested volume was adjusted to collect 10^9 cells (100 mL for T1, 10 mL for T2, and 5 mL for T3). For RNA extraction, cells were harvested by centrifugation at 10,000 rpm for 2 min at the temperature of the culture when the target OD_{600} was reached. The pellet was then immersed in liquid nitrogen and stored at −80°C before extraction. For TEM observations, cells were harvested by centrifugation at 3,000 rpm for 10 min to preserve cell integrity and fixed immediately.

2.3 RNA extraction

Total RNA was extracted by the Trizol™ reagent method (Invitrogen™, Thermo Fisher Scientific) with some adaptations (Toledo-Arana et al., 2009). Pellets were resuspended in 1 mL of Trizol™ and transferred into a screw-cap tube containing 0–50 µm glass beads for cell disruption by bead beating for 40 s at 6 m/s (FastPrep-24 instrument, MP Biomedicals, Illkirch, France). RNA contained in the upper aqueous phase was washed with chloroform, precipitated with cold isopropanol, and finally washed twice with 75% ethanol (Sigma Aldrich). The RNA pellet was dried for 30 s at 63°C and resuspended in 20 µL of nuclease-free water (Invitrogen™, Thermo Fisher Scientific). Total RNA was treated by DNaseI and purified using the Monarch® RNA Cleanup kit (New England BioLabs® Inc., Evry-Courcouronnes, France). Before RNA sequencing and RT-qPCR, Nanodrop (2000 NanoDrop™ spectrophotometer, Thermo Fisher Scientific) and Qubit™ (Qubit™ 3 Fluorometer with the Qubit™ RNA HS assay kit, Invitrogen™, Thermo Fisher Scientific), respectively, were used to quantify RNA. Samples with a concentration above 10 ng/µL were used. RNA quality was assessed with a BioAnalyzer (Agilent 2100 Bioanalyzer System with RNA 6000 Nano kit, Agilent, Santa Clara, United States) and Nanodrop (2000 NanoDrop™ spectrophotometer, Thermo Fisher Scientific). Samples with an RNA Integrity Number (RIN) above 7.5 were used for RNA sequencing. Samples with $A_{260/280}$ and $A_{260/230}$ ratios above 2.0 and 1.8, respectively, were used for the RT-qPCR. Three to six biological replicates were performed for each of the conditions.

2.4 RNA sequencing and data analysis

RNA sequencing was performed by the I2BC platform (Paris-Saclay University, Gif-sur-Yvette, France, https://www.i2bc.paris-saclay.fr/) on an Illumina NextSeq sequencer NS500446. Primary data processing was performed by the I2BC platform with the following data analysis pipeline: demultiplexing (with bcl2fastq2 v.2.18.12), adapter trimming (with Cutadapt v.1.15), quality control (with FastQC v.0.11.5) resulting in 20 M to 34 M reads per sample, post QC and trimming. Reads were mapped to

the Lm208 genome (BioProject PRJNA1074122, SRR29855365) using the BWA 0.6.2-r126. This generated between 8 M and 13 M uniquely mapped reads per sample. Mapping efficiency ranged from 77.86 to 84.34% for individual reads. FeatureCounts (v.1.5.2) was used to assign sequence reads to genomic features. The downstream data was analyzed using the R programming language with the Rstudio interface (v.4.1.2) and R package DESeq2 (v.1.30.1) (Love et al., 2014) based on the raw counts provided by the I2BC platform. First, 83 genes with fewer than 10 counts in the three biological replicates were excluded. Then, raw counts were normalized according to gene length and the total number of mapped reads by calculating FPKM (Fragments per Kilobase per Million Mapped reads) using the library-size estimation method implemented in R package DESeq2 (v.1.30.1) (Love et al., 2014). These FPKM values were used as expression level values for each gene in each condition. To assess the overall reproducibility, normalized counts of samples (log2(fpkm+5)) were compared by computing pairwise Pearson coefficients (r) and distances ($1-r$). The distances were computed by average-linkage clustering using the "hclust" function (v.3.6.2) and summarized by an ascending hierarchical classification.

2.5 Clustering of gene expression profile

To detect differentially expressed genes (DEG) between pairs of conditions, log2 fold-change (log2FC) and p-values (p) were calculated using DESeq2. Four pairwise comparisons were considered: "FA5.vs.C5"; "FA37.vs.C37"; "C5.vs.C37"; "FA5.vs.FA37" where "FA" and "C" refer to the conditions with #C18:1 or without (control), respectively, and "5" and "37" refer to the culture temperature in °C. To control the false discovery rate of each pairwise comparison, the R package "fdrtool" (v.1.2.17) (Strimmer, 2008) with the vector of p-values was used to estimate q-values (q). The DEG reported for the four pairwise comparisons were based on a q-value ≤ 0.05 and $|\log2FC| \geq 1$. The expression profiles of the DEG across all samples were compared by computing pairwise Pearson distances (on log2(fpkm+5)) and subjected to average-link hierarchical classification with "hclust" function (v.3.6.2).

Clusters of DE genes, called DE No. (1 to 35), were defined by cutting the hierarchical clustering tree at a Pearson distance of 0.3 (average Pearson correlation above 0.7). Clusters were labeled according to their size, DE1 being the largest cluster. The resulting dendrogram and heatmap were produced with the "heatmap.2" function included in the R package "gplots" (v.3.1.1), using a specific color palette with red for up-regulated genes ($q \leq 0.05$ and log2FC ≥ 1) and blue for down-regulated genes ($q \leq 0.05$ and log2FC ≤ -1). The whole transcriptomic dataset has been deposited in GEO (accession number GSE268246).

2.6 Functional categorization of differentially expressed genes

Functional categories were assigned to the genes of DE clusters using the Listeriomics database (Bécavin et al., 2017) and the SmartTables tool based on the BioCyc database[1] (Caspi et al., 2016) with *Listeria monocytogenes* EGD-e genome as a reference. For each DE cluster, the percentage of functional categories was calculated and is summarized in Supplementary Table S1.

2.7 RT-qPCR and data analysis

Primers were designed using Primer-Blast[2] (Table 1) with the following settings: a PCR amplicon size between 100 and 120 pb in the first third of the coding sequence, a melting temperature between 57.0 and 63.0°C with an optimum of 60°C and a difference of 3°C. Primer pair efficiency was assessed by performing the qPCR reaction on serial dilutions of gDNA extracted from Lm208 (64, 32; 16; 8 and 1.6 ng/µL). The efficiency was assessed twice with independent gDNA dilutions and calculated as described by Pfaffl (2001). Primer pairs with an efficiency above 1.8 were selected for RT-qPCR. The reference gene was chosen among six genes selected for their stable expression in all conditions of the transcriptomic analysis and tested for stability in RT-qPCR. Stability was assessed twice with or without different #FA (#C14, #C18:1, or #C18:2) at 5°C by calculating a stability M value using the geNorm module integrated in the qbase+ software (v.3.4). The smaller the M value (below 0.5) the greater the gene stability: *lmo208__02104* (*lmo2262* in *L. monocytogenes* EGD-e, hypothetical protein), which had the smallest M value ($M = 0.120$ and 0.157 for the two replicates), was selected as reference gene (Vandesompele et al., 2002; Hellemans et al., 2007). For each sample, 1 µg of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) to obtain cDNA. cDNA was diluted at 1:10 and qPCR was conducted in a StepOne™ Real-Time PCR System (Thermo Fisher Scientific) using SsoAdvanced™ Universal SYBR Green® Supermix (BioRad, Hercules, United States) with 250 nM for each primer. The transcript levels of genes of interest were normalized to the mean of the transcript levels of *lmo208__02104* (reference gene) in all conditions. The relative expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method by calculating Δ_{CT} and $\Delta\Delta_{CT}$ according to the two formulas below (Livak and Schmittgen, 2001):

$$\Delta_{C_T} = C_{T_{\text{gene of interest}}} - C_{T_{\text{reference gene}}} \qquad \Delta\Delta_{C_T} = \Delta_{C_{T\#FA}} - \Delta_{C_{T\text{control}}}$$

The relative expression levels of each gene in the different conditions with #C14, #C18:1, or #C18:2 were compared to the control without #FA at each time point. The control is fixed to a relative expression level of 1.

2.8 Membrane fatty acid analysis

Bacterial pellets were washed twice with 0.1% Triton X-100 to remove unincorporated #FA. FA extraction and methylation were performed as previously described (Dubois-Brissonnet et al., 2016; Touche et al., 2023). Briefly, FA were saponified and methylated with methanolic NaOH and methanolic HCl solutions (1st step: 1 mL 3.75 M NaOH in 50% v/v methanol solution for 30 min at 100°C; 2nd step: addition of 2 mL 3.25 M HCl in 45% v/v methanol solution for 10 min at 80°C). FA methyl esters (FAME) were extracted with a

1 https://biocyc.org/

2 https://www.ncbi.nlm.nih.gov/tools/primer-blast/

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TABLE 1 Primers used for quantitative PCR.

Lm208 locus tag	Gene name	Product	Primers 5′ → 3′		Amplicon size (bp)	Efficiency
lmo208__02104	NA*	Hypothetical protein	F	TGGCTATTTACTTGCGCAACG	100	2.064
			R	TGTAGCCTGGTCTTCCGGAT		
lmo208__00517	*cheY*	Chemotaxis response regulator	F	GCGGAAAATGGACTGGAAGC	105	1.978
			R	CGCAAGTGCTTCTAAGCCATC		
lmo208__00516	*flaA*	Flagellin	F	TGATGACGCTGCTGGTCTTG	119	1.989
			R	GAGCTGAATCCGCTGTTTGT		
lmo208__02013	*fabK2*	Enoyl-ACP-reductase	F	TACAAGGGGCAATGGCACAA	102	2.002
			R	TGTCTGCTGACATTCCACCA		
lmo208__02015	*pct*	Propionate CoA transferase	F	GGGCTCCGCGTAATCTTACA	115	1.898
			R	GCAATTCCACCAATCCAGCG		
lmo208__02018	*fabG*	3-ketoacyl-ACP-reductase	F	GGCGCAAAAGTAGTAGTGGC	105	1.918
			R	TACATTAGCTGCGACGGCAA		

*NA, not applicable.

diethyl ether/cyclohexane solution (1:1 v/v), and the organic phase was finally washed with a dilute NaOH solution (0.3 M NaOH). Reagents were purchased from Sigma Aldrich, Merck KGaA. Analytical gas chromatography of FAME was carried out on a GC-MS Trace 1300/ISQ 7000 (Thermo Fisher Scientific) equipped with a BPX70 capillary column (25 m, 0.22 mm id) (SGE™, Victoria, Australia). The column temperature was set at 100°C for 1 min and then increased to 170°C at the rate of 2°C/min. FAME were expressed as a percentage of the total area and grouped into classes: saturated fatty acids (SFA), unsaturated fatty acids (UFA), *iso-and anteiso*-branched-chain fatty acids (*i*-BFA and *a*-BFA).

2.9 Transmission electron microscopy

Bacterial pellets were fixed for 1 h at room temperature with 2% glutaraldehyde (Delta Microscopies, Mauressac, France) and centrifuged at 3,000 rpm for 10 min at 5°C. Cells were resuspended in 0.1 M cacodylate buffer (Delta Microscopies) and stored at 4°C (maximum 3 weeks). Cells were loaded onto a carbon film membrane on a 300-mesh copper grid which was rinsed twice with 0.1 M cacodylate buffer and stained with 1% uranyl acetate (Delta Microscopies). Finally, the grid was observed with a Hitachi HT7700 80 kV transmission electron microscope (TEM) at the MIMA2 platform (Paris-Saclay University, INRAE, AgroParisTech, GABI, 78350 Jouy-en-Josas, France, https://www6.jouy.inrae.fr/mima2/). Images were acquired with a charge-coupled device (CDD) camera (AMT Imaging, Woburn, United States). Cell and flagella lengths were measured on all the original micrographs using NIH ImageJ software (V1.54d). In each image, the total flagellar length was measured using the free-hand line tool and reported to the number of bacteria in the image to obtain an average flagellar length per bacterium.

2.10 Statistics

Statistical analysis was performed using GraphPad Software (v.10.1.2, Prism, United States). All experiments were performed with independent subcultures and the number of replicates is specified in the legend of each figure. Two-ways ANOVA with Tukey's multiple comparison test (95% confidence interval) was performed on the data of RT-qPCR and fatty acid composition. One-way ANOVA with Tukey's multiple comparison test (95% confidence interval) was performed on the data of bacterial cell and flagella lengths. According to the *p*-value, results were reported as significantly different *if $p < 0.0332$; ** if $p < 0.0021$; *** if $p < 0.0002$; **** if $p < 0.0001$.

3 Results

3.1 Global assessment of the *Listeria monocytogenes* transcriptome

20 M to 34 M of high quality paired reads were generated and assigned to the genome of Lm208. Of 2,879 genes, 97.1% were expressed (83 genes with fewer than 10 counts in the three biological replicates in all conditions were excluded). Ascending hierarchical clustering of the samples was performed to assess the quality and reproducibility of the RNA-seq data (Figure 1; Supplementary Figure S1). The resulting dendrogram reveals that samples are first divided by the impact of temperature, and then by the impact of the presence of #C18:1. According to Pearson distance, replicates with #C18:1 were more heterogenous than control samples at each temperature. The presence of #C18:1 had a greater effect at 5°C than at 37°C.

Of all genes, 1,068 were differentially expressed in at least one of the four pairwise comparisons considering a *q*-value ≤0.05 and |log2FC| ≥1 (Figure 2). To explore the impact of oleic acid at low temperature, we mainly focused on the pairwise comparison FA5.vs.C5: 94 and 88 genes were up-and down-regulated, respectively. Moreover, the impact of the temperature without #FA was investigated with the pairwise comparison C5.vs.C37: 408 and 414 genes were up-and down-regulated, respectively. The comparison FA37.vs.C37 allowed us to point out the impact of #FA at 37°C: 62 and 6 genes were up-and down-regulated, respectively. Finally, the comparison FA5.vs.FA37 compared the temperature effect in the presence of #C18:1: 337 and 341 genes were up-and down-regulated, respectively.

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The DEG were grouped according to the expression profiles of the four pairwise comparisons into 35 DE clusters. In the 12 DE containing more than 10 genes (Figure 2), the three largest clusters, DE1, DE2, and DE3, presented more than half of the 1,068 DEG, with 377, 312, and 77 genes, respectively. These genes were mainly involved in general bacterial metabolism. In contrast, in the other DE, the genes were mainly involved in more specific pathways with one or two major functional categories (Supplementary Table S1). The genes in DE1 were down-regulated at low temperature. They include a cold shock protein (*cspB*), a propionate-CoA-transferase (*lmo2172* called here *pct*) involved in lipid transport and metabolism, and genes involved in virulence (*prfA*, *plcA*, *hly*, *actA*, and *plcB*). In the presence of #C18:1 at low temperature, *cspB* and *lmo2172* were up-regulated, with a log2FC = 1.43 and 2.26, respectively, but genes involved in virulence, as most of the genes from DE1, showed no significant difference in their expression profile. The genes clustered in DE2 and DE3 were up-regulated at low temperature. DE2 included *cspA* and genes encoding for transporters involved in cryoprotection (*opuCABCD*, and *gbuABC* genes). DE3 included genes involved in general function and carbohydrate metabolism (*glpK* and *glpD*). With #C18:1 at low temperature, most of the genes from these three DE showed no significant difference in their expression profiles.

30.4% of DE4 genes are involved in inorganic ion transport and metabolism, such as the genes *tatAC*, *hbp1* and *hbp2*, *isdGEF*, *efeOBU*, and *fhuCBG*. The genes in DE4 were up-regulated at low temperature, mostly with a log2FC >2, but only in the absence of #C18:1 (C5.vs.C37). In the presence of #C18:1 at low temperature (FA5.vs.C5), these genes were also down-regulated, mostly with a log2FC < −2.

56.4% of DE5 genes are involved in cell motility, in particular in flagellar assembly. Genes involved in flagellum metabolism are regulated by the TCS CheAY and the response regulator DegU. They are distributed in two large operons (operon 112 in DE5 and operon 111 not classified) with the *flaA* gene encoding the flagellin protein in between (Figure 3A). The sensory *cheA* and its corresponding response regulatory *cheY* were down-regulated at low temperature (log2FC of −0.67 and −0.70, respectively), whereas they were up-regulated with #C18:1 at 5°C with a log2FC of 1.48 and 1.54, respectively (Figure 3B). Similarly, the genes of the operon 112 and *flaA* (Figure 3A, represented respectively in green and yellow), which are involved in the assembly of the filament, the hook, and the MS-ring of the basal body, were down-regulated at 5°C and up-regulated when #C18:1 was present (mainly *fliF*; *fliG*; *fliH*; *fliI*, and *flaA*). The genes of the operon 111 (Figure 3A, represented in blue) involved in the motor switch and the export apparatus were

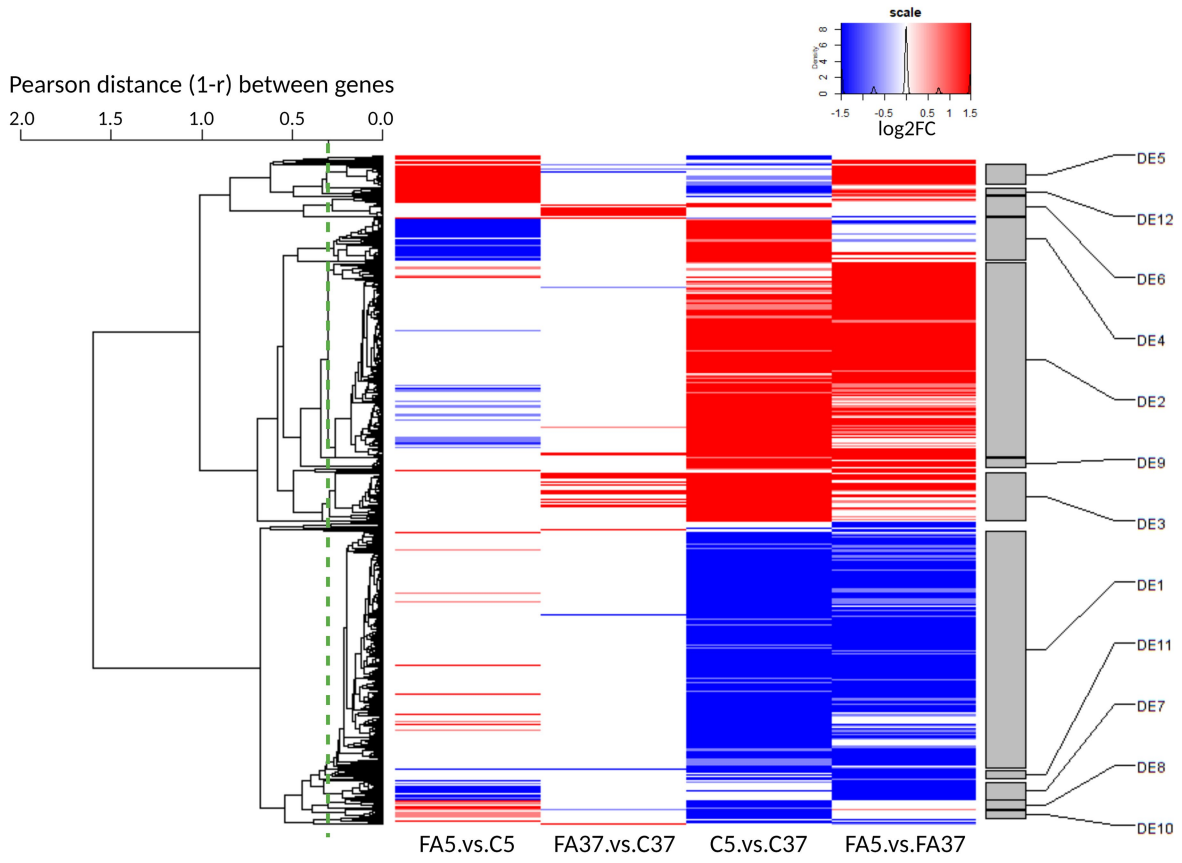

FIGURE 2
Global heatmap representation of the relative variations of expression level across 4 pairwise comparisons (C and FA refer to control cultures and cultures grown with #C18:1, 5 and 37 refer to the culture temperature) for differentially expressed genes. The dendrogram on the left side of the heatmap represents the hierarchical clustering of the 1,068 gene expression profiles across all samples. The dendrogram was cut at an average Pearson correlation of 0.7 (dashed green line) to define the differential expression (DE) clusters on the right side of the heatmap. Clusters were named from DE1 to DE35 by decreasing size. Only clusters with more than 10 genes are represented. Up-regulated genes are reported in red ($q \leq 0.05$ and log2FC ≥1) and down-regulated genes in blue ($q \leq 0.05$ and log2FC ≤ −1).

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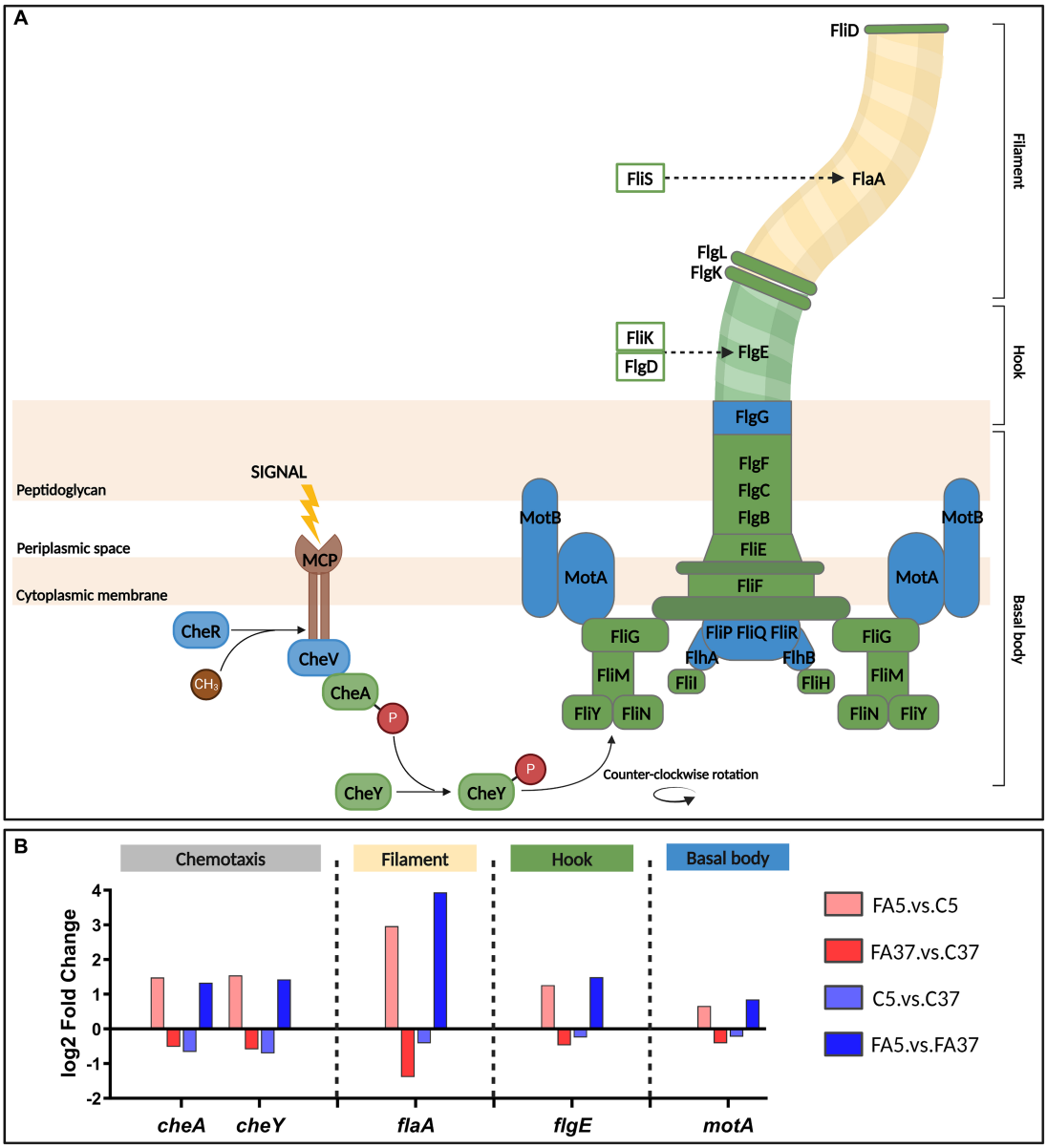

FIGURE 3
Chemotaxis and flagellar assembly in *L. monocytogenes* and the transcriptome of key genes at 5°C or 37°C with #C18:1 or without. **(A)** Chemotaxis signal transduction and organization of flagellar proteins [adapted from Liu and Ochman (2007), Porter et al. (2011), and Cheng et al. (2018)]. Proteins from operon 111, 112 and FlaA are in blue, green and yellow, respectively. **(B)** Differential expression (log2 fold change) of genes of interest. The figure was created with BioRender.com.

up-regulated only when temperature decreased in the presence of #C18:1 (FA5.vs.FA37).

46.7% of DE9 genes are involved in amino acid transport and metabolism. DE9 genes were up-regulated (log2FC >1) at 5°C, but the expression of these genes did not change significantly with #C18:1 at this temperature.

3.2 Deep assessment of the FASII pathway in the global transcriptome

Here, we focused on the genes involved in the well-described lipid metabolism pathways leading to the synthesis of SFA and BFA (Figure 4A). Genes involved in BFA biosynthesis, forming operon 360 (*ilvABCDN* and *leuABCD*), were clustered in DE2. These genes were up-regulated at 5°C (C5.vs.C37) with a log2FC of 2.79 and 1.67 for *ilvB* and *leuA*, respectively (Figure 4B and Supplementary Table S2), but the presence of #C18:1 at the same temperature (FA5.vs.C5) did not induce significant differential expression (Figure 4B and Supplementary Table S2). No significant difference in gene expression was observed for *ilvE* and genes involved in the branched-chain α-ketoacid dehydrogenase complex formation, whatever the culture conditions (Supplementary Table S2). Among genes involved in the FASII initiation module, *pct* (*lmo2172*) was down-regulated at 5°C (log2FC = −3.34) but up-regulated with #C18:1 at the same temperature (log2FC = 2.26) (Figure 4B). In contrast, *accABCD* genes, involved in the

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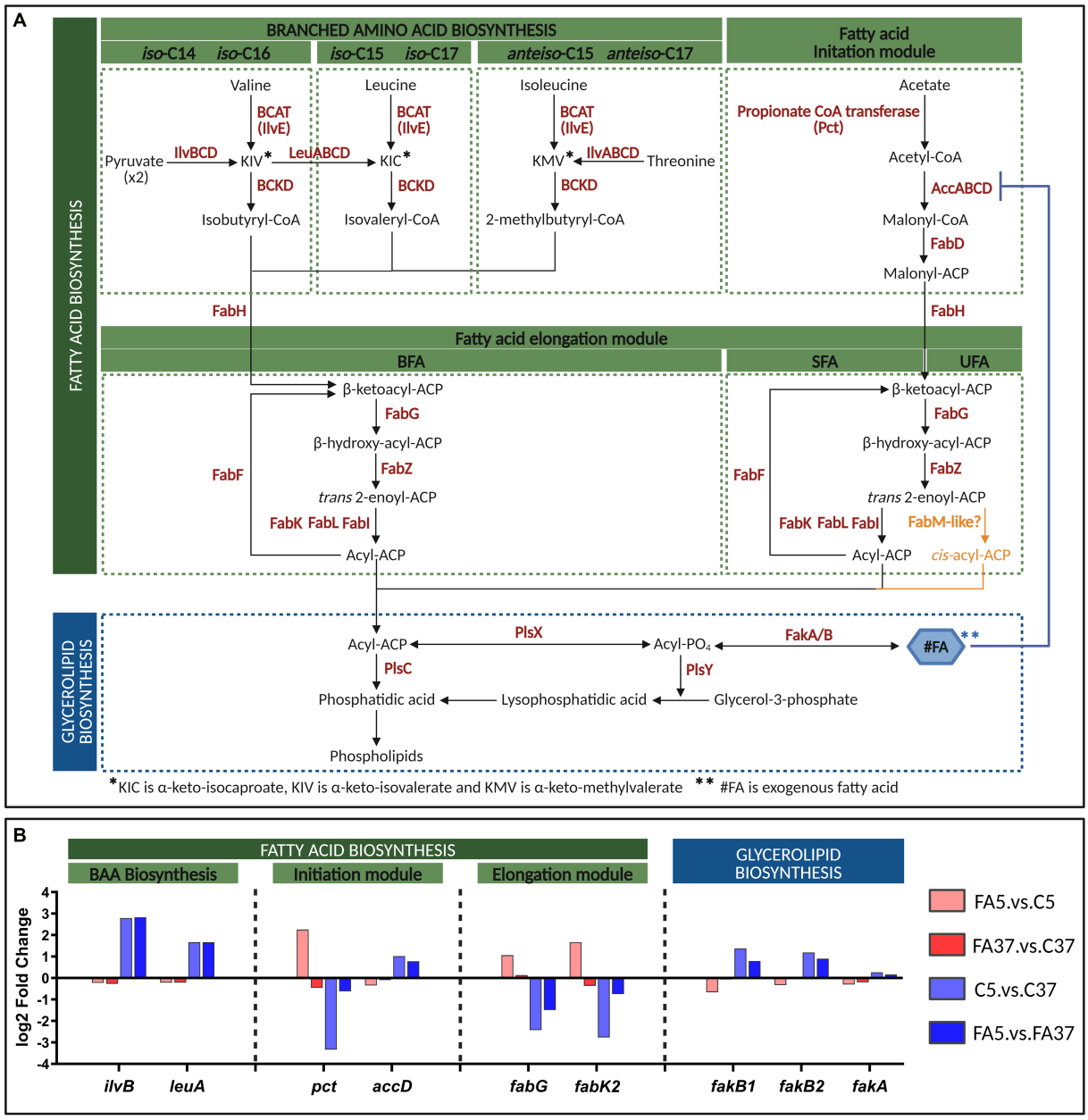

FIGURE 4
Fatty acid biosynthesis of *L. monocytogenes* and transcriptome of key genes at 5°C or 37°C with or without #C18:1. **(A)** Pathways of FASII and glycerolipid biosynthesis in *L. monocytogenes* [adapted from Parsons and Rock (2013) and Yao and Rock (2017)]. A hypothetical pathway for UFA synthesis is depicted in orange. **(B)** Differential expression (log2 fold change) of genes of interest. The figure was created with BioRender.com.

formation of the ACC (acetyl-CoA carboxylase) complex, were up-regulated at low temperature (log2FC = 1.02 for *accD*), but not differentially expressed in the presence of #C18:1 at the same temperature (Figure 4B and Supplementary Table S2). Among genes involved in the elongation module of FA biosynthesis, two genes showed noticeable opposite differential expressions (Figure 4B). At low temperature, *fabK2* (*lmo2170*) and *fabG* (*lmo2175*) were down-regulated (log2FC = −2.77 and −2.43, respectively), while the addition of #C18:1 induced their up-regulation (log2FC = 1.66 and 1.06, respectively) (Figure 4B). Genes involved in #FA uptake, in particular the genes *fakB1* and *fakB2* encoding FA-binding proteins, were up-regulated at low temperature (log2FC = 1.38 and 1.19, respectively), while their differential expressions in the presence of #C18:1 at the same temperature were down-regulated (*fakB1*) or not significant (*fakB2*) (Figure 4B). No significant differences were observed for the gene *fakA* encoding the FA kinase, whatever the conditions (Figure 4B). Genes involved in glycerolipid biosynthesis, *plsX*, *plsY*, and *plsC*, showed no variation in their expression profile, whatever the conditions (Figure 4B).

3.3 Expression profile of five genes of interest by RT-qPCR

To extend the results of the global transcriptome analysis, five genes whose expression was impacted by oleic acid supplementation at low temperature (inverse differential expression between C5.vs.C37 and

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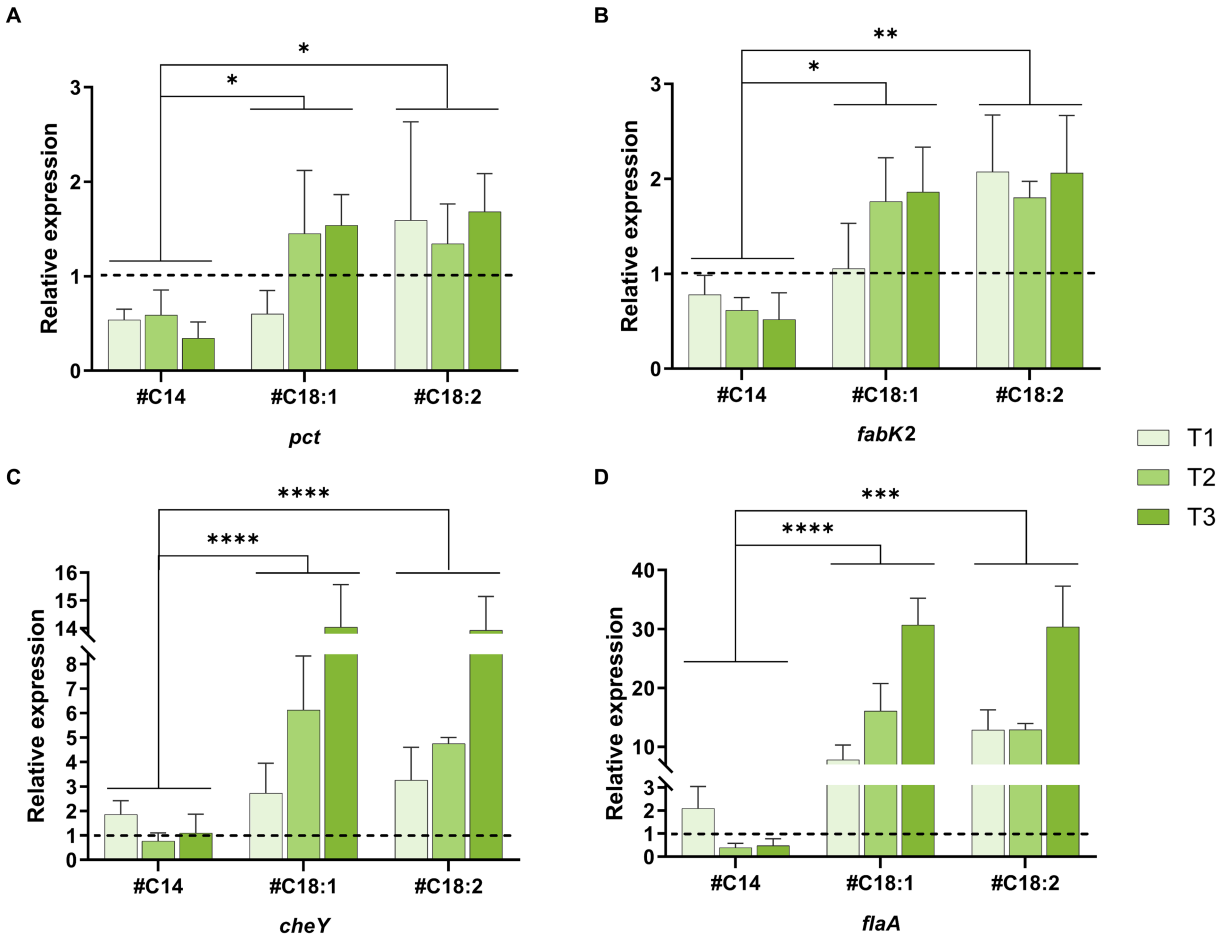

FIGURE 5
Quantitative RT-qPCR analysis of four genes of interest. Relative expression of *pct* **(A)**, *fabK2* **(B)**, *cheY* **(C)** and *flaA* **(D)** of *L. monocytogenes* at 5°C with #C14, #C18:1, #C18:2 at time points T1, T2, T3. The control is fixed to a relative expression level of 1 (dashed black line). Mean and standard deviation are represented (n = 3 to 6).

FA5.vs.C5) were selected for targeted expression analysis using RT-qPCR. These genes are involved in FA biosynthesis (*pct*, *fabG*, and *fabK2*) and chemotaxis and flagellar assembly (*cheY*, *flaA*). Their levels of relative expression were compared at low temperature in the presence of different #FA (#C14, #C18:1, or #C18:2) and at different time points in the growth phase (T1, T2, T3). First of all, the RT-qPCR results are consistent with the global transcriptome analysis (in presence of #C18:1 at T1).

The relative expressions ($2^{-\Delta\Delta CT}$) of *pct* in comparison to the control was higher than 1 with #C18:1 and #C18:2 (except T1 in the presence of #C18:1) while it is lower than 1 with #C14 (Figure 5A). No significant difference was observed between cultures with the two #UFA. Furthermore, the difference in relative expression between cultures with #UFA and #C14 was significant (#C18:1: p-value = 0.0339 and #C18:2: p-value = 0.0234). The relative expressions ($2^{-\Delta\Delta CT}$) of *fabK2* in comparison to the control was higher than 1 with #C18:1 and #C18:2 while it is lower than 1 with #C14 (Figure 5B). No significant difference was observed between the two cultures with #UFA. Furthermore, the difference in relative expression between cultures with #UFA and cultures with #C14 was significant (#C18:1; p-value = 0.0339 and #C18:2; p-value = 0.0043). *fabG* was up-regulated with #C18:1 at T1 compared to the control ($2^{-\Delta\Delta CT}$ = 1.35), but not in all other conditions (data not shown).

The relative expressions ($2^{-\Delta\Delta CT}$) of *cheY* and *flaA* in comparison to the control were higher than 1 when the medium was supplemented with #C18:1 or #C18:2 (Figures 5C,D). For each #UFA, the relative expressions of *cheY* and *flaA* significantly increased over time (from T1 to T3) and reached more than 14 and 30, respectively. No significant difference was observed between cultures with the two #UFA. In the presence of #C14, a significant difference in the relative expression level of both genes was only observed with the control at T1. The difference in relative expression between cultures with #UFA and #C14 was overall highly significant (p-value <0.0001). Unlike *flaA* up-regulated at all time points, the relative expression level of *cheY* depended on the growth phase. Its relative expression at T3 with #C18:1 or #C18:2 was significantly different from the other growth phases (#C18:1: p-value <0.0001 and 0.004, respectively, and #C18:2: p-value <0.0001).

3.4 Membrane fatty acid profile

The FA profiles of Lm208 were compared at low temperature in the presence of different #FA (#C14, #C18:1, or #C18:2) and at different time points in the growth phase (T1, T2, T3). The FA

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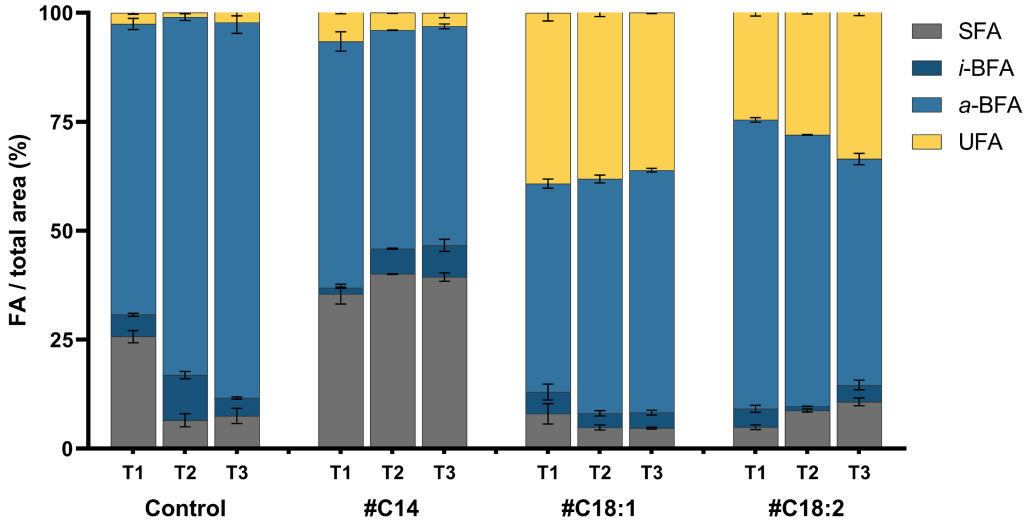

FIGURE 6
Fatty acid composition expressed in four FA categories (SFA, *i*-BFA, *a*-BFA and UFA) of *L. monocytogenes* at 5°C with #C14, #C18:1, #C18:2 or without (Control) at time points T1, T2, T3. Mean and standard deviation are represented ($n = 3$).

composition of Lm208 without #FA was predominantly composed of BFA with 71.7, 92.5, and 90.3% of the total FA at T1, T2, and T3, respectively. The most abundant *a*-BFA were *a*-C15:0 (61.5% average over all the growth phases) and *a*-C13:0 (16.2% average over all the growth phases). The proportion of total SFA was significantly lower at T2 (6.5%) and T3 (7.5%) in comparison to T1 (25.7%). The high proportion at T1 was due to a high proportion of C16:0 (11.6% at T1 versus 3.7 and 4.5% at T2 and T3, respectively). Total UFA represented less than 3% of membrane composition whatever the growth phase (Figure 6). In the presence of #C14, the level of SFA in the membrane increased significantly with a level of incorporation of 9.7, 33.5, and 31.9% at T1, T2, and T3, respectively, compared to the control. The membrane of #C14 cultures contained 31.9% of C14 versus 4.2% in the control at T1. No significant differences were observed between the different time points of the growth phase (Figure 6). In the presence of #C18:1, the proportion of UFA was significantly higher compared to the control (39.2, 36.8, and 36.1% at T1, T2, and T3, respectively). The increased proportion was almost exclusively due to the presence of #C18:1 in the membrane. The incorporation of #C18:1 did not significantly change over time. With #C18:2, the results had the same trend as those obtained with #C18:1, but the incorporation of #C18:2 significantly increased with time (21.6%, 24.4, and 30.1% at T1, T2, and T3, respectively) (p-value <0.0001 between T1 and T3).

3.5 Cell and flagella morphologies by TEM

Cell and flagella morphologies were compared at 5°C with #C14, #C18:1, or #C18:2 or without #FA at the three time points, and at 37°C with #C18:1 or without #FA at T1. Bacterial cell and flagellar lengths per bacterium were measured in each condition from an overall number of more than 190 images. One representative TEM image of each condition is represented in Figure 7A. For all culture conditions, the time point in the growth phase did not indicate quantitative differences in bacterial and flagellar lengths per bacterium (data not shown). Without #FA, bacterial cell length significantly decreased at 5°C when compared to 37°C, while flagellar length per bacterium remained similar. With #C18:1, bacterial length was similar to the control at each temperature (Figure 7B). Conversely, flagella per cell were significantly longer with #C18:1 at 5°C but not at 37°C. With #C18:2, the results were similar to those with #C18:1 at 5°C. Moreover, many flagella form multiple loops in the images of the cultures grown with each #UFA at low temperature. There was also high variability in terms of flagella length when #UFA were present. With #C14 at 5°C, bacterial length was significantly shorter than the control, but the flagellar length was similar (Figure 7B).

4 Discussion

In our previous paper (Touche et al., 2023), we demonstrated that exogenous UFA (#UFA), but not exogenous SFA (#SFA), are growth promoters of *Listeria monocytogenes* at low temperature. We also showed that # UFA and #SFA are both over-incorporated at 5°C in comparison with 37°C. In the present study, we aimed to understand the impact of #FA on the behavior of *L. monocytogenes* at low temperature using untargeted and targeted transcriptomics together with the characterization of membrane FA profiles, and cell and flagellar morphologies.

Our results confirm that *L. monocytogenes*, like numerous other bacteria, can incorporate #FA into their membrane. Moreover, we showed here that, when *L. monocytogenes* was grown in the presence of #C14, #C18:1, or #C18:2, the three FA were highly incorporated since the first time point (mid-exponential phase). Bacterial FA profiles did not differ significantly at the subsequent time points of the growth phase (T2 and T3) in the presence of #C14 or #C18:1. The genes encoding FakB1 and FakB2, the FA-binding proteins for SFA and mono-UFA, are up-regulated at low temperature, but the presence of #C18:1 did not induce the increase of expression we could expect according to the corresponding incorporation. These

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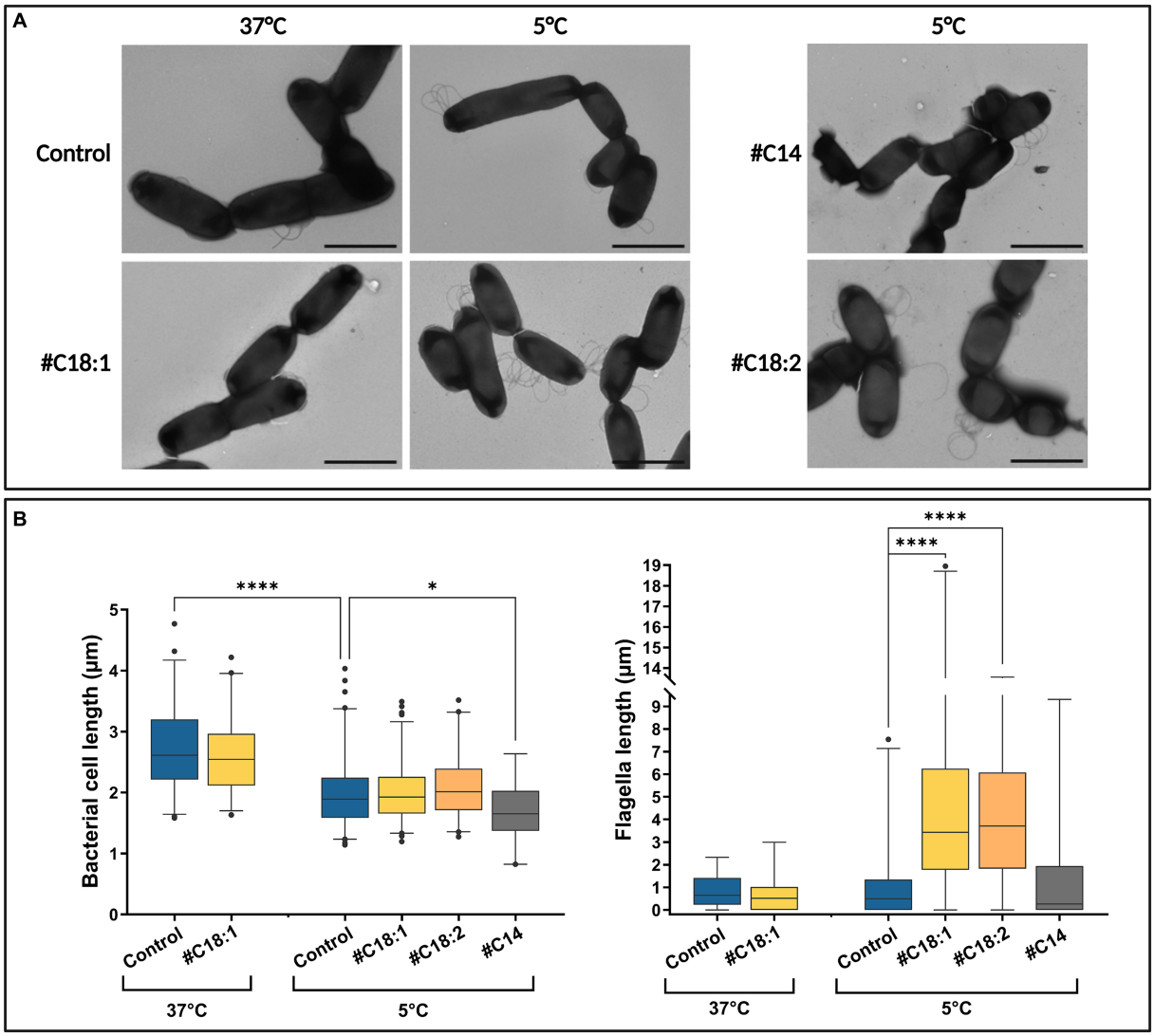

FIGURE 7
(A) TEM images and **(B)** bacterial cell and flagellar lengths of *L. monocytogenes* grown at 5°C or 37°C with #C18:1, #C18:2, #C14 or without (scale bar = 2 µm).

results suggest that the up-regulation of *fakB1* and *fakB2* probably occurs at the early stage of the exponential growth phase, which is consistent with the early FA incorporation and with the changes in growth rate starting from the very beginning of growth (Touche et al., 2023). Furthermore, we can notice that the incorporation of #C18:2 at 5°C significantly increases with time as shown in the bacterial FA profiles. A *fakB3-like* gene encoding for a poly-UFA binding protein probably exists due to the ability of the strain to incorporate #C18:2. Nevertheless, the dynamics of its expression are still unknown because we did not identify it in the genome of Lm208 by BLAST and sequence comparison.

We also analyzed the impact of #FA on the regulation of the FA metabolism. The untargeted transcriptomic analysis showed that genes involved in FASII are greatly affected by both temperature and the presence of #C18:1. Genes involved in BFA synthesis (*ilvABCDN*, *leuABCD*) are up-regulated at low temperature (Figure 4 and Supplementary Table S2). The corresponding over-synthesis of BFA at the expense of SFA when temperature decreases is very well described in the literature (Chihib et al., 2003; Touche et al., 2023). Moreover, some genes involved in FA metabolism are down-regulated at low temperature but up-regulated with #C18:1, especially *pct* (*lmo2172*), encoding propionate-CoA-transferase, which is responsible for the first step of the initiation module of straight chain FA, and *fabK2* involved in FA *de novo* synthesis. The targeted transcriptomic of *pct* and *fabK2* conducted at low temperature with another #UFA (#C18:2) and one SFA (#C14) showed that both #UFA induced their up-regulation but not #SFA. This highlights the specificity of the regulation of these genes according to the nature of exogenous FA. *pct* is involved at the very beginning of the initiation module of FASII, but it is also involved in other pathways of bacterial synthesis, such as pyruvate metabolism. It is thus quite difficult to draw more hypotheses about its involvement in the uptake of #UFA. *fabK* regulates *de novo* synthesis of SFA and BFA in the membrane. Two *fabK* were identified in Lm208. Only *fabK2 (lmo2170)* expression was modulated according to the type of #FA: the presence of #SFA and #UFA in the medium induces the decrease and increase of *fabK2* expression, respectively.

fabK1 (lmo0814) expression is only modulated by the temperature. In *Streptococcus pneumoniae*, it was shown that FabK competes with FabM for the same substrate (*trans*-2-decenoyl-ACP) to modulate the ratio of UFA/SFA in the membrane (Parsons and Rock, 2013). *fabK* expression has to be weakened to allow UFA synthesis to proceed (Dong and Cronan, 2022). Here, even if no *fabM* and *fabN* coding sequences or corresponding homologous protein sequences were not found in the *L. monocytogenes* genome (see Supplementary Table S2), we hypothesize that Lm208 possesses a *fabM/N-like* gene for UFA *de novo* synthesis because its FA membrane profile contains UFA when grown without #UFA (Touche et al., 2023; this study). We also hypothesize that *fabM/N* could compete with *fabK2* for modulating the ratio SFA/UFA in the membrane, according to the type of #FA available in its environment. Similarly, Zheng et al. (2005) showed that some #FA, but not all, inhibited FabI in *S. aureus* at 37°C. FabI, similarly to FabK, is an enoyl-ACP reductase that catalyzes the final step of the chain elongation process of the FASII. The modulation between the two pathways for SFA and UFA syntheses does not exist in *S. aureus* which cannot synthesize UFA. #UFA, but not #SFA, inhibited FabI, increased the fluidity of the *S. aureus* membrane, and showed antibacterial activity at 37°C (Zheng et al., 2005). The temperature probably plays here a very important role in the regulation of FASII pathways in the presence of #FA because of its direct impact on membrane fluidity.

In addition, in several bacterial strains, UFA are produced by a post-synthesis mechanism using a desaturase system. In *B. cereus*, two desaturases have been identified, DesA and DesB, which catalyze the formation of double bonds in the acyl chain of FA (Diomandé et al., 2015). To achieve double bond formation, desaturases recruit and activate molecular oxygen with the use of an active-site di-iron cluster (Diaz et al., 2002). No desaturase was found in the genome of Lm208 (Supplementary Table S2), but our results show that genes involved in iron uptake, such as *fhuCBG* and *tatAC*, are up-regulated when temperature decreases and down-regulated with #C18:1 at low temperature. This is compatible with the hypothesis, already suggested by Hingston et al. (2017) and Touche et al. (2023), that *L. monocytogenes* could possess an iron-dependent desaturase-like system which is activated by low temperature, but not when UFA are incorporated in the membrane.

Interestingly, we shed light on an unexpected connection between the presence of #UFA at low temperature and the expression of chemotaxis and flagellar assembly genes. At low temperature without #FA, most chemotaxis and flagellar assembly genes are not differentially expressed (*flaA*, operon 111 and 114) or down-regulated (operon 112), as already observed by Hingston et al. (2017). The impact of the temperature on *L. monocytogenes* is well known, this bacterium being motile below 25°C but not at 37°C (Liu et al., 2002; ANSES, 2020). The presence of #C18:1 at low temperature leads to the up-regulation of all genes in operon 112, *motA*, *gamR*, *cheV* in operon 111, and *flaA*, in association with the appearance of a remarkable bacterial phenotype trait, being numerous and long-looped flagella. More specifically, the targeted transcriptomic analysis revealed that *cheY* and *flaA* were also up-regulated in the presence of another #UFA (#C18:2), but not in the presence of #SFA (#C14). TEM observations confirm the increase in flagellar length per bacterium in the presence of #UFA, but not with #SFA. In addition, this phenotype appeared with #C18:1 only at 5°C but not at 37°C. We thus hypothesize a role of #UFA in the regulation of genes involved in motility and flagella biosynthesis at low temperature. In *L. monocytogenes*, Δ*cheAY* and Δ*degU* mutants are respectively non-motile and non-flagellated (Dons et al., 2004; Williams et al., 2005). As in most firmicutes, DegU is the temperature-response regulator of motility and chemotaxis in *L. monocytogenes*. When active, at lower temperature than 25°C, DegU directly represses its own expression by binding its promoter and activates chemotaxis and flagellar motility gene expression by binding the upstream region of operon 111 and the *flaA* gene (Knudsen et al., 2004; Williams et al., 2005; Gueriri et al., 2008). Gueriri et al. (2008) have shown that *degU* also controls the expression of two uncharacterized operons (operons 114 and 295) each of them encoding one methyl-accepting chemotaxis protein (MCP). In our results, these two operons, like operon 112 and *flaA*, are up-regulated in the presence of #C18:1 at low temperature. MCP are transmembrane proteins able to sense chemical and physical signals and are the starting point of the phosphorylation cascade activating the response regulator of a two-component system (TCS). The MCP of operon 114 (*lmo0723*) has been reported as part of CheAY TCS in which it transduces the external signal from extracellular to cytoplasmic histidine kinase (Lacal et al., 2010; Casey et al., 2014). Nevertheless, the cognate DegS kinase of DegU is noticeably absent in the genome of *L. monocytogenes* (Gueriri et al., 2008). The signal sensing system and the nature of the signal remain unknown. We hypothesize here that MCP in operon 295 (*lmo1699*) could be involved in DegU phosphorylation and that the presence of #UFA could be a chemical signal that activates the system. The chemotaxis signal detected by MCP could be due either to the physical state of the membrane or the specific chemical signal of #UFA.

5 Conclusions and perspectives

In the present study, we first demonstrated that oleic acid supplementation at low temperature significantly impacts the gene expression of *L. monocytogenes*. Analysis of the expression of genes involved in FA biosynthesis suggests the presence of two potential UFA biosynthesis pathways in *L. monocytogenes*. To identify the genes that could support our hypotheses, such as a *fabM*-like, *des*-like or *fakB3*-like, a bank of deletion mutants will be constructed and submitted to different FA types at low temperature. In addition, we demonstrate that *L. monocytogenes* grown in the presence of UFA but not SFA at low temperature overproduces flagella. These results raise new questions to be addressed, such as the ability of these bacteria to adhere to inert surfaces and produce biofilms in food processing environments. Moreover, as we noted heterogeneity in terms of flagellar length when #UFA are present in the environment, it could be interesting to monitor the expression level of genes of interest at the single-cell level with fluorescent reporters to evaluate the heterogeneity of gene expression in the whole population. Addressing these new questions will help improve understanding of the behavior of *L. monocytogenes* at low temperature and thus help find ways to control this pathogen in foods and food environments.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

AQ: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. MD: Data curation, Investigation, Methodology, Supervision, Writing – review & editing. AP: Formal analysis, Investigation, Methodology, Writing – review & editing. AA: Data curation, Investigation, Writing – review & editing. PN: Data curation, Software, Writing – review & editing. FD-B: Conceptualization, Data curation, Funding acquisition, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1441784/full#supplementary-material

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Maple compounds prevent biofilm formation in *Listeria monocytogenes* via sortase inhibition

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The Pss exopolysaccharide (EPS) enhances the ability of the foodborne pathogen *Listeria monocytogenes* to colonize and persist on surfaces of fresh fruits and vegetables. Eradicating listeria within EPS-rich biofilms is challenging due to their increased tolerance to disinfectants, desiccation, and other stressors. Recently, we discovered that extracts of maple wood, including maple sap, are a potent source of antibiofilm agents. Maple lignans, such as nortrachelogenin-8′-O-β-D-glucopyranoside and lariciresinol, were found to inhibit the formation of, and promote the dispersion of pre-formed *L. monocytogenes* EPS biofilms. However, the mechanism remained unknown. Here, we report that these lignans do not affect Pss EPS synthesis or degradation. Instead, they promote EPS detachment, likely by interfering with an unidentified lectin that keeps EPS attached to the cell surfaces. Furthermore, the maple lignans inhibit the activity of *L. monocytogenes* sortase A (SrtA) *in vitro*. SrtA is a transpeptidase that covalently anchors surface proteins, including the Pss-specific lectin, to the cell wall peptidoglycan. Consistent with this, deletion of the *srtA* gene results in Pss EPS detachment from listerial cells. We also identified several additional maple compounds, including epicatechin gallate, isoscopoletin, scopoletin, and abscisic acid, which inhibit *L. monocytogenes* SrtA activity *in vitro* and prevent biofilm formation. Molecular modelling indicates that, despite their structural diversity, these compounds preferentially bind to the SrtA active site. Since maple products are abundant and safe for consumption, our finding that they prevent biofilm formation in *L. monocytogenes* offers a viable source for protecting fresh produce from this foodborne pathogen.

KEYWORDS

biofilm, *Listeria (L.) monocytogenes*, antibiofilm, sortase A inhibitor, maple, exopolysaccharide, surface attachment, fresh produce and foodborne illness

Introduction

Fresh produce contaminated with the bacterial foodborne pathogen *Listeria monocytogenes* has been linked to several listeriosis outbreaks in recent decades, rivaling traditional sources of contamination such as deli meat, poultry, fish, and dairy products (Zhu et al., 2017). According to the Centers for Disease Control and Prevention (CDC), recent outbreaks in the USA have originated from contaminated whole cantaloupes (rock melons), frozen vegetables, cut celery, packaged salads, bean sprouts, caramelized apples, and mushrooms [Centers for

Disease Control and Prevention (CDC), 2022]. In otherwise healthy individuals, listerial infections typically remain confined to the gastrointestinal tract and are rarely life-threatening. However, in individuals with compromised immune systems, the elderly, pregnant women, fetuses, and young children, these infections can progress to systemic listeriosis. The mortality rates for those who do develop listeriosis are alarmingly high, reaching 15–20% in Western countries. As a result, strict regulations are in place regarding acceptable levels of *L. monocytogenes* in ready-to-eat food products. In the USA, there is a "zero tolerance" policy (Archer, 2018), while the European Union allows less than 100 colony-forming units (CFUs) per 100 grams of food (European Commission, 2005). Frequent product recalls due to confirmed or suspected contamination make *L. monocytogenes* a costly foodborne pathogen (Hoffmann and Ahn, 2021).

Completely avoiding contamination of fresh produce is challenging, if not impossible, because *L. monocytogenes* is ubiquitous in the environment (Marik et al., 2020). Additionally, the relative importance of various contamination sources is not fully understood. For instance, a recent metagenomic analysis revealed that significant percentages of both farm animals and, surprisingly, humans may carry *L. monocytogenes* asymptomatically (Hafner et al., 2021). However, in most cases, fresh produce contamination occurs postharvest, particularly at storage and processing facilities (Ferreira et al., 2014; Garner and Kathariou, 2016; Rodríguez-López et al., 2018; Marik et al., 2020). The cleaning and disinfection protocols at these facilities often fail to eliminate *L. monocytogenes*, especially if biofilms are present on the fresh produce or in hard-to-reach areas of processing equipment. To effectively eradicate *L. monocytogenes* biofilms, more than just cleaning and disinfection may be required; specific antibiofilm agents may need to be applied (Fagerlund et al., 2020).

The Pss exopolysaccharide (EPS) is a recently identified component of listerial biofilms that plays a crucial role in their resilience. *L. monocytogenes* produces Pss when intracellular levels of c-di-GMP are elevated (Chen et al., 2014; Köseoğlu et al., 2015). C-di-GMP, a second messenger, activates EPS synthesis and promotes biofilm formation in various bacteria (Römling et al., 2013; Poulin and Kuperman, 2021). The Pss EPS enhances the colonization of plant surfaces, including fruits and vegetables, but does not significantly improve colonization of manmade materials. Strains that overproduce Pss colonize rough plant surfaces, such as cantaloupe rind, more than 10-fold more efficiently than strains with impaired Pss synthesis. On smooth surfaces, such as cantaloupe flesh, the difference is less pronounced, approximately 2-fold (Fulano et al., 2023). Importantly, bacteria within Pss-containing biofilms exhibit greater tolerance to desiccation, disinfectants, and hydrochloric acid (Chen et al., 2014; Fulano et al., 2023). These stresses are particularly relevant to the storage, transportation, and consumption of fresh produce (Tennant et al., 2008; Esbelin et al., 2018). Consequently, Pss EPS-synthesizing strains have a significant advantage, approximately 10^2 to 10^4 times greater, in reaching the small intestines of consumers, which are the primary infection sites for this pathogen (Fulano et al., 2023). This highlights Pss EPS as a risk factor for fresh produce safety.

The Pss EPS has a distinct composition and structure. It consists of a chain of N-acetyl mannosamine (ManNAc) disaccharide units linked by (1–4)-β-glycosidic bonds, with every other ManNAc residue decorated with α-galactose {4)-β-ManpNAc-(1–4)-[α-Galp-(1–6)]-β-ManpNAc-(1-} (Köseoğlu et al., 2015). The significance of Pss EPS for the survival of *Listeria* in the environment is underscored by the fact that the *pss* operon, which encodes the Pss biosynthetic machinery (Chen et al., 2014), is part of the core genome of *L. monocytogenes*, as revealed by the genomes of over a thousand sequenced isolates (Moura et al., 2016). The high conservation of the *pss* operon among nonpathogenic *Listeria* species (Chen et al., 2014) further emphasizes its importance for environmental survival.

Recently, we discovered that aqueous extracts from maple wood, including maple sap and syrup, have strong anti-EPS properties. For instance, dilutions of commercially available maple syrup at 1:200 or higher effectively prevented biofilm formation by Pss-overproducing strains on various fruits and vegetables, and caused the dispersion of existing EPS biofilms. We found that two lignans in maple wood products — nortrachelogenin-8'-O-β-D-glucopyranoside (NTG) (Wan et al., 2012) and lariciresinol (LR) (St-Pierre et al., 2014) — are responsible for this anti-EPS activity (Elbakush et al., 2023). However, the exact mechanism by which these compounds work has remained unknown. Elucidating such a mechanism has been the major goal of this study. Additionally, we identified several non-lignan antibiofilm compounds in maple. Given the abundance, affordability, and lack of toxicity of maple products, along with the newly discovered antibiofilm mechanism, there is great potential for their use in protecting fresh produce from listerial contamination.

Results

Assessing the spectrum of antibiofilm activity of maple compounds

Before investigating the mechanism of action of maple lignans, we assessed whether their antibiofilm activity extends to other fresh produce pathogens beyond *L. monocytogenes*. Specifically, we tested the impact of maple compounds on biofilm formation by another common foodborne pathogen, *Salmonella enterica* subsp. Typhimurium. Unlike *L. monocytogenes*, a monoderm belonging to the Bacillota (Firmicutes) phylum, *S. typhimurium* is a diderm gammaproteobacterium from the Pseudomonadota (Proteobacteria) phylum.

We incubated *S. typhimurium* strains in minimal liquid media (HTM/G) with sterile pieces of cantaloupe rind or cut celery under the same conditions used in recent experiments with *L. monocytogenes* (Elbakush et al., 2023). The high c-di-GMP strain MAE97 overproduces solely the primary *S. typhimurium* EPS, phosphoethanolamine-modified cellulose (PEAC) (Zogaj et al., 2001; Yaron and Römling, 2014; Thongsomboon et al., 2018). Similar to the Pss-overproducing *L. monocytogenes* strain, the PEAC-overproducing strain formed clumps in the HTM/G medium (Figure 1A). However, neither diluted maple syrup nor the lignan NTG prevented PEAC-mediated cell aggregation in this strain (Figure 1A). Consistent with the lack of antibiofilm activity against *S. typhimurium*, there was no significant reduction in the CFUs of *S. typhimurium* attached to cantaloupe or cut celery surfaces, whether in the PEAC overexpressing strain, MAE97, or the wild type strain, UMR1 (Figure 1B). Furthermore, maple wood extracts did not reduce biofilm formation of *S. typhimurium* on polystyrene microtiter plates in rich LB medium lacking NaCl (Supplementary Figure S1). These findings suggest that the antibiofilm activity of maple compounds is specific to *L. monocytogenes* or potentially to other members of the Bacillota phylum.

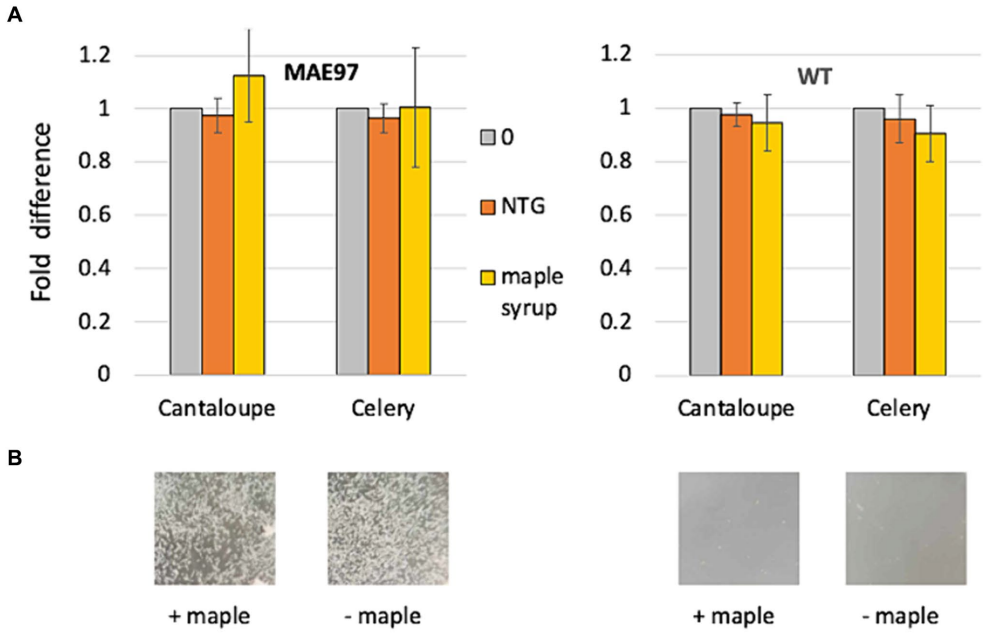

FIGURE 1
Maple compounds do not inhibit colonization of fresh produce by *S. typhimurium*. Standard cantaloupe rind-containing round coupons and pieces of cut celery were incubated in liquid HTM/G medium for 48 h at 30°C in the presence *S. typhimurium* strains, MAE97 (high c-di-GMP strain) and UMR1 (wild type). The following compounds were added at the time of inoculation -- NTG (120 μM, final concentration), maple syrup (1:200 dilution), or DMSO (0, negative control). The numbers of attached bacteria were counted following rinsing and maceration in PBS of the cantaloupe and celery biomass, and plating of diluted suspension on LB. **(A)** Fold-difference in CFUs of *S. typhimurium* attached to the cantaloupe rind-containing coupons and celery pieces. CFUs in the presence of DMSO were set at 1. Results of two experiments, each involving three produce pieces. **(B)** Representative images of the appearance of bacterial cultures incubated with DMSO ("– maple") and maple syrup ("+ maple") at the end of 48-h incubation. Note that clumps formed by the high c-di-GMP strain are not affected by the presence of maple syrup.

Search for the target of the antibiofilm activity of maple lignans

In investigating the potential mechanism behind the antibiofilm activity of maple lignans in *L. monocytogenes*, we initially considered two possibilities: (i) inhibition of Pss synthesis or (ii) activation of Pss hydrolysis. To test the effect on Pss synthesis, we used a Congo red assay. Congo red is a dye that stains various types of EPS, including Pss (Chen et al., 2014). We observed that colonies of the high c-di-GMP strain, *ΔpdeB/C/D*, grown with diluted maple syrup, LR (Figure 2A), or NTG (not shown) retained their red color, similar to colonies grown without these compounds. This indicates that Pss synthesis is largely unaffected by the maple compounds.

We then explored whether maple compounds might activate Pss hydrolysis, leading to smaller Pss fragments that do not support cell adhesion. To this end, we measured the impact of diluted maple syrup on the abundance and activity of the Pss hydrolase, PssZ, known to degrade Pss EPS (Köseoğlu et al., 2015). Due to the lack of PssZ-specific antibodies, we assessed *pssZ* transcript levels using quantitative RT-PCR. The maple lignans did not significantly increase *pssZ* mRNA (Figure 2B), suggesting that they are unlikely to drastically elevate PssZ abundance.

To determine if maple syrup enhances PssZ activity, we conducted a clump dispersion assay. This assay monitors the release of bacteria from preformed clumps by measuring the increase in absorbance of bacterial suspensions over time after the undispersed clumps settle (Köseoğlu et al., 2015; Elbakush et al., 2023). If maple compounds activated PssZ, we would expect faster dispersion in the presence of maple syrup. However, we found that the addition of diluted maple syrup resulted in only modest, immediate clump dispersion (Figure 2C), with no significant change over the 30-min experimental period. This suggests that maple compounds do not enhance PssZ activity. Note that we could not use a *pssZ* null mutant as a control because this mutant is impaired in Pss production (Köseoğlu et al., 2015).

To further investigate the hypothesis that maple compounds activate PssZ-dependent Pss hydrolysis, we measured clump dispersion with the addition of purified PssZ protein (Köseoğlu et al., 2015). As expected, the addition of exogenous PssZ promoted clump dispersion. However, when PssZ was combined with maple syrup, clump dispersion was not accelerated (Figure 2C, PssZ versus PssZ-1x). This suggests that maple compounds do not activate PssZ. Increasing the concentration of maple syrup threefold also failed to enhance dispersion (Figure 2C, PssZ-1x versus PssZ-3x), reinforcing our conclusion. Similar results were obtained with NTG instead of maple syrup, leading us to reject the hypothesis that maple compounds activate PssZ-mediated Pss hydrolysis.

Given that maple compounds did not affect Pss synthesis or degradation, we explored the possibility that they influence the attachment of Pss EPS to bacterial cells. If attachment were impaired, we would expect Pss to accumulate in the supernatant of liquid cultures, while remaining within bacterial colonies on solid media, which would explain the red colony appearance in the presence of Congo red dye (Figure 2A). To test this, we grew the high c-di-GMP

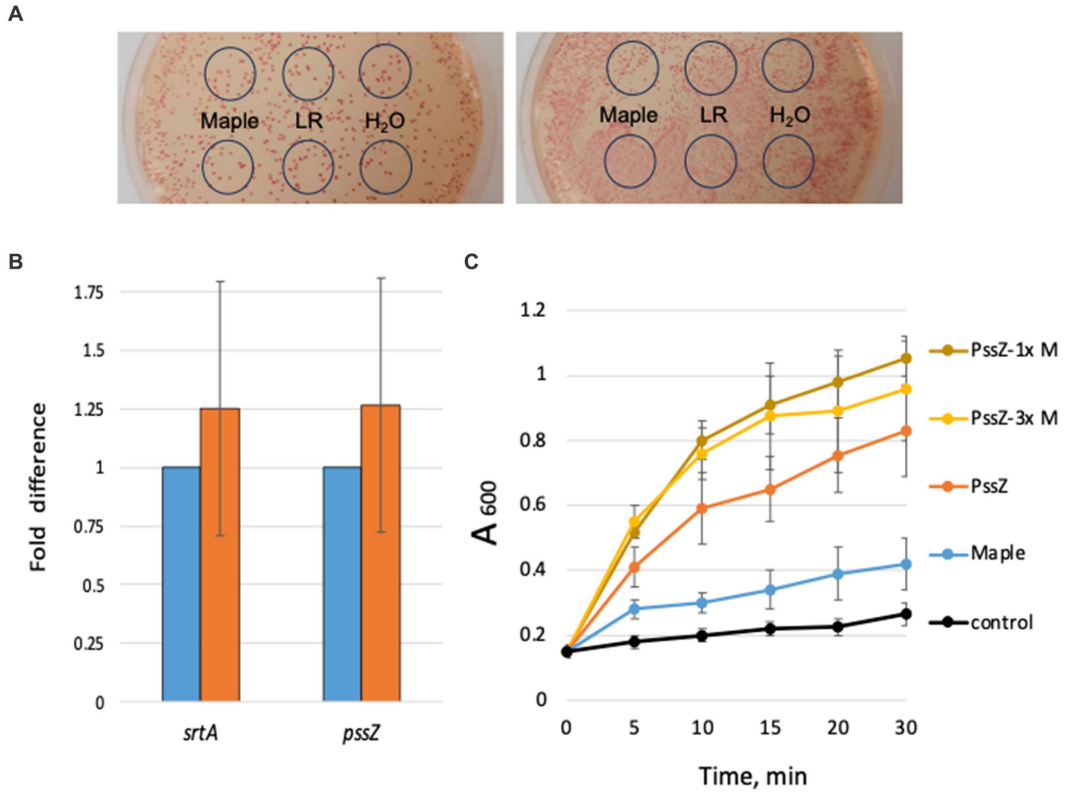

FIGURE 2
Maple compounds affect neither Pss synthesis nor hydrolysis. **(A)** Maple compounds do not appear to affect Pss EPS synthesis. The high c-di-GMP strain, Δ*pdeB/C/D*, was plated on the HTM/G agar medium containing Congo red dye. Two representative plates have a 10-fold difference in colony numbers. Five μL of diluted (1:200) maple syrup ("Maple"), 80 μM LR solution, or H_2O were spotted inside the marked circles after plating. The Congo red staining of colonies does not differ in the presence of maple compounds. **(B)** Maple compounds do not significantly affect abundance of the *pssZ* or *srtA* mRNA. Results of qRT-PCR based on RNA purified from the Δ*pdeB/C/D* strain (light-blue bars; value set at 1.0) and Δ*pdeB/C/D* grown in the presence of 1:200 maple syrup (orange bars). **(C)** Maple compounds do not accelerate PssZ-mediated Pss hydrolysis. Clump dispersion assay was performed as described earlier (Elbakush et al., 2023). Control, HTM/G medium; PssZ, 0.13 μg mL^{-1} PssZ::His6; Maple, 1:200 maple syrup; PssZ-1x, PssZ plus 1:200 maple syrup; PssZ-3x, PssZ plus 1:70 maple syrup.

strain in liquid medium with and without maple syrup, then centrifuged the bacterial biomass and precipitated the soluble extracellular EPS from the supernatant using cold ethanol (Köseoğlu et al., 2015). Consistent with our hypothesis, the amount of ethanol-precipitated material from the *ΔpdeB/C/D* strain grown with maple syrup was significantly higher than without (Figure 3A). Both the wild type and the *ΔpdeB/C/D ΔpssC* mutant, which do not synthesize Pss (Chen et al., 2014), produced no cell-attached EPS, as indicated by the absence of Congo red staining of their biomass (Figure 3A). The small amount of precipitated soluble EPS in these strains likely represents lipoteichoic acids shed from the cell surfaces, as previously shown (Köseoğlu et al., 2015).

Maple lignans target the *Listeria monocytogenes* sortase SrtA

The detachment of Pss from bacterial cell surfaces induced by maple compounds could be explained by two potential mechanisms: (i) these compounds may interfere with Pss EPS binding to the Pss-specific lectin on the surface of listeria, or (ii) they may reduce the abundance of the lectin on cell surfaces. Given that the identity of the Pss-specific lectin is currently unknown, we focused on the second possibility. Most surface proteins in *L. monocytogenes* are covalently anchored to the cell wall peptidoglycan by transpeptidases known as sortases (Schneewind and Missiakas, 2014). If maple compounds inhibit sortase activity or reduce sortase levels, the abundance of the Pss-specific lectin on the cell surface could be decreased.

L. monocytogenes has two sortases: SrtA, which anchors the majority of surface proteins, and SrtB, which anchors proteins involved in heme uptake (Bierne et al., 2002; Bierne et al., 2004). If SrtA were targeted by maple lignans, a *ΔsrtA* deletion in the high c-di-GMP strain would be expected to mimic the effect of maple syrup on Pss detachment. Indeed, the constructed here *ΔpdeB/C/D ΔsrtA* mutant produced significantly more soluble EPS, resembling the effect of maple compounds (Figure 3B). To complement the *ΔsrtA* mutation, we inserted the intact *srtA* gene into the chromosome of the *ΔpdeB/C/D ΔsrtA* strain via the integrative vector pIMK2. Complementation resulted in the restoration the mutant *ΔpdeB/C/D* phenotype, i.e., retention of the Pss EPS on listerial cells (Figure 3B).

We then investigated whether maple lignans affect the abundance or activity of SrtA. Quantitative RT-PCR

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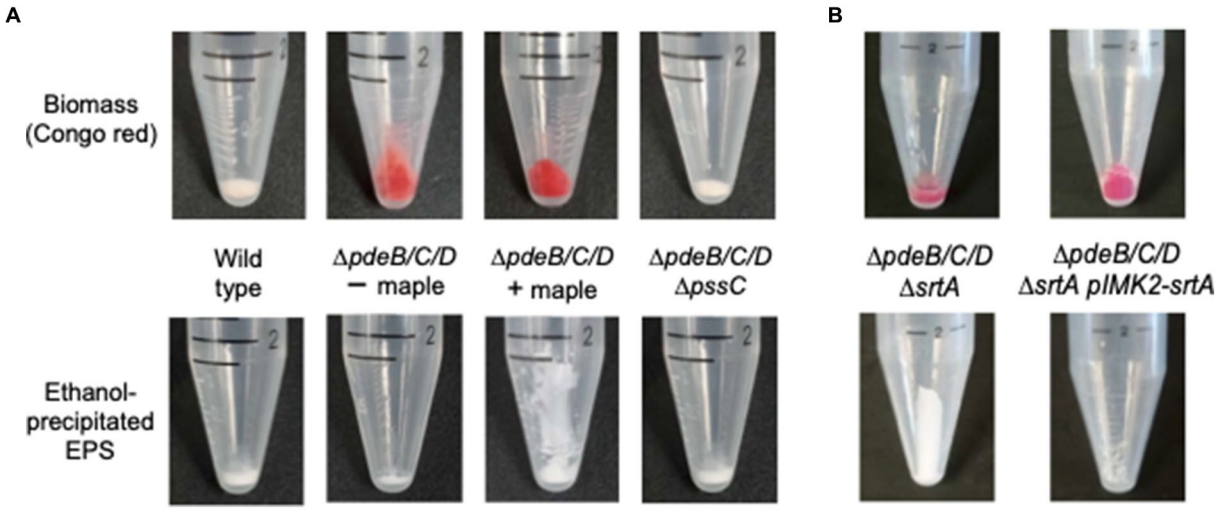

FIGURE 3
Maple lignans induce Pss detachment from bacterial cell surfaces. **(A)** The biomass and ethanol-precipitated EPS from *L. monocytogenes* wild type, EGD-e, and its high-c-di-GMP derivative, Δ*pdeB/C/D*, grown in the presence ("+ maple") or absence ("- maple") of maple compounds. Congo red was added to stain the EPS associated with the biomass. **(B)** Congo red stained biomass and ethanol-precipitated EPS from the *srtA* mutant of the high c-di-GMP strain, Δ*pdeB/C/D* Δ*srtA*, and the Δ*pdeB/C/D* Δ*srtA* strain complemented by the chromosome-intgrated intact *srtA* gene.

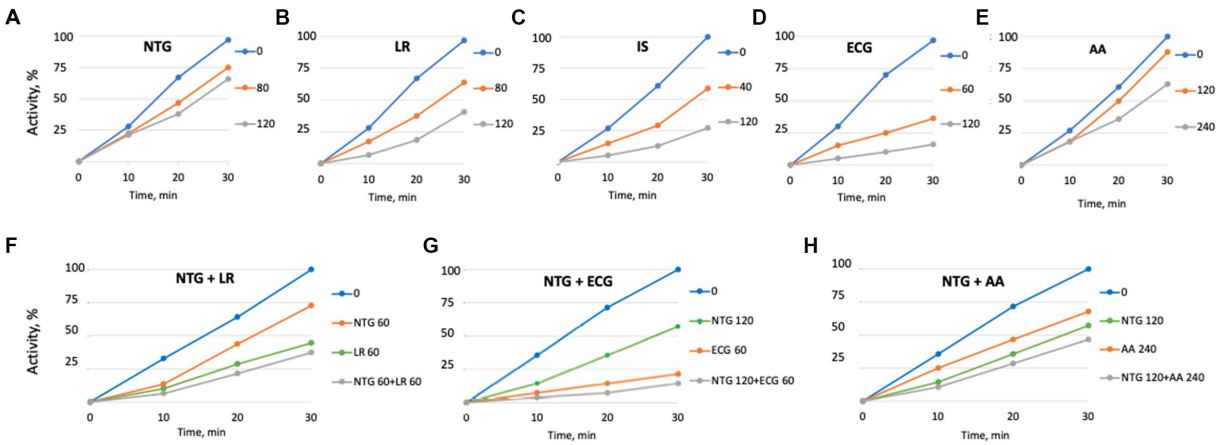

FIGURE 4
Sortase A is the target of maple lignans. SrtA inhibition by various maple compounds. Activity of the purified membrane-less SrtA::His_6 was tested using the fluorescently labelled LPxTG-containing peptide substrates. **(A)** nortrachelogenin-8′-O-β-D-glucoside (NTG); **(B)** lariciresinol (LR); **(C)** isoscopoletin (IS); **(D)** (−)-epicatechin gallate (ECG); **(E)** abscisic acid (AA); **(F)** NTG plus LR; **(G)** NTG plus ECG; **(H)** NTG plus AA. Concentrations (in μM) are shown in the legends. The average value of two experiments is displayed. Standard deviations did not exceed 14% of the average values.

analysis showed no significant differences in *srtA* mRNA levels in cultures grown with or without maple compounds (Figure 2B), suggesting that maple compounds do not drastically reduce SrtA protein levels. To assess the inhibitory effects of maple lignans on SrtA, we purified the truncated SrtA protein that lacks the N-terminal membrane-spanning domain (Li et al., 2016). The peptidase activity of SrtA was measured by monitoring the proteolysis of a fluorescently labeled peptide containing the SrtA-specific LPxTG motif. As shown in Figure 4A, both maple lignans, NTG and LR, moderately inhibited SrtA activity in a dose-dependent manner, indicating that SrtA is a direct target of the maple lignans.

Multiple maple compounds inhibit *Listeria monocytogenes* SrtA

While the inhibition of SrtA activity *in vitro* by NTG and LR aligns with their observed antibiofilm effects, an intriguing discrepancy remained: diluted maple syrup exhibited greater antibiofilm potency than anticipated. Specifically, a 1:200 dilution of maple syrup should not contain lignan concentrations in the ten-to-hundred micromolar range used in the *in vitro* assays. For instance, the concentration of LR in undiluted maple syrup has been reported to be approximately 54 μM (St-Pierre et al., 2014). After a 1:200 dilution, the LR concentration would drop to the sub-micromolar range.

To resolve this discrepancy, we hypothesized that additional compounds in maple syrup might also inhibit SrtA and that their effectiveness could surpass that of the lignans. We tested several polyphenolic compounds known to be present in maple syrup and maple wood extracts (Ramadan et al., 2021; Wan et al., 2012). Among these, we identified several compounds with potent anti-SrtA activities. (−)-Epicatechin gallate (ECG), isoscopoletin (IS), and scopoletin showed stronger SrtA inhibition *in vitro* than NTG and LR (Figures 4A–D), while abscisic acid (AA), which is relatively abundant in maple syrup (Ramadan et al., 2021), exhibited lower SrtA inhibitory activity (Figure 4E). Interestingly, clump dispersion by ECG is more effective at lower concentrations (30–60 μM) than the concentration (120 μM) originally used for screening of maple compounds (Elbakush et al., 2023). This explains why ECG was not prioritized by us earlier, though the reason for this unexpected phenomenon is not immediately clear.

We next assessed whether the anti-SrtA activity of these newly identified maple compounds correlated with their ability to inhibit listerial EPS-biofilms. We inoculated the high c-di-GMP strain in the presence of 60 μM of each compound and measured antibiofilm activity using the clump dispersion assay (Elbakush et al., 2023). As shown in Figures 5A,B, all three compounds effectively inhibited clumping. These findings suggest that the high potency of maple extracts as listerial antibiofilm agents is largely attributable to the presence of several SrtA inhibitors.

SrtA inhibitory activity *in vitro* does not necessarily correlate with antibiofilm activity

To further investigate whether *in vitro* SrtA inhibitory activity correlates with anti-EPS effects, we tested previously identified SrtA inhibitors of *L. monocytogenes*: genistin (Liu et al., 2023), chalcone (Li et al., 2016), and baicalein (Lu et al., 2019) at concentrations of 60 and 120 μM. None of these compounds prevented listerial clump formation. Notably, chalcone was found to be insoluble and precipitated out of the growth medium (Supplementary Figure S2A).

We also evaluated several inhibitors of SrtA enzymes from other pathogens of the Bacillota (Firmicutes) phylum that had not been previously tested against *L. monocytogenes* SrtA. These included astibin (Wang et al., 2019), curcumin (Hu et al., 2013), and morin (Kang et al., 2006; Huang et al., 2014). All these compounds were ineffective in preventing *L. monocytogenes* EPS-biofilm formation (Supplementary Figure S2B). These results indicate that *in vitro* SrtA inhibitory activity does not necessarily translate to protection against listerial EPS-biofilms.

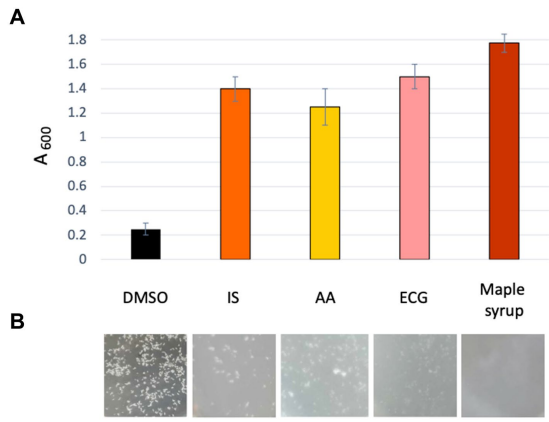

FIGURE 5
Newly identified maple compounds with anti-SrtA activity inhibit EPS formation. **(A)** Dispersion of EPS-clumps of the *L. monocytogenes* high-c-di-GMP strain, Δ*pdeB/C/D*, by the newly identified SrtA inhibitors from maple. Absorbance (A_{600}) of bacterial culture is measured at the end of the 48-h incubation in the presence of the indicated compounds. A_{600} was measured after a 2-min pause needed for spontaneous precipitation of undispersed clumps (Elbakush et al., 2023). The higher the absorbance the more potent dispersion. **(B)** Representative images of the appearance of bacterial cultures after 48-h incubation in the presence of the maple compounds or 1:200 diluted maple syrup.

Molecular modelling of the *Listeria monocytogenes* SrtA interactions with maple compounds

The maple compounds that inhibit SrtA belong to various chemical classes: NTG and LR are lignans, ECG is a catechin, IS is a coumarin, and AA is an abscisic acid derivative. The effectiveness of these structurally diverse compounds in inhibiting *L. monocytogenes* SrtA is somewhat surprising. To understand how these compounds might inhibit SrtA, we conducted *in silico* modelling using the X-ray structure of *L. monocytogenes* SrtA (Li et al., 2016) and the protein-ligand docking software Autodock Vina (Trott and Olson, 2010). Our molecular models indicated that all maple compounds with anti-SrtA activity bind within the catalytic site of SrtA (Figures 6A–E) with negative ΔG values ranging from-5.0 to-7.4 kcal/mol (Table 1). The most potent SrtA inhibitor *in vitro*, ECG, has the most negative ΔG value and is predicted to form hydrogen bonds with each of the catalytic triad residues of SrtA: H127, C188, and R197 (Li et al., 2016) (Figure 6E). Notably, baicalein, another SrtA inhibitor, has a ΔG value similar to that of ECG (Table 1; Figure 6F). However, the ΔG values of other maple compounds did not directly correlate with their potency as SrtA inhibitors *in vitro* or their antibiofilm activity *in vivo*. For instance, the second most potent SrtA inhibitor and antibiofilm agent, IS (Figures 4A, 5), has a lower absolute ΔG value compared to other antibiofilm compounds (Table 1). Like baicalein (Figure 6F) and LR (Figure 6B), IS forms mostly hydrophobic interactions within the catalytic site (Figure 6C). In contrast, AA has a better ΔG value than IS (Table 1) and forms hydrogen bonds with each residue of the SrtA catalytic triad (Figure 6E), yet it is less effective as an SrtA inhibitor *in vitro* and *in vivo* compared to IS (Figures 4A, 5).

Since the *in silico* models predict that all inhibitors bind within the catalytic site, adding two inhibitors at nonsaturating concentrations was expected to have an additive effect. We tested this by examining selected inhibitor pairs and observed additive inhibitory effects (Figures 4F–H). This finding does not preclude the possibility of synergistic effects with other combinations of SrtA inhibitors or with combinations of inhibitors and non-inhibitory maple compounds. Interestingly, some SrtA inhibitors might bind to secondary sites outside the catalytic site (Supplementary Figure S3). These peripheral interactions could potentially impair SrtA's conformational flexibility and enhance the inhibition by compounds that bind within the catalytic site.

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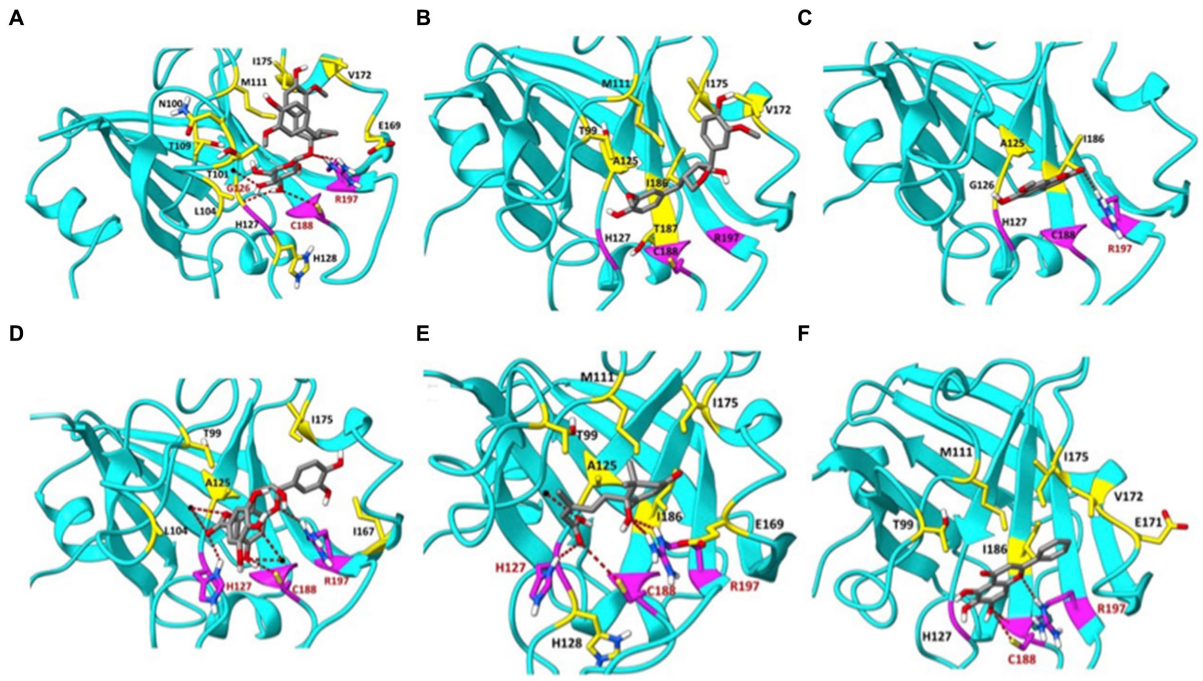

FIGURE 6
Molecular modelling of the SrtA interactions with selective maple compounds. The X-ray structure of the *L. monocytogenes* SrtA (PDB: 5HU4) was used to predict binding modes of the maple compounds using the Autodoc Vina software (Trott and Olson, 2010). The predicted binding sites and chemical bonds are shown on the panels corresponding to individual compounds. Grid Box measurements were established with AutoDockTools-1.5.7. Box coordinates: X: 5.117 Å, Y: 20.439 Å, Z: 11.52 Å; Box Size: X: 52 Å, Y: 58 Å, Z: 58 Å, with default spacing 0.375, Mode Number: 3, Energy Range: 5, Exhaustiveness: 8. The amino acid residues of the catalytic triad of SrtA (H127, C188, R197) are colored in magenta. H-bonds are shown in red. Amino acids forming hydrophobic interactions with maple compounds are shown in yellow, except for hydrophobic interactions with the amino acids of the catalytic triad, which are shown in black. **(A)** nortrachelogenin-8'-O-β-D-glucoside (NTG); **(B)** lariciresinol (LR); **(C)** isoscopoletin (IS); **(D)** (−)-epicatechin gallate (ECG); **(E)** abscisic acid (AA); **(F)** baicalein.

TABLE 1 **Predicted ΔG of binding of SrtA inhibitors.**

Compound	ΔG, kcal/mol
Nortrachelogenin-8'-O-β-D-glucoside (NTG)	−7.2
Lariciresinol (LR)	−6.2
Isoscopoletin (IS)	−5.0
(−)-Epicatechin gallate (ECG)	−7.4
Abscisic acid (AA)	−5.9
Baicalein	−7.4

Maple syrup, sap and aqueous maple wood extracts inhibit *Listeria monocytogenes* SrtA *in vitro*

We wanted to invvestigate whether diluted maple syrup exhibits SrtA inhibitory activity *in vitro*. A 1:200 dilution of Amber syrup inhibited SrtA activity by approximately 50% (Figure 7A). We were unable to test less diluted solutions due to high autofluorescence from the compounds in the maple syrup, which interfered with the fluorescence-based activity assay. Further, aqueous extracts from maple wood and diluted maple sap also demonstrated anti-SrtA activity (Figures 7A,B). These findings support the conclusion that SrtA inhibition is a key factor in the antibiofilm activity of aqueous maple wood extracts against *L. monocytogenes*.

Discussion

Washing fresh fruits and vegetables and cleaning and disinfecting produce processing equipment might not always be sufficient for removing *L. monocytogenes* biofilms or preventing their formation (Oloketuyi and Khan, 2017; Fagerlund et al., 2020). Therefore, using safe and affordable natural products that inhibit biofilm formation and promote biofilm dispersion may be necessary. In our previous work, we identified aqueous extracts from maple, hickory, pecan, and star jasmine wood as potent antibiofilm agents against *L. monocytogenes* EPS-biofilms (Fulano et al., 2023). Producing these extracts from wood byproducts like shavings, chips, sawdust, and bark is cost-effective, particularly where wood is locally available. Additionally, the maple syrup industry represents a large source of antibiofilm compounds, with approximately one billion gallons of maple sap collected annually in Canada and the USA (Statistics Canada, 2023; USDA and National Agricultural Statistics Service, 2023). Given the long history of safe human consumption of maple sap and syrup, using maple wood-based products to prevent listerial biofilms seems promising.

In this study, we deciphered the mechanism of action of maple lignans, NTG and LR, and identified several new antibiofilm compounds. We show that maple wood extracts, including commercially available maple syrup, contain a cocktail of antibiofilm compounds. These compounds do not affect the synthesis or hydrolysis of Pss EPS (Figure 2) but inhibit its attachment to bacterial

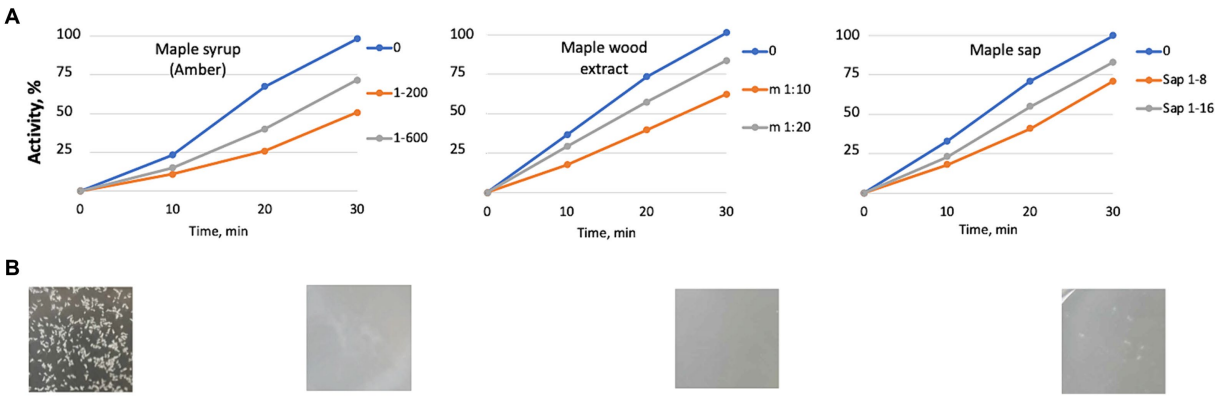

FIGURE 7
Maple products inhibiting cell aggregation in *L. monocytogenes* possess the anti-SrtA activity *in vitro*. **(A)** inhibition of the SrtA activity *in vitro*. Maple syrup at 1:200 and 1:600 dilutions; aqueous maple wood extract prepared as described in Elbakush et al. (2023); and maple sap. The averages of two experiments are displayed. Standard deviations did not exceed 17% of the average values. **(B)** Representative images of the appearance of bacterial cultures after 48-h incubation in the presence of the maple products.

cells by targeting sortase A (SrtA) (Figure 3). We hypothesize that this effect is due to interference with the SrtA-mediated peptidoglycan anchoring of the Pss-specific lectin, which remains to be identified. Inhibiting SrtA causes the detachment of the lectin and Pss EPS from bacterial cells, reducing bacterial adherence to fresh produce and preventing clumping.

This mechanism of SrtA inhibition explains several observations that alternative antibiofilm mechanisms cannot. For example, maple compounds inhibit listeria attachment not only in Pss-overproducing strains but also in strains that do not synthesize Pss (Elbakush et al., 2023). This suggests that SrtA inhibition affects not only the Pss-lectin but also other surface proteins involved in attachment. In strain EGD-e, such proteins are not yet characterized, but in a related strain, 1040S, the *Listeria* cellulose-binding protein (Lcp) has been identified (Bae et al., 2013). Lcp homolog Lmo0842, encoded in the EGD-e genome, likely serves the same function. Since Lcp/Lmo0842 has the LPxTG sequence characteristic of SrtA targets, its anchoring to the peptidoglycan must be impaired by SrtA inhibition. This hypothesis is currently being tested.

Another observation supporting the SrtA inhibition mechanism is the relatively slow rate at which preformed Pss EPS-biofilms are dispersed by maple compounds. As shown in Figure 2C, diluted maple syrup causes an immediate but modest dispersion of listerial clumps, with no further increase over a 30-min period. This suggests that SrtA-dependent dispersion affects only newly synthesized EPS, while already anchored EPS is less affected. The rapid, modest clump dispersion also indicates an additional, SrtA-independent antibiofilm activity of maple compounds.

In addition to previously reported lignans NTG and LR (Elbakush et al., 2023), we found that ECG, IS, and its isomer scopoletin, also possess antibiofilm activity and act as potent SrtA inhibitors. Further, the common phytohormone AA, abundant in maple syrup, has moderate SrtA inhibitory activity (Figures 4A–E). Molecular modelling of SrtA interactions with these inhibitors showed that, despite structural differences, all bind within the catalytic site with negative ΔG values (Figure 6). Interestingly, binding modalities vary. ECG, the most potent SrtA inhibitor *in vitro* (Figure 4D) and *in vivo* (Figures 5A,B), primarily forms hydrogen bonds with the SrtA catalytic triad, while IS, the second most potent inhibitor, forms hydrophobic interactions with non-catalytic residues (Figures 6C,D). We found that the correlation between ΔG values and activity for other compounds is not straightforward. For example, previously described SrtA inhibitors, such as genistin (Liu et al., 2023), chalcone (Li et al., 2016), and baicalein (Lu et al., 2019), as well as inhibitors of SrtA from related pathogens, did not show antibiofilm activity (Supplementary Figure S2). This suggests that factors like extracellular matrix sequestration, poor solubility, or inactivation by media components might limit the effectiveness of these compounds.

Our work suggests that the antibiofilm potency of maple extracts is due to a cumulative effect of SrtA inhibitors (Figure 7). In the limited pairwise testings of SrtA inhibitors, we observed additive, but not synergistic, effects. However, other combinations of SrtA inhibitors or inhibitors with non-inhibitory maple compounds might yield synergistic effects. Some SrtA inhibitors may also bind to sites outside the catalytic pocket (Supplementary Figure S3), potentially enhancing inhibition by stabilizing SrtA in an inactive conformation.

It is worth noting that maple compounds are ineffective against bacteria that lack SrtA, like *S. typhimurium* (Figure 1), which support the idea that SrtA is the primary target. Sortases are attractive targets for antibacterial agents because they anchor cell wall adhesins involved in interactions with surfaces and cells, and their extracellular localization makes them accessible. Importantly, *srtA* mutants in *Listeria*, and also in *Streptococcus* and *Staphylococcus*, show reduced virulence (Schneewind and Missiakas, 2014). If maple products can inhibit sortases A in all pathogens from the Bacillota phylum, their utility may extend beyond combating *L. monocytogenes* biofilms.

Materials and methods

Bacterial strains, plasmids and growth conditions

The strains used in this study are listed in Table 2. The *L. monocytogenes* wild type strain, EGD-e, and its derivatives were grown in the liquid minimum HTM medium (Tsai and Hodgson, 2003) containing 3% glucose, HTM/G, at 30°C under shaking (220 rpm). For enumerating CFUs, cultures were plated onto Brain Heart Infusion (BHI) agar (Millipore Sigma) and incubated at 37°C for

TABLE 2 Strains and plasmids used in this study.

Strains and plasmids	Relevant genotype or description	Reference or source
Strains		
Escherichia coli		
DH10β	Strain used for plasmid maintenance	New England BioLabs
Listeria monocytogenes		
EGD-e	Wild type	ATCC BAA-679[†]
Δ*pdeB/C/D*	EGD-e containing deletions in the *pdeB, pdeC* and *pdeD* genes	Chen et al. (2014)
Δ*pdeB/C/D* Δ*pssC*	Δ*pdeB/C/D* and in-frame deletion in *pssC*	Köseoğlu et al. (2015)
Δ*pdeB/C/D* Δ*srtA*	Δ*pdeB/C/D* and in-frame deletion in *srtA*	This work
Δ*pdeB/C/D* Δ*srtA*::pIMK2-*srtA*	Complementation of the Δ*srtA* mutation by the chromosome-integrated wild-type *srtA* gene	This work
Salmonella enterica subsp. Typhimurium		
UMR1	Wild-type (a $rdar_{28C}$ positive colony of ATCC14028) Nal^r	Zogaj et al. (2001)
MAE97	High c-di-GMP strain overexpressing EPS. *UMR1 PcsgD1 ΔcsgBA102*	Römling et al. (2000)
Plasmids		
pET23a	Plasmid for His_6-tagged protein overexpression	Invitrogen
pET23a-*srtA*	pET23a::*srtA*-His_6	This study
pLR16-*pheS**	Suicide vector for allelic exchange in *L. monocytogenes*	Argov et al. (2017)
pLR16-*pheS**-Δ*srtA*	Plasmid for the in-frame *srtA* deletion	This study
pIMK2	Integration vector in *L. monocytogenes*	Monk et al. (2008)

[†]ATCC, American Type Culture Collection.

24 h. The *Salmonella enterica* subsp. Typhimurium strains were also grown in HTM/G for biofilm experiments with pieces of fresh produce, and in Luria–Bertani (LB) for biofilm experiments in microtiter plates.

Maple wood products and phytochemicals

Maple syrup (Amber grade) from Crown Maple (NY, United States) was diluted with sterile water and used at the indicated final dilutions, usually 1 part to 200 parts (1:200) of HTM/G medium or reaction buffer. Phytochemicals were purchased from the following suppliers: (−)-epicatechin gallate (ECG) from Aobius Inc. (MA, United States), nortrachelogenin-8'-O-β-D-glucopyranoside, lariciresinol, (+)-abscisic acid, scopoletin and isoscopoletin from Targetmol (MA, United States).

Construction and complementation of the *srtA* mutant

The *srtA* gene was deleted by using the suicide vector for allelic exchange, pLR16-pheS* (Argov et al., 2017). Approximately 800-bp fragments upstream and downstream from the deleted regions of the *srtA* gene were separately amplified by PCR using primers pairs designated A and B, and C and D (Supplementary Table S1). The pLR16-pheS* plasmid was linearized with XhoI and KnpI and the two PCR fragments were cloned by Gibson assembly (NEB), and transformed into *Escherichia coli* NEB 10β. The pLR16-*pheS**-Δ*srtA* plasmid was purified, sequenced and transformed in the *L. monocytogenes* high c-di-GMP strain, Δ*pdeB/C/D*. Two transformants were grown at 41°C in the presence of 10 μg chloramphenicol (Cm) mL^{-1} to ensure integration of the plasmid into the genome via homologous recombination, and plated on BHI agar containing Cm at 41°C overnight. Five colonies were picked, grown at 37°C overnight with no antibiotics and plated on BHI agar. Colonies were screened on BHI agar supplemented with 18 mM fenclonine (MedChemExpress), which selects against the inserted plasmid. In-frame *srtA* deletion mutants were identified by colony-PCR. To complement the Δ*srtA* mutation, the wild-type *srtA* gene, including its promoter, was cloned into the integrative vector pIMK2 (Monk et al., 2008). The primers used in this study are shown in Supplementary Table S1. The pIMK2-*srtA* plasmid was transformed in the Δ*pdeB/C/D* Δ*srtA* strain. The chromosomal integrants were identified by kanamycin resistance. The restoration of the *srtA* gene was verified by PCR-amplification of the *srtA* gene fragment from the inserted pIMK2 and sequencing.

Preparation and inoculation of pieces of fresh produce

Overnight *S. typhimurium* cultures were diluted 1:100 into 10 mL HTM/G medium in 125-mL flasks and grown at 30°C until optical density, A_{600}, ~ 0.4, at which point pieces of fresh produce were added, and cultivation was continued for 48 h. Cantaloupe coupons (20 mm diameter × 4 mm thickness) were obtained by using a cork borer. Celery strands of approximately the same diameter was cut in pieces of ~20 mm in length. Preparation of the sterile cantaloupe and celery pieces was done as described earlier (Fulano et al., 2023). Following incubation for 48 h, produce pieces were aseptically withdrawn, rinsed in HTM/G twice to remove loosely bound biofilms, and mechanically macerated in the homogenizer (Stomacher® 80 Biomaster, Seward, UK), as described

earlier (Fulano et al., 2023). The serial dilutions of the homogenates were plated onto BHI agar plates for CFU enumeration after 24 h.

RNA purification and quantitative RT-PCR, qRT-PCR

Total RNA was extracted by using Quick-RNA Fungal/Bacterial Miniprep Kit (Zymo Research, R2014) as instructed by the manufacturer. High c-di-GMP listerial strains were grown in 10 mL HTM/G medium with and without 1:200 diluted maple syrup for 48 h at 30°C. After brief centrifugation, cell pellets were resuspended in the RNA lysis buffer and disrupted mechanically with a BEAD BUG cell disrupter (Benchmark Scientific, 50 s, speed 400). Following RNA extraction, Turbo DNA-free kit (Invitrogen) was used to remove remaining chromosomal DNA. One μg of purified DNA-free RNA was converted to cDNA by using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed by using IQ SYBR green Supermix (Bio-Rad). The mRNA levels of the *pssZ* gene and *srtA* genes were normalized to the mRNA level of *rpoB* used as a reference transcript (Supplementary Table S2).

Proteins overexpression and purification

The *L. monocytogenes* PssZ protein was overexpressed in *E. coli* BL21(DE3) containing the overexpression plasmid, pET23a::*pssZ*-His_6, as described previously (Köseoğlu et al., 2015). The DNA fragment encoding the *L. monocytogenes* SrtA protein lacking the first 70 amino acids was PCR-amplified from the genomic DNA using primers specified in Supplementary Table S1. The amplified fragment was digested with BamHI and NdeI and cloned into the pET23a vector. The recombinant protein containing the C-terminal His_6-tag was overexpressed in *E. coli* BL21(DE3). Cultures of BL21(DE3) harboring the pET23a::*srtA*-His_6 were grown in LB medium supplemented with ampicillin (100 mg/L) at 37°C with shaking until the absorbance reached an A_{600}, 0.6–0.8. IPTG was then added to a final concentration of 1 mM, and the culture was grown for a further 12 h at room temperature. The cells were collected by centrifugation at 4°C, and the pellets were resuspended in the buffer containing 300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, and protease inhibitors (APExBIO) (pH 7.4). The cells were disrupted by using a French press minicell (Spectronic Instruments, NJ). The crude cell extracts were centrifuged at 15,000 × g for 10 min. Soluble protein fractions were collected, mixed with pre-equilibrated Co^{2+} charger resin (TALON-metal affinity resin; TaKaRa) for 3 h at 4°C, and then placed into a column and extensively washed with resuspension buffer containing 25 mM imidazole. The SrtA::His_6 protein was subsequently eluted by using 250 mM imidazole. Protein purity was assessed by SDS-PAGE, and the protein concentration was determined by using a Bradford protein assay (BIO-RAD).

SrtA activity assays

SrtA activity was measured following previously described protocol (Li et al., 2016). Reactions (120 μL) were performed in the buffer (50 mM Tris–HCl, 150 mM NaCl, and 5 mM $CaCl_2$; pH 8) containing 24 μg SrtA::His_6 and 1 μg (8 nM) substrate, Dabcyl-LPETG-Edans (AnaSpec). Quantification of fluorometric intensity (350 nm excitation, 520 nm emission) was done using a microplate reader (SYNERGY H4, BioTek).

Statistical analysis

Microsoft Excel was used for data processing and analysis. The bar charts display a mean ± standard deviation from three-to-five independent experiments, each performed in at least two replicates. Unpaired Student's t-tests were performed using Prism 9 for Mac (GraphPad). The sortase activity measurements were performed in duplicate, and the averages are displayed.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Requests to access the datasets should be directed to Mark Gomelsky, gomelsky@uwyo.edu.

Author contributions

AE: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. OT: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. SS: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. UR: Investigation, Supervision, Writing – original draft, Writing – review & editing, Formal analysis. MG: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

AE and MG are listed as inventors on patent applications filed by the University of Wyoming on the use of maple products for the prevention of biofilms via sortase inhibition.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1436476/full#supplementary-material

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Current methodologies available to evaluate the virulence potential among *Listeria monocytogenes* clonal complexes

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Listeria monocytogenes is a foodborne pathogen that causes listeriosis in humans, the severity of which depends on multiple factors, including intrinsic characteristics of the affected individuals and the pathogen itself. Additionally, emerging evidence suggests that epigenetic modifications may also modulate host susceptibility to infection. Therefore, different clinical outcomes can be expected, ranging from self-limiting gastroenteritis to severe central nervous system and maternal-neonatal infections, and bacteremia. Furthermore, *L. monocytogenes* is a genetically and phenotypically diverse species, resulting in a large variation in virulence potential between strains. Multilocus sequence typing (MLST) has been widely used to categorize the clonal structure of bacterial species and to define clonal complexes (CCs) of genetically related isolates. The combination of MLST and epidemiological data allows to distinguish hypervirulent CCs, which are notably more prevalent in clinical cases and typically associated with severe forms of the disease. Conversely, other CCs, termed hypovirulent, are predominantly isolated from food and food processing environments and are associated with the occurrence of listeriosis in immunosuppressed individuals. Reports of genetic traits associated with this diversity have been described. The Food and Agriculture Organization (FAO) is encouraging the search for virulence biomarkers to rapidly identify the main strains of concern to reduce food waste and economical losses. The aim of this review is to comprehensively collect, describe and discuss the methodologies used to discriminate the virulence potential of *L. monocytogenes* CCs. From the exploration of *in vitro* and *in vivo* models to the study of expression of virulence genes, each approach is critically explored to better understand its applicability and efficiency in distinguishing the virulence potential of the pathogen.

KEYWORDS

listeriosis, virulence, risk assessment, CC, infection

1 Introduction

Within the genus *Listeria*, twenty-eight species are recognized; however, only two are considered pathogenic: *Listeria ivanovii* and *Listeria monocytogenes* (Raufu et al., 2022; Vázquez-Boland et al., 2001). *Both L. ivanovii* and *L. monocytogenes* can cause listeriosis, but the majority of cases are attributed to *L. monocytogenes* and only a few to *L. ivanovii.* Although much rarer than those caused by *L. monocytogenes* and *L. ivanovii*, *L. innocua* infections have been reported in humans and ruminants (Favaro et al., 2014; Moura et al., 2019; Perrin et al.,

2003; Rocha et al., 2013; Walker et al., 1994). Moura et al. (2019) demonstrated the virulence potential of atypical haemolytic *L. innocua* strains.

Human listeriosis, primarily caused by the consumption of contaminated food, is a severe illness that can manifest in one of two forms: non-invasive gastrointestinal infection in immunocompetent individuals or invasive listeriosis in risk groups, including pregnant women and newborns, the elderly and immunocompromised individuals (Vázquez-Boland et al., 2001; World Health Organization & Food and Agriculture Organization of the United Nations, 2004). In the invasive form, the pathogen surpasses the blood–brain and placental barriers, resulting in septicaemia, meningitis, spontaneous abortion and stillbirth (Lecuit, 2005). In 2022, the European Union reported 2,738 confirmed cases of listeriosis, which is 50 times fewer cases than the predominant gastrointestinal infection reported in humans, campylobacteriosis. Among the surveyed zoonotic pathogens, L. monocytogenes had the highest rates of hospitalization (96%) and case fatalities (18.1%) (EFSA and ECDC, 2023). These highlights the gravity of this major public health issue in developed nations. In addition to posing a significant public health risk, contamination of foods with this pathogen leads to disruptions in production, distribution, and recalls. As a result, it is receiving considerable attention from the food industry and authorities due to the significant economic losses and food waste involved (Li et al., 2022).

The study of *L. monocytogenes* bacterial model is of undoubtable importance; however, scientific research cannot be directly performed in humans. The investigation of this foodborne pathogen infection in humans has been mainly through reported clinical cases, epidemiological data, genome analysis and the use of infection models. In addition, *L. monocytogenes* has relatively low incidence in humans and extended incubation periods can be challenging in listeriosis studies, hindering the identification of causing pathogen and contamination routes (Hoelzer et al., 2012; Vázquez-Boland et al., 2001). Although different methods have evolved to better characterize *L. monocytogenes*, this species is genetically heterogeneous and different typing methods (discussed below) can be used to subtype this species at different levels. Due to the great variety of typing methods available, comparative analysis between studies can be challenging (Koopmans et al., 2023). Additionally, the study of *L. monocytogenes* virulence potential can be conducted through different host species used as infection models, however, differences in the selected model, infection dose, incubation time, etc. can be difficult when comparing between studies. Several phenotypic and genotypic tools as well as *in vitro* and *in vivo* models have been used to evaluate the uneven virulence potential among distinct strains. Given the diversity of *L. monocytogenes* studies, it is challenging to define criteria that are universally objective, consistent and applicable. Therefore, in this review we aim to explore and analyse the current methodologies utilized for evaluating differences in the virulence potential among

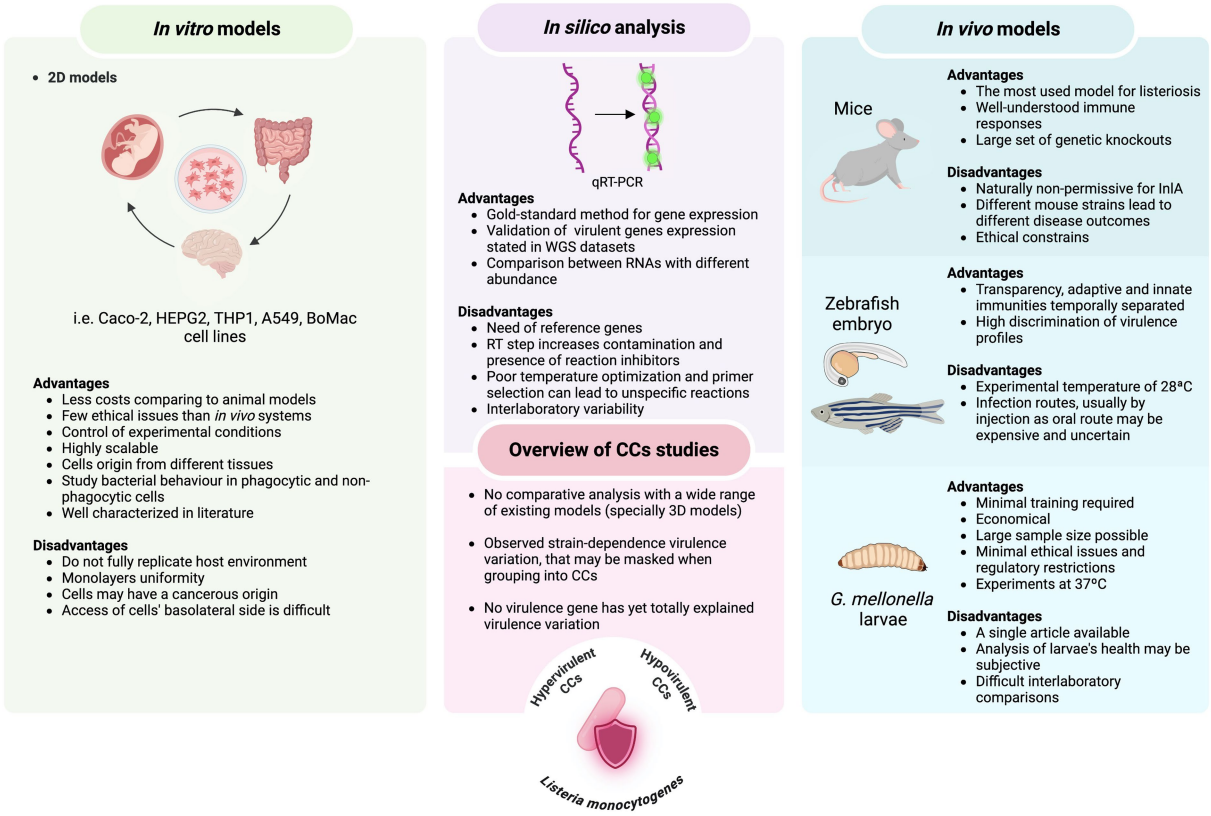

FIGURE 1
Overview of advantages and disadvantages of infection systems (both *in vitro* and *in vivo* models) and molecular approaches used to assess the virulence potential among *L. monocytogenes* clonal complexes.

strains of distinct CCs (summarized in Figure 1), giving readers an overview of the available literature.

2 Typing of *Listeria monocytogenes*

Typing of *L. monocytogenes* has been essential in epidemiological studies of listeriosis, allowing for the establishment of clonal relatedness among collected isolates. Over the decades, the development and implementation of pheno-and geno-typing methods have made it possible to confirm outbreaks, trace sources of contamination and identify transmission routes within the food chain. Additionally, the increasing adoption of standardized typing methods has facilitated the establishment of effective national and international surveillance systems, enabling the monitoring of evolutionary trends and the generation of comparisons across different geographical regions. This has indubitably had a major influence on the responses and strategies of public health systems worldwide. On the other hand, these methods have massively enhanced our perception of the remarkable biodiversity within *L. monocytogenes* species and their distribution in different environments. The first method, largely employed in epidemiological studies, was based on the serological antigen structure of the bacterium, specifically on the agglutinating activity of somatic (O) and flagellar (H) antigens (Seeliger and Höhne, 1979; Seeliger and Langer, 1989). This method was gradually replaced by more expeditious methods – namely, a gel-based multiplex-polymerase chain reaction (PCR serogroup) that differentiates, between four major serogroups, including the serovars more frequently isolated from food and patients (> 98%, i.e., 1/2a, 1/2b, 1/2c, and 4b): serogroup IVb (comprising serovars 4b, 4d, 4e), serogroup IIa (comprising serovar 1/2a, 3a); IIb (comprising serovars 1/2b, 3b, 7); and serogroup IIc (comprising serovars 1/2c, 3c) (Doumith et al., 2004). Later, a real-time triplex-PCR assay that differentiates these groups was made available (Vitullo et al., 2013). Although many *L. monocytogenes* serotypes have been discovered, three major serovars (1/2a, 1/2b, and 4b) are responsible for a substantial fraction of listeriosis cases (about 90 to 95% of human infections) (Schiavano et al., 2022).

Several typing methods have been used for multiple purposes, with genotypic methods being particularly highlighted due to their higher discriminatory power (e.g., amplified fragment length polymorphism (AFLP), multilocus variable-number tandem repeat analysis (MLVA) or ribotyping) compared to phenotypic methods. In the specific case of epidemiological studies, pulsed-field gel electrophoresis (PFGE), based on the analysis of DNA restriction patterns, has been considered the "gold standard" technique for typing *L. monocytogenes* for many years (Graves and Swaminathan, 2001). However, PFGE has some drawbacks, such as the difficulty of standardizing the analysis of fingerprints, which poses a challenge for inter-laboratory and inter-country comparisons. In addition, while it is valuable for assessing genetic relatedness between isolates, pinpointing sources of contamination and identifying outbreaks, it is not sufficient for establishing comprehensive phylogenetic relationships between strains. Sequence-based typing methods such as multilocus sequence typing (MLST) or multi-virulence-locus sequence typing (MLVST) are more appropriate for this purpose (Maiden et al., 1998; Salcedo et al., 2003; Zhang et al., 2004). Currently, MLST is widely used as a reference method to categorize the clonal structure of bacterial species and to define clonal complexes (CCs) of genetically related isolates, i.e., those descended from the same ancestor. In *L. monocytogenes*, MLST is based on the sequencing of seven housekeeping genes (*acbZ, bglA, cat, dapE, dat, ldh*, and *lhkA*), that allow the determination of sequence types (STs) (Bergholz et al., 2018; Orsi et al., 2011; Ragon et al., 2008). Additionally, Ragon et al. (2008) grouped these STs within CCs, with strains sharing at least six out of seven MLST alleles being assigned to the same CC. Currently, the preferred method for epidemiological and phylogenetic studies has shifted to whole genome sequencing (WGS), which has become more accessible to a broader range of laboratories due to technological advances and reduced costs (Gerner-Smidt et al., 2019). Whole genome sequencing of *Listeria* provides high-resolution data that not only allows phylogenetic relationships between strains to be determined, but also provides in-depth knowledge of the genomic structure of a given strain, including information on specific virulence factors and other genes that contribute to pathogenesis, as well as potential antibiotic resistance prediction (Hurley et al., 2019; Moura et al., 2024).

This species presents a diverse genetic pool and its virulence potential is very heterogeneous, resulting in an uneven capacity of strains to cause disease (Pyz-Łukasik et al., 2022). Currently, this species is divided into four major evolutionary lineages (I-IV), comparable to subspecies (Liu, 2006; Orsi et al., 2008; Rasmussen et al., 1995; Roberts et al., 2006; Ward et al., 2008; Wiedmann et al., 1997); lineage I includes serotypes 1/2b, 3b, 4b, 4d, and 4e and 7, and is significantly overrepresented in human listeriosis cases (Gray et al., 2004; Orsi et al., 2011); lineage II includes serotypes 1/2a, 1/2c, 3a, and 3c, prevalent among isolates from environmental samples, food, and animal listeriosis cases (Nightingale et al., 2005b; Sauders et al., 2006), and contribute significantly to sporadic cases of human listeriosis (Jeffers et al., 2001); and, lineage III and IV include serotypes 4a, 4c and atypical serotype 4b isolates, which are rare and are mainly associated with listeriosis in animals (Liu, 2006). Clonal complexes are grouped within lineages, for example, CC1, CC2, CC4 and CC6 (serotype 4b, lineage I) and CC121 and CC9 (serotypes 1/2a and 1/2c, respectively, lineage II) (Maury et al., 2016). A methodology for cloning *L. monocytogenes* and assessing potential human infectivity has been patented (WO2017009198A1).

More than one hundred CCs have been reported globally. The predominance of particular CCs is highly heterogeneous among different sources and regions. In 2011, Chenal-Francisque et al. (2011) characterized the genotypic profile of three hundred isolates collected from 42 countries on five distinct continents, and these isolates were distributed within 111 STs, assembled into only 17 CCs. This reinforces the idea that there is an irregular geographical distribution, with a few prevailing CCs (Chenal-Francisque et al., 2011; Wagner et al., 2022). However, these isolates were collected between 1933 and 2007, and it has been established that the distribution of CCs tends to change over time with some CCs, such as CC9, CC121, CC5, and CC6, emerging more recently (Bergholz et al., 2018). Information on STs/CCs associated with listeriosis outbreaks in European countries during the last decade are presented in Table 1. When molecular characterization of outbreak strains was not available from publications, additional information

TABLE 1 Reported listeriosis outbreaks in Europe for the last decade.

Year	Country	Source[a]	Sequence type/ clonal complex	No. of cases	Deceased[b]	[c]References
2009	Denmark	Beef meat – meals-on-wheels delivery	ST9/CC9	8	2	Smith et al. (2011), Jensen et al. (2016), and Moura et al. (2016)
2009–2010	Austria, Germany, Czech Republic	"Quargel" cheese	ST398/CC398 ST403/CC403 ST777/CC403	34	8	Fretz et al. (2010a,b), Harter et al. (2019), and Chen et al. (2016)
2009–2012	Portugal	Cheese	ST388/CC388	30	2	Magalhaes et al. (2015) and Ferreira et al. (2018)
2011	Belgium	Hard Cheese	ST37	12	4	Yde et al. (2012)
2011–2013	Austria and Germany	Unaged soft cheese and shrink-wrapped deli meat – plausible suspects	ST398/CC398 ST403/CC403	7	2	Schmid et al. (2014) and Moura et al. (2016)
2012–2015	Germany	Smoked pork belly	ST8/CC8	66	6	Ruppitsch et al. (2015) and Kleta et al. (2017)
2013–2014	Denmark	Deli meat products (mainly spiced meat roll)	ST224	41	17	Kvistholm et al. (2016)
2013–2014	Switzerland	RTE salad	ST4/CC4	31	4	Tasara et al. (2015) and Stephan et al. (2015)
2013–2015	Denmark	cold smoked salmon	ST391	10	4	Gillesberg et al. (2016)
2013–2015	Denmark	cold smoked halibut and trout	ST6	10	3	Gillesberg et al. (2016)
2013–2018	Germany	RTE meatballs	CC5 CC7	83	5	Wilking et al. (2021) and Lüth et al. (2020)
2014–2019	Multicountry outbreak: Estonia, Denmark, Finland, France, Sweden	Fish products	ST1247/CC8	22	5	Mäesaar and Roasto (2020), ECDC and EFSA (2019), and Mäesaar et al. (2021)
2014–2019	Germany	RTE meat sausages	ST8	39	18	Lachmann et al. (2021) and Fischer et al. (2021)
2015	Italy	Cheese	ST29/CC29, ST1/CC1, ST7/CC7, ST398/ CC398	6	1	Comandatore et al. (2017)
2015–2017	Cross-border: Denmark and France	Cold-smoked salmon	ST8/CC8	7	1	Schjørring et al. (2017)
2015–2017	Austria	Meat processing company (sliced pizza ham)	ST155/CC155	7	n/d	Pietzka et al. (2019)
2015–2018	Multicountry outbreak: Austria, Denmark, Finland, United Kingdom, Sweden	Frozen vegetables (corn)	ST6/CC6	47	9	EFSA and ECDC (2018), EFSA et al. (2020), and McLauchlin et al. (2021)
2016	Italy	Sliced cold beef ham	ST1/CC1	40	n/d	Maurella et al. (2018)
2018	Austria	Liver pâté	ST4/CC4	13	n/d	Cabal et al. (2019)
2018–2019	Germany	Blood sausage	ST6/CC6	112	7	Halbedel et al. (2020) and Wilking et al. (2021)
2018–2020	Switzerland	Cheese	ST6/CC6	34	10	Nüesch-Inderbinen et al. (2021)
2019	Spain	Stuffed pork	ST388/CC388	207	3	Fernández-Martínez et al. (2022) and Domínguez et al. (2023)
2020–2021	Multicountry outbreak: Germany, Austria, Denmark, and Switzerland	Smoked rainbow trout filets	ST394	55	3	Halbedel et al. (2023)

[a]RTE, ready-to-eat.

[b]n/d, no data available.

[c]For a comprehensive overview of listeriosis outbreaks worldwide between 1969 and 2022, we refer to the recent review by Koopmans et al. (2023).

was collected from the Institut Pasteur MLST database.[1] CC6 and CC8 were the two main CCs, accounting for 17.4% of the total of 23 outbreaks identified. Many studies across European countries have also reported that some clonal complexes, such as CC1, CC2, CC3, CC4, CC5, CC6, CC8, CC9, CC37, CC121, and CC388 are globally prevalent with some geographical disparities (Domínguez et al., 2023; Félix et al., 2022; Maury et al., 2016; Painset et al., 2019). To better characterize this heterogeneity between strains from different CCs, two independent terms have been established: CCs with a high frequency in human clinical cases are considered hypervirulent; conversely, CCs associated with food, persistence in food manufacturing environments, and with a lower frequency in human listerioses cases are considered hypovirulent (Maury et al., 2016). Therefore, CC1, CC2, CC4, and CC6 (lineage I) are considered to be hypervirulent clones since they are clinically related and mainly infect individuals with low or no comorbidities. Contrarily, strains belonging to CC9 and CC121 (lineage II), recognized as hypovirulent clones, are regularly isolated from food and food processing environments. The latter are often associated with individuals with a compromised immune system (Maury et al., 2016). There is also an intermediate classification for those clones that may be in transition from their host-associated lifestyle due to loss of virulence and acquisition of stress resistance genes (FAO and WHO, 2022). In 2018, Fritsch and co-workers also established three different levels of virulence among CCs and STs for risk characterization: hypovirulence, medium virulence, and hypervirulence. The latter, includes, in addition to the previously mentioned hypervirulent CCs, CC224, ST54, CC101 + 90, ST87, ST451, ST504, CC220, ST388, and CC207 (Fritsch et al., 2018). Hypovirulent CCs include CC9 and CC121, as well as CC11, CC19, CC31, CC193, CC199, CC204, and ST124.

It is important to note that, although hypovirulent CCs such as CC9 and CC121 are mainly associated with food and food processing environments, cases of invasive listeriosis caused by these CCs have also been reported. For instance, CC121 was considered the second most common CC isolated from human clinical cases in Norway and in France (Fagerlund et al., 2022; Maury et al., 2016).

Despite the potential ability to predict the risk of a specific strain of *L. monocytogenes* causing disease after consumption of contaminated food, most regulatory authorities worldwide take action when any *L. monocytogenes* is found in ready-to-eat (RTE) food that is capable of supporting growth, regardless of its strain characteristics. This approach is recommended by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) (2022), although in some countries risk managers are permitted to use information on *L. monocytogenes* subtypes to guide risk management decisions. However, the FAO and the WHO encourage the search for other virulence markers to predict, based on genetic virulence profiles (CCs characterization) (FAO and WHO, 2022). The discovery of one or multiple biomarkers that would allow to predict the real virulence potential of a given strain, and a clear distinction between hypo-and hypervirulence would be of great value to reassess the risks associated with different *L. monocytogenes* strains and to develop appropriate policies that neither overstate nor underestimate the risk posed by each strain. Ultimately, this finding would also contribute massively to the reduction of costs associated with the recall and destruction of contaminated food products and to reduced food waste and its social and economic consequences.

3 Putative virulence biomarkers (core and accessory genome)

The *L. monocytogenes* infection cycle comprises various steps: adhesion and invasion, lysis and escape from the vacuole, cytosolic multiplication, actin-tails polymerization, spread to neighbouring cells, and rupture of a double-membrane vacuole (Luque-Sastre et al., 2018; Pizarro-Cerdá et al., 2012). Some virulence genes are important for infection, such as, InlA-E-cadherin and/or InlB-C-Met (*L. monocytogenes* internalins-host receptors) for invasion, listeriolysin O (LLO) and phospholipases A and B (PlcA and PlcB) for both primary and double-layer vacuoles disruption, ActA for actin tail polymerization and intracellular motility (Quereda et al., 2021; Radoshevich and Cossart, 2018).

Considering all the above information, a detailed investigation regarding the putative virulence markers linked to both hyper-and hypovirulence is still ongoing, and some interesting findings have been reported. Regarding the core genome, inlA is normally present and expressed as a full-length form within clinical isolates (Lecuit et al., 2001). Premature stop codons mutations (PMSCs) have been found in the inlA gene, resulting in a truncated non-functional internalin in food isolates. In some studies, these PMSCs have been found amid strains from hypovirulent CCs, such as CC9 and CC121, and thus it is hypothesized that in some way, the lower virulence potential of these strains can be justified by the InlA truncation, leading to a reduced capacity to cross the intestinal barrier (Jacquet et al., 2004; Lachtara et al., 2022; Moura et al., 2016). The significant role of InlA-mediated crossing of *L. monocytogenes* through the intestinal barrier has been described. However, some studies have showed that the inoculation of Δ*inlA* mutants still resulted in *L. monocytogenes* infection (Bierne et al., 2002; Bou Ghanem et al., 2012; Disson et al., 2008). Therefore, it was hypothesized that *L. monocytogenes* employes alternative routes to cross the intestinal barrier. Besides the M cell-mediated translocation in Peyer's patches, Drolia et al. (2018, 2024) showed that the linkage between Listeria adhesion protein (LAP) and its surface receptor Hsp60 promotes cell disruption by using the cell innate system, consequently leading to bacterial translocation (Drolia et al., 2024; Drolia et al., 2018). These studies have shown that *L. monocytogenes* can cross the intestine through InlA-independent routes, which could explain the isolation of strains belonging to hypovirulent CCs (normally associated with the production of truncated inlA) in clinical cases. However, to our knowledge no comparative studies have investigated LAP or other InlA-independent invasion factors as putative candidate to distinguish hyper-or hypovirulent strains of *L. monocytogenes*.

All strains of *L. monocytogenes* carry the Listeria Pathogenicity Island 1 (LIPI-1), which clusters several fundamental genes for *L. monocytogenes* pathogenicity (Moura et al., 2016; Vázquez-Boland et al., 2001). These include the hly gene, which encodes a hemolysin – LLO – that provides the capacity to lyse erythrocytes. As mentioned

1 http://bigsdb.pasteur.fr/

above, this toxin can form a pore and allow the bacteria to escape from the internalization vacuole; thus, this virulence factor is detrimental to the virulence of *L. monocytogenes*. Another important virulence factor is PrfA, known as the main regulator of virulence genes in *L. monocytogenes*, such as the *prfA*, *actA* and *hly* genes. However, some studies have reported the existence of non-hemolytic *L. monocytogenes* strains, belonging to both lineages I and II, that have mutations in either the *prfA* or *hly* genes, and consequently a lower virulence potential (Maury et al., 2017).

Regarding the accessory genome, the pathogenicity island LIPI-3 carries eight genes. Listeriolysin S (LLS) encoded by *llsA*, functions as a bacteriocin with the capacity to modify the composition of the intestinal microbiota by eliminating or hindering the growth of neighbouring bacteria. This virulence cluster is often present within lineage I isolates, especially those from CC1, CC2 and CC6 – constituting a potential marker of hypervirulence (Cotter et al., 2008; Moura et al., 2016; Quereda et al., 2016). Additionally, Maury et al. (2016) identified a novel virulence cluster termed LIPI-4, which aggregates six genes that encode a cellobiose family phosphotransferase system (PTS). This gene cluster is strongly associated with strains of CC4, which are highly relevant to human brain and placental infections (Maury et al., 2016; Moura et al., 2016). Furthermore, it was thought that this pathogenic island was exclusively related to CC4 strains, but isolates from CC87 in China also displayed this locus (Wang et al., 2019; Zhang et al., 2020). These findings suggest that this could be a putative marker of hypervirulence, although it was found that this island was also present in *L. innocua* – a non-pathogenic species – and thus its role in hypervirulence is still controversial, reinforcing the need for further studies. Another intriguing gene is *lmo2776*, which acts as a bacteriocin and plays an important role in modulating the intestinal microbiome, mainly targeting *Prevotella copri* – a common gut commensal that has the capacity to modify the intestinal mucus layer and potentially intensify gut infection. The critical aspect is its significant presence in lineage I strains compared to its low frequency in lineage II strains. Curiously, deletion of *lmo2776* resulted in a better spread of the bacteria to the liver and spleen – the primary target organs of *L. monocytogenes* after crossing the intestinal barrier. This can be explained by the capacity of *L. monocytogenes* to discriminate between *P. copri*, preventing exorbitant inflammation and leading to longer periods of infection (Rolhion et al., 2019).

4 Models to study *Listeria monocytogenes* clonal complexes

4.1 *In vivo* infection models

In virulence studies, both pathogen characteristics and host physiology and anatomy must be considered, as microbial infections result from interactions between pathogens, hosts and the surrounding environment (Prescott, 2022). Preclinical trials using *in vivo* and *in vitro* biological models, have provided valuable insights into host-pathogen interactions (Anju et al., 2020; Khan et al., 2018). *In vivo* systems, used for various purposes from drug development to investigating physiological processes, complement *in vitro* studies by providing a more comprehensive understanding of biological responses. However, neither system alone is sufficient to make absolute predictions (Khan et al., 2018). Some of these models will be detailed in the following sections (Table 2).

In order to improve human health research, both mammalian and non-mammalian models are used due to ethical constraints with experiments involving humans (World Medical Association, 2013). The broad host range of *L. monocytogenes* allows the use of various animal models, such as *Drosophila melanogaster* (fly), *Galleria mellonella* (moth), *Caenorhabditis elegans* (nematode), *Mus musculus* (mouse), *Cavia porcellus* (guinea pig), *Oryctolagus cuniculus* (rabbit), among others (Anju et al., 2020; Prescott, 2022) – some of which will be discussed further. Animal models offer advantages that make them invaluable for human health research: they have identical biological processes, anatomical similarities (especially in vertebrates animals) – which are difficult to replicate in *in vitro* systems – compatible diseases such as cancer and diabetes, short life cycle and some can be easily genetically transformed to acquire some fundamental characteristics to express the disease phenotype (Kiani et al., 2022). Additionally, *in vivo* models are essential because they possess some unique characteristics when compared to *in vitro* models, for instance, the immunity associated with commensals and the intestinal mucosa throughout infection (Eng and Pearson, 2021). Depending on the final objective of the study, several aspects must be considered when selecting the ideal animal model: (1) the pathogen should have a similar tissue and cell affinity as in humans; (2) it should reveal the identical observable disease outcome and immunopathological harm; and (3) it should be susceptible to genetic manipulation (Lecuit, 2007). In addition to animal features, to study *L. monocytogenes* virulence, understanding listeriosis pathophysiology is crucial to select the adequate animal model. As already mentioned, *L. monocytogenes* can cross the intestinal, blood–brain and placental barriers. Therefore, pregnant, non-pregnant and geriatric animal models have been used in the study of *L. monocytogenes* pathogenesis, this was exhaustively described by Hoelzer et al. (2012). Animal models have played crucial roles in the characterization the virulence of *L. monocytogenes*. Generally, insightful data about the different pathways of bacterial translocation through host's defensive barriers, the exploitation of host's immunity to improve disease, performance of dose-dependent assays, the complex host immune responses to infection, the species specificity, virulence factors and strains virulence potential have been emerged from animal models studies (Hoelzer et al., 2012; Koopmans et al., 2023; Lecuit, 2007; Maury et al., 2016; Tsai et al., 2013). To our knowledge, an ideal animal model for listeriosis has not been established. Continuous new insights into animal physiology have increase the possibilities for infection systems, yet no single animal model completely aggregates the desirable characteristics to study human listeriosis. Therefore, the selection of an *in vivo* system is according to the specific objectives of the research being conducted.

Although animal models bring unquestionable insights into the study of infectious diseases, their extensive and indiscriminate use is strongly condemned by the European commission. This authority bases its policy on the Three R's principle (Replacement, Reduction and Refinement), which aims to replace the use of animals with non-animal strategies, to use a reduced number of animals per experiment without compromising the ultimate aim of the research, and to improve practices that contribute to the welfare of animals from birth to death. When animal replacement is not possible, the use of animals must follow strict guidelines set out in EU Directive 2010/63/EU (European Parliament, 2010; Zuang et al., 2024).

TABLE 2 Infection models used to study virulence potential among *L. monocytogenes* CCs.

Model		CC/ST/serotypes (no. of strains)	Source	Year	Reference/ control strains	Reference
In vivo	Mice	CC1 ($n = 3$)	Ganges river, agricultural soil and human placenta bit	2017	ATCC19115 and MTC-C1143	Soni et al. (2017)
		CC388 ($n = 1$), CC1 ($n = 1$), CC4 ($n = 4$)	Meat and retail products – ooutbreak strain in Spain (CC388)	2019	ATCC19115 (CC2)	Domínguez et al. (2023)
		CC1 ($n = 1$), CC4 ($n = 1$), CC6 ($n = 1$)	N/A	2022	EGDe (CC9)	Maudet et al. (2022)
	G. mellonella larvae	CC1 ($n = 6$), CC6 ($n = 5$), CC7 ($n = 9$), CC9 ($n = 4$), CC14 ($n = 6$), CC37 ($n = 1$), CC204 ($n = 3$)	Bovine ($n = 16$), human ($n = 12$), goat ($n = 2$), faeces ($n = 1$), rabbit ($n = 1$), silage ($n = 1$)	2019	–	Cardenas-Alvarez et al. (2019)
	Zebrafish	CC121 ($n = 1$), CC9 ($n = 2$), CC31 ($n = 1$), CC3 ($n = 1$), ST213 ($n = 1$), CC218 ($n = 1$)	Food (meat, vegetables), environmental swab,	2019	EGDe (CC9)	Hurley et al. (2019)
		CC1 ($n = 9$), CC2 ($n = 6$), CC4 ($n = 6$), CC6 ($n = 4$), CC8 ($n = 5$), CC9 ($n = 10$),	Human ($n = 14$), food (meat, milk cheese, RTE-salads, plant associated, ham) ($n = 25$), rabbits ($n = 1$)	2022	–	Muchaamba et al. (2022)
In vitro	Caco-2 cells	CC1 ($n = 3$), CC7 ($n = 1$), CC9 ($n = 1$), CC31 ($n = 1$), CC101 ($n = 1$), CC121 ($n = 2$)	Human (blood, CSF) ($n = 3$) and food (head cheese, fresh salami, salami, spit roasted pork) ($n = 6$)	2022	*L. innocua* ATCC 33090	Schiavano et al. (2022)
		CC1 ($n = 3$), CC2 ($n = 2$), CC3 ($n = 3$), CC315 ($n = 3$), CC5 ($n = 3$), CC121 ($n = 5$), CC14 ($n = 3$), CC19 ($n = 4$), CC403 ($n = 3$), CC415 ($n = 3$), CC7 ($n = 6$), CC8 ($n = 4$), CC9 ($n = 4$)	Food (salmon and meat)	2022	EGDe	Wagner et al. (2022)
		CC7 ($n = 5$)	Salmon and meat processing environment, dairy	2024	EGDe	Møretrø et al. (2024)
		CC1 ($n = 1$) and respective mutant strains	Rhombencephalitis in cattle	2017	EGDe, *L. innocua* (CCUG15531)	Rupp et al. (2017)
	HEPG2 hepatocytes	CC14 ($n = 3$), CC9 ($n = 2$)[a], CC121 ($n = 1$)[a]	Salmon	2022	EGDe	Wagner et al. (2022)
		CC7 ($n = 5$)	Salmon and meat processing environment, dairy	2024	EGDe	Møretrø et al. (2024)
	Macrophage-like THP1 cells	CC14 ($n = 3$)	Salmon	2022	EGDe	Wagner et al. (2022)
		CC7 ($n = 5$)	Salmon and meat processing environment, dairy	2024	EGDe	Møretrø et al. (2024)
	A549 cells	CC388 ($n = 1$), CC1 ($n = 1$), CC4 ($n = 4$)	Meat and retail products – outbreak strain in Spain (CC388)	2019	ATCC19115 (CC2)	Domínguez et al. (2023)
	Macrophage-like BoMac cells	ST1, ST4, ST412, ST18, ST37	Cattle	2016	–	Dreyer et al. (2016)
		CC1 ($n = 1$) and respective mutant strains	Rhombencephalitis in cattle	2017	EGDe, *L. innocua* (CCUG15531)	Rupp et al. (2017)
	Human macrophages differentiated from peripheral blood monocytes.	EGDeΔinlB supplemented with idInlB$_{CC1}$[b], idInlB$_{CC7}$ and idInlB$_{CC9}$	–	2023	–	Chalenko et al. (2023)
	Intestinal organoid from mice	1/2a ($n = 1$), 4a ($n = 1$)	–	2022	–	Zhou et al. (2022b)
Molecular approaches	RT-qPCR	CC1 ($n = 1$)	Rhombencephalitis in cattle	2017	EGDe, *L. innocua* (CCUG15531)	Rupp et al. (2017)
		CC14 ($n = 2$), CC9 ($n = 2$)[a], CC121 ($n = 1$)[a]	Salmon	2022	EGDe	Wagner et al. (2022)

N/A, not available; CSF, cerebrospinal fluid.
[a]Reconstructed strains with inlA gene.
[b]Receptor-binding domains of InlB.

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4.1.1 Mammalian models (mice)

The establishment of Robert Koch postulates to determine the etiological agent of an infectious disease, marked the inception of using mammalian species, phylogenetically related to humans, as healthy susceptible models (Kaito et al., 2020; Short and MacInnes, 2022).

Listeria monocytogenes is a ubiquitous microorganism, which enables it to infect a wide range of animals (Kammoun et al., 2022). However, in addition to humans, it mainly causes disease in ruminants, which, in an immediate and logical thought, should be the primary models to study listeriosis. However, this brings up many limitations. Thus, mice are the standard *in vivo* model to study listeriosis due to their size, ease of breeding and reproduction, rapid acclimation to confinement and an equivalent physiology when compared to humans (Lecuit, 2007). Commonly, mice are intravenously infected with the pathogen, and the role of some virulence factors, such as ActA and LLO, have emanated from this technique (Disson et al., 2009). Although mice are widely used in *L. monocytogenes* studies, the efficacy of oral infection is low due to the species-specific associated of with mammalian cells. As mentioned above, InlA binds to the E-cad receptor, which is a specific linkage for each species, depending on its 16th amino-acid type. Permissive species, such as guinea pigs, rabbits, humans and gerbils, have a proline in this position while non-permissive species have a glutamic acid – mice and rats have the glutamic acid and, consequently do not allow InlA binding (Lecuit et al., 1999). On the other hand, InlB naturally binds to C-Met in mice, humans and gerbils (Khelef et al., 2006). Theoretically, animals that naturally possess the imperative requirements to be bound to *L. monocytogenes* internalins, such as ruminants, non-human primates, and gerbils, should be selected to study listeriosis (Kammoun et al., 2022). Nonetheless, the ethical hurdles do not allow their wide application, so humanized mice have surged to overcome this limitation (Disson et al., 2008; Lecuit et al., 2001). Additionally, a "murinized" *L. monocytogenes* strain was developed to interact more closely with mouse E-cadherin. This modification involved altering the *inlA* gene in the *L. monocytogenes* EGDe strain to successfully infect wild-type mice (Wollert et al., 2007). Although this species-specific limitation was overcome, it was further discovered that the altered InlA was able to interact with both E-cadherin and N-cadherin in mice, luminally accessible in goblet and M-cells respectively, leading the bacteria to target both cells, increasing gut inflammation and consequently, hindering the capacity of *L. monocytogenes* to spread in the host (Tsai et al., 2013).

Currently there has not been a published comparative analysis of clonal complexes and their virulence in some animal models, such as gerbils, non-human primates, guinea pigs or rats. Although gerbils are permissive to both receptors, their use in listeriosis studies is limited. This may be related to the decreased sensitive to oral infection with *L. monocytogenes* when compared to other models, insufficient characterization when compared to mice and guinea pigs, absence of genetic models, and limited specific reagents and antibodies. The guinea pig infection model is advantageous in maternal-fetal studies as its placenta is the most comparable to human placenta among all rodents and has equivalent placental tropism. However, its narrow use may be related not only to species-specificity but also due to different disease symptoms from human listeriosis, with weak central nervous system tropism. Guinea pigs also present long gestation periods compared to mice, lack of gene deletion and transgenic models, and their larger size is more costly, limiting the number of animals per experiment. Despite the similarities of rats to mice, this infection model has shown low susceptibility to infection, requiring high infection doses to provoke disease. The use of non-human primates, as expected, is limited due to extended gestation periods, reduced number of available animals per study, and limited gene libraries compared to mice. Additionally, all these models are most costly when compared to mice (Cossart, 2011; D'Orazio, 2014; Eallonardo and Freitag, 2024; Hoelzer et al., 2012; Khelef et al., 2006; Roulo et al., 2014; Yan et al., 2023).

Considering this, very few articles have employed mice to investigate this phylogenetic association, either directly or indirectly (Domínguez et al., 2023; Maudet et al., 2022; Soni et al., 2017). Although the objective was not to compare strains from different CCs, Soni et al. (2017) inoculated three strains from CC1 in mice and observed a varying disease-causing capacity. One strain did not kill any mouse, while the other two presented 60 and 100% relative virulence. This highlights that although CCs are a more thorough classification, strains within a single CC can exhibit different virulence potentials. They also observed that the three strains harboured the major virulence genes, with the strain showing the lower pathogenicity presenting mutations in crucial virulence factors, such as listeriolysin O. However, no conclusion has been reached as to which mutation or genes better explain this unequal pathogenicity between phylogenetically close strains (Soni et al., 2017). Furthermore, in 2019, a large outbreak of listeriosis occurred in the Andalusian region, causing 207 cases, which was later associated with the strain ST388 from CC388 (Ministerio de Sanidad Consumo y Bienestar Social de España, 2019). Domínguez and her colleagues proceeded to investigate the virulence potential of this strain by comparing it with other strains from hypervirulent CCs (CC1 and CC4). *In vivo* infection assays were performed, and mice were infected intravenously with four strains (reference ATCC® 19115™, CC1, CC4, and CC388 strains). The results showed no significant differences between the CC388 strain and the other hypervirulent strains, as CC4 and CC388 isolates exhibited identical infection and spread ability (Domínguez et al., 2023). Maudet et al. (2022) selected strains from CC1, CC4, and CC6 – previously characterized as highly neuroinvasive CCs (Maury et al., 2016) – to perform infection assays in humanized KIE16P mice. The comparative analyses performed between these hypervirulent CCs and EGDe strain (CC9), corroborated the increased capacity of hypervirulent CCs to invade mice brains. Additionally, gene expression assays showed that hypervirulent strains presented upregulated levels of the *inlAB* operon, when compared to EGDe. Throughout these experiments different Δ*inlB* mutant strains were constructed to validate its relevance in the neuroinvasion capacity of *L. monocytogenes*. Despite the reduced neuroinvasion levels of EGDe when compared to CC4 strains, this study showed that whether using hypovirulent or hypervirulent strains, the *inlB* gene deletion reduced bacterial loads in the brain, confirming the need of overexpressing the *inlB* gene in *L. monocytogenes* neuroinvasiveness. Furthermore, the authors reported that InlB has immunosuppressive properties that are crucial to protect infected cells from host immune responses, resulting in an increase of infected monocytes' lifespan and *L. monocytogenes* propagation to the brain. Additionally, as hypervirulent strains exhibit overexpression of *inlB* and are mainly associated with infections in immunocompetent individuals, this article highlights the need to continuously study hypervirulent CCs to improve our perspective

regarding the bacterial factors employed in *L. monocytogenes* infection mechanism. Altogether, these finding showed that regardless of some reports suggesting strain-dependence in *L. monocytogenes* virulence studies, strains from hypervirulent CCs confer a significant concern to human health with distinct virulence factors that allows them to evade the host immune system. Moreover, mice models have proven to be a reliable tool to study *L. monocytogenes* infection cycle in mammals and we believe they will continue to be useful in future works.

4.1.2 Non-mammalian model organisms

Although mammalian models are the paradigm for studying host-pathogen interactions, they still present many obstacles, such as ethical issues due to animal welfare, high costs, adequate facilities and differentiated training requirements. Therefore, alternative models are needed for *in vivo* experiments that are less costly, easier to manipulate, with a short life cycle and are ethically acceptable. The complexity and relevance of these models lie between the sophisticated humanized mice and the simplicity of *in vitro* approaches (Lecuit, 2007). A variety of invertebrate and vertebrate models have been used to study the virulence potential of pathogens and the host immune response (Ahlawat and Sharma, 2022; Mylonakis et al., 2007). In *L. monocytogenes* studies, we highlight the use of *G. mellonella* larvae, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Danio rerio* (zebrafish), which have given valuable insights in the study of listeriosis. For instance, *C. elegans* model was previously used to evaluate the effects and toxicity of antimicrobial or antibiofilm substances in host-pathogen interactions and study nitrogen metabolism of *L. monocytogenes* after nematodes gut colonization (Kern et al., 2016; Muthulakshmi et al., 2022; Silva et al., 2015; Sivaranjani et al., 2016). The pathogenesis of *L. monocytogenes* has also been explored in *D. melanogaster* model, focusing in host immune system modulation, fly's metabolism alterations upon infection, association between the bacterial growth dynamics and host's genotypes (Chambers et al., 2012a,b; Hotson and Schneider, 2015; Mansfield et al., 2003; Taillebourg et al., 2014). Besides the widely use of both *C. elegans* and *D. melanogaster* models in the study of pathogenic bacteria, to our knowledge there are no comparative studies between *L. monocytogenes* CCs. In the fly model this can be related to the fact that the favourable temperature to flies is between 22 and 25°C, however, listeriosis studies are mainly conducted at 30–37°C and its inadequacy to distinguish between avirulent and virulent *Listeria* spp. after bacterial injection into the flies thorax (Jensen et al., 2007). On the other hand, *C. elegans* limited use may be associated to the fact that the deletion of some bacterial virulence genes (i.e., ActA) did not affect nematode's death and that the *C. elegans* intestine architecture may be different from mammals, since neither cell junction during cell extrusion or in goblet cells lumen are common in this nematode (Balla and Troemel, 2013; Thomsen et al., 2006). Considering this, we focused on both zebrafish and waxworms to give an overview about the study of virulence potential between *L. monocytogenes* strains/CCs in non-mammals.

4.1.2.1 Insect models

In the past, it was thought that insects were not a good *in vivo* model to study microorganisms that cause disease in humans since they are not phylogenetically close. However, they share a few physiological aspects with humans. Human pathogens present an analogous virulence capacity in humans and insects, with similar virulence factors involved (Mansfield et al., 2003; Martinez et al., 2017; Mukherjee et al., 2010; Tsai et al., 2016). In addition, the pathogen follows similar infection cycle steps in both hosts. Consequently, insects have evolved some defence mechanisms that are shared between mammals and insect hosts, for instance, the innate immune system with physical and phagocytic barriers, that have a homologous function (Kemp and Massey, 2007; Peterson et al., 2008). However, insects lack the capacity to develop an adaptive immune response, which is a common feature in vertebrates (Ahlawat and Sharma, 2022; Tsai et al., 2016). Hence, insects as host models have been a convenient alternative to mammals for infectious disease research.

4.1.2.1.1 *Galleria mellonella* as an infection model

Experiments with *Galleria mellonella* larvae have been carried out for some time, with increasing interest in recent year as a potential surrogate model to explore pathogen infections (Dinh et al., 2021). Besides being small, cheap, short life cycle, easy to maintain and to obtain in large numbers, it is also adapted to temperatures from 25°C to 37°C – the optimum growth temperature for the vast majority of human pathogens (Dinh et al., 2021; Mylonakis et al., 2005). The wax worm is selected stage to be utilized as a model, with infection normally occurring by injection, which requires minimal training (Singkum et al., 2019; Tsai et al., 2016). Its whole genome has recently been sequenced, enabling the search for further novel insights (Lange et al., 2018). Moreover, these insects possess a relatively advanced innate immune system, comprising two main components – the cellular and humoral immune response. The primer is composed of hemocytes – phagocytic cells that prevail in the hemolymph, and they are also capable of encapsulation and nodulation of pathogens. The humoral response results from the production of lytic enzymes, antimicrobial peptides (AMPs), opsonins and melanin upon microbial exposure (Boman and Hultmark, 1987; Kavanagh and Reeves, 2004; Pereira et al., 2018). It has been reported that *G. mellonella* larvae infected with *L. monocytogenes* are prone to produce AMPs such as galiomycin, lysozyme, gallerimycin, insect metalloproteinase inhibitor (IMPI) and cecropin D (Mukherjee et al., 2011; Mukherjee et al., 2010). Beside the analysis of host's immune modulation through hemocytes enumeration and variations in AMPs expression, the melanization, survival capacity, development of cocoon, motion ability can be evaluated in infected larvae with *L. monocytogenes* (Kavanagh and Sheehan, 2018).

Galleria mellonella has been utilized as an infection model to study the virulence potential of *L. monocytogenes* through comparative studies with different *Listeria* species or comparisons between *L. monocytogenes* serotypes (Martinez et al., 2017; Mukherjee et al., 2010; Pan et al., 2024; Rakic Martinez et al., 2020). Mukherjee and co-workers explored the ability of this insect model to discriminate between non-pathogenic and pathogenic *Listeria* species. When injected with 10^6 CFU/larva, strains belonging to non-pathogenic species, such as *L. innocua* and *L. seeligeri* were observed to have a lower infection capacity than the *L. monocytogenes* EGD-e strain; and, although *L. ivanovii* caused a significant but slightly higher mortality than the non-pathogenic species, it presented a reduced pathogenicity efficiency compared to *L. monocytogenes* (Mukherjee et al., 2010). These results were corroborated by Martinez et al. (2017), who observed that, at the same inoculum level, the *L. monocytogenes*

LS1209 reference strain displayed a LT_{50} (lethal time to kill 50% of larvae) 4 to 6 times lower than the non-pathogenic *Listeria* strains (Martinez et al., 2017).

The wax model was used to test the virulence potential of *L. monocytogenes* strains of different serotypes. The serotype 4b strain, commonly associated with clinical cases, expressed the highest larvae killing rate and was more pathogenic than the serotype 1/2a strain, usually related to food isolates. Other serotypes tested, 4a, 4c and 4d, also showed a lower pathogenic potential (Mukherjee et al., 2010). However, the study conducted by Martinez et al. (2017) showed that strains from different serotypes (1/2a, 4b, 1/2b) resulted in similar larvae mortality and identical LT_{50} at 24 h when administered at 10^6 CFU/larva (Martinez et al., 2017). This lack of correlation between serotypes and virulence potential was in clear contrast to the findings of the former study, highlighting the importance of considering potential confounding. These may include differences in the dose of bacteria injected (10^6 CFU/larva in the first study, whereas three different concentrations – 10^6 CFU/larva, 10^5 CFU/larva and 10^4 CFU/larva – were used in the second) and the parameters analysed (Mukherjee et al. monitored the % survival along 7 days, whereas Martinez et al. focused on LT_{50} at 24 h and % mortality – not specifying its progression over the infection period). Nonetheless, it was concluded in both studies that the virulence potential of *L. monocytogenes* is dose and strain dependent, so these different results could be explained by the use of different *L. monocytogenes* strains. Another factor that could externally influence on the observed results is the larvae's diet, since no information was available on the rearing of the larvae used in the Martinez et al. research. Previous studies have shown the importance of the diet in the larvae development, health, hemolymph volume and hemocyte concentration, which subsequently affect the immune response of *G. mellonella* (Jorjão et al., 2018; Kwadha et al., 2017). It has also been published that the diet of worms has an important impact in microbiological studies (Banville et al., 2012; Jorjão et al., 2018). Hence, standardization of diets could reduce external biases on results allowing for interlaboratory comparisons.

To date, virulence evaluation of different *L. monocytogenes* CCs using *G. mellonella* has only been performed by Cardenas-Alvarez et al. (2019). This insect model was used to compare the pathogenic potential of CC1, CC6, CC7, CC9, CC14, CC37, and CC204 strains. Briefly, differences were observed between strains from different CCs, with strains from the putatively hypervirulent CCs, CC1, and CC14, causing a reduced average survival rate (33.2 and 29.1%, respectively). Oppositely, isolates from CC9, widely accepted as hypovirulent CC, presented the highest survival rate (53.5%). In addition, the remaining CCs (6, 7, 37 and 204) showed an intermediate range of survival rates from 40 to 50%. Another parameter evaluated was the LD_{50} value (median lethal dose) – calculated from the colonies counted on plates and the number of larvae killed per day – lower values were observed for CC14, meaning that fewer cells of the pathogen are needed to kill *G. mellonella*. Cytotoxicity was also evaluated by measuring the level of lactate dehydrogenase (LDH), which is a signal of cell damage after bacterial infection. CC14 strains caused significantly less cytotoxicity than other CCs (CC6 and CC7). A positive correlation was found between LD_{50} and cytotoxicity, therefore CC14, strains by having a reduced LD_{50}, also caused less injuries to host cells, which is hypothesized to be a defence mechanism to escape the host immune system and successfully spread (Cardenas-Alvarez et al., 2019). Considering these results, *G. mellonella* as an infection model, besides the capacity to differentiate non-pathogenic from the pathogenic *Listeria* species, has the potential to distinguish between virulent and attenuated *L. monocytogenes* strains from different CCs, validating its ability to discriminate the virulence potential of *L. monocytogenes*.

4.1.2.2 Zebrafish model

The non-mammalian vertebrate *Danio rerio*, known as the zebrafish, is an *in vivo* model that has been gradually catching the attention of researchers for the study of infectious diseases as it meets the ideal features of vertebrate and mammalian models (Shan et al., 2015). As other models already described in this review, zebrafish is more easily applicable and economically and ethically acceptable than most mammalian models. Being a vertebrate, its morphological and genetic similarities with humans are more pronounced than with invertebrate (Pont and Blanc-Potard, 2021). In addition to the zebrafish's large clutch dimension, *ex-utero* growth and small size, its transparency makes zebrafish a distinctive mode, allowing observation of the early stages of growth and enabling the real-time observation of bacterial infections (Pont and Blanc-Potard, 2021; Shan et al., 2015; van der Sar et al., 2004). Another interesting peculiarity is that the innate and the adaptive immune systems are temporally separated, where the primer acts singularly during early weeks while the latter is perceived just during the 4–6 weeks post-fertilization (Herbomel et al., 1999; Herbomel et al., 2001; Lam et al., 2004; Trede et al., 2004).

The use of this vertebrate model in the study of host-pathogen interactions began in 1999, when Philippe Herbomel et al., reported that primitive macrophages – which evolve during the embryo's development and subsequently give rise to hematopoietic stem cells – develop in the zebrafish embryos at 22 h post-fertilization (Herbomel et al., 1999). Therefore, zebrafish have been used to explore host-pathogen interactions and provide new insights into the capacity of *L. monocytogenes* to cause disease in this *in vivo* model (Levraud et al., 2009; Shan et al., 2015; Zakrzewski et al., 2020). The different stages of development of zebrafish are used for research and have their advantages, but infection assays have only been performed in zebrafish's embryos to study the association of clonal complexes with hyper-and hypovirulence of *L. monocytogenes* strains, thus, our review will merely focus on this developmental stage. Among these, Hurley et al. (2019) made use of *L. monocytogenes* strains collected from three meat and vegetable processing facilities over 4 years. Genome analysis on these isolates reported distinct virulence genotypes and grouped them into hypervirulent, hypovirulent and unknown virulence groups (Hurley et al., 2019). This classification was slightly different from that previously described by Maury et al. (2016), as the isolates commonly associated with clinical cases (strains from CC1, CC2 and CC6) were underrepresented among the isolates collected. Therefore, hypervirulent strains were selected based on the presence of additional virulence factors such as listeriolysin S from LIPI-3 or LIPI-4. Selected hypovirulent strains (CC121, CC9, CC31) harboured PMSC mutation in the *inlA* gene and some of them had a deletion on the *actA* gene, which is associated with a decrease in intracellular spread. Isolates with integral virulence factors or with minimal mutations in some genes were classified as having unknown virulence capacity (CC3). Zebrafish embryos infected with putatively hypervirulent strains presented only a 3% survival rate, followed by zebrafish embryos infected with isolates of unknown virulence (20% survival rate), while

hypovirulent strains caused a higher survival rate of 53–83%, requiring 72 h post-infection to cause this decline. Using the zebrafish infection model, Hurley et al. (2019) were able to discriminate the different virulence phenotypes and confirm the previous virulence genotypes obtained by WGS (Hurley et al., 2019). Muchaamba et al. (2022) also performed infection assays using the zebrafish embryo model, comparing the virulence potential of *L. monocytogenes* strains by lineage, serotype, and clonal complex. When the strains were grouped by CC, the researchers observed virulence discrepancies by CC and strain-specific intra-clonal complex. Embryos infected with CCs that are generally considered hypervirulent showed higher mortality than isolates from CC9 or CC8. Within some CCs, such as CC1 and CC9, strain-dependent virulence variation was observed – three CC1 strains required more than 24 h post-infection to cause 100% mortality, and while two CC9 strains exhibited no virulence, the other three CC9 strains presented variable levels of virulence. The conclusion of the *in vivo* assays was that the virulence potential of this pathogen varies with genotype, serotype and strain (Muchaamba et al., 2022). Therefore, both studies confirmed the previous categorization of hypervirulent and hypovirulent *L. monocytogenes* CCs using the zebrafish embryo infection model. This underscores the model's relevance as an *in vivo* tool for further elucidating the virulence phenotypes of *L. monocytogenes* strains.

4.2 *In vitro* infection models

The *in vitro* systems represent alternative processes to study bacterial virulence as they mimic the infectious mechanism, allowing, for example, screening of pathogen gene expression and how the deletion of some genes affects the behaviour of strains in physiological environments mimicking *in vivo* conditions. *In vitro* assays are based on the assumption that pathogens, such as *L. monocytogenes*, have the ability to infect hosts by attachment, invasion, multiplication and subsequent dissemination in either phagocytic or non-phagocytic cells through the production of virulence factors (Liu et al., 2007). Although these systems do not precisely replicate the full features of the host-pathogen interaction, as infectious agents may encounter unfavourable conditions and the host immune system, when compared to *in vivo* models they are less expensive, less time consuming and less ethically demanding, allowing large-scale experiments. Additionally, the ability to control experimental conditions allows to unravel favouring factors in disease. Therefore, their use is recommended for preliminary studies to find new virulence factors, after which *in vivo* models can be used on a limited scale to confirm the results (Lehr, 2002; McCoy et al., 2024; Chiang et al., 1999). For these reasons many different *in vitro* models have been developed. The standard *in vitro* system, that has been used for decades, is the 2D monolayer culture of immortalized human cells. More recently, in a bioengineering context, there has been an increase in the use of different systems based on *in vitro* and *ex vivo* models, such as organoids and 3D cell cultures, to improve the monolayer model (Taebnia et al., 2023). The choice of an appropriate *in vitro* model should focus on the definite biological issue, for example, cell lines are more adequate to study precise interaction processes of pathogens. The addition of unneeded complexity can be disadvantageous, shrouding relevant host-pathogen interactions (McCoy et al., 2024). Therefore, many tissue culture experiments to study adhesion, invasion, cell to cell spread in different cell lines, survival in macrophages, evaluation of cytotoxicity and pathogens activity upon different host environmental conditions (e.g., pH and temperature) have been reported to describe and determine novel virulence concepts of bacteria (Conte et al., 1994; Hasebe et al., 2017; Wagner et al., 2022). In *L. monocytogenes*, *in vitro* models have been used to investigate host-pathogen interactions at either an intestinal, cerebral or placental level. These models brought significant knowledge regarding the *L. monocytogenes* intracellular cycle, invasion at cell extrusion sites, the role of putative virulent genes in cell invasion, required internalins (InlA, InlB and InlP) to placental invasion, *L. monocytogenes* bacteriocins in intestinal commensals, pathogen's routes to invade the brain and other related aspects (Banović et al., 2020; Cabanes et al., 2004; Cabanes et al., 2005; Lamond and Freitag, 2018; Pentecost et al., 2006; Rolhion et al., 2019).

4.2.1 Tissue culture assays for adhesion, invasion, intracellular growth and cell-to-cell spread

In 1948 the first cell line based on subcutaneous mouse tissues was developed. Thenceforth, various mammalian cell lines have been developed and used as the primary *in vitro* model to investigate infectious diseases, since they mimic host defence mechanisms (Magdalena, 2017). In *L. monocytogenes*, the human colorectal adenocarcinoma cell line Caco-2 is one of the most popular cell models that replicate the intestinal barrier, along with HT-29, Henle-407, HeLa, and many other cell lines (Liu et al., 2007; Pizarro-Cerdá et al., 2012). Different cell lines used in listeriosis studies were represented in Table 3.

The main limitation of cell models is their uniformity, not truly mimicking environment and morphology of epithelial tissues where a panoply of distinct cells can be found (Hidalgo, 1996; Pearce et al., 2018). One way to overcome this limitation is to co-culture different cell lines. However, to our knowledge, this strategy has not been commonly used to study the virulence potential of *L. monocytogenes* (Laparra and Sanz, 2009; Wikman-Larhed and Artursson, 1995). Another limitation of these cell models is their cancer origin, which makes it difficult to extrapolate the data since they may not reflect the actual physiological context. Additionally, the static conditions in which these monolayers are performed lead to very rapid bacteria overgrowth, thus compromising the duration of the culture and the search for new insights about the interaction between the host and its microbiome (Rodriguez, 2018; Taebnia et al., 2023).

Summing up, these *in vitro* cell models have been widely used to study the virulence potential of *L. monocytogenes* and have contributed to expand the current knowledge of the virulence mechanism of this bacterium. Consequently, this research has led to the development of strategies to control the dissemination of listeriosis. To date, very few studies have used these models to differentiate between strains from different CCs (Domínguez et al., 2023; Møretrø et al., 2024; Schiavano et al., 2022; Wagner et al., 2022). Schiavano et al. (2022) performed adhesion and invasion assays using the Caco-2 cell line in order to do a comparative analysis between human clinical isolates and food isolates. They observed that two out of three clinical strains (from CC1 and CC101) expressed a relatively high adhesion regarding to *L. innocua*. However, the invasion efficiency was not significantly higher than that of the non-pathogenic strain. On the other hand, the food isolates showed a variable adhesion capacity, with strains from

TABLE 3 Examples of cell lines used to study human listeriosis, representing the intestinal, placental and brain barriers.

Barrier	Cell line	Cell type	Reference
Intestinal	Caco-2	Human colorectal adenocarcinoma	Cajnko et al. (2015), Nightingale et al. (2005a), and Rousseaux et al. (2004)
	HT-29	Human colon adenocarcinoma	Roche et al. (2001)
	Henle-407	Human papillomavirus-related cervical adenocarcinoma	Czuczman et al. (2014)
	HeLa	Human cervix carcinoma	Bierne et al. (2005), Czuczman et al. (2014), Dhanda et al. (2021), Quereda and Pucciarelli (2014), Quereda et al. (2019), and Tham et al. (2010)
Placental	BeWo	Human placenta choriocarcinoma	Bakardjiev et al. (2004), Desai et al. (2013), Lecuit et al. (2004), Phelps et al. (2018), and Zeldovich et al. (2011)
	JEG-3	Human placenta choriocarcinoma	Bonazzi et al. (2008), Dhanda et al. (2021), and Quereda and Pucciarelli (2014)
	Primary trophoblast		Bakardjiev et al. (2004) and Lecuit et al. (2004)
Brain	HBMEC	Human brain microvascular endothelial cells	Greiffenberg et al. (2000) and Greiffenberg et al. (1998)
	HIBCPP	Human epithelial choroid plexus papilloma	Dinner et al. (2017) and Gründler et al. (2013)
	hCMEC/D3	Human cerebral microvascular endothelial cells	Ghosh et al. (2018) and Shi et al. (2024)

CC7, CC121 and CC1 showing significantly higher values than *L. innocua*.

For invasiveness, strains from CC7 and CC1 displayed significant higher capacity than *L. innocua*. No correlation was found between adhesion and invasion for food-derived strains. Curiously, one clinical strain (566 strain) did not show high levels of both adhesion and invasion. However, this strain belongs to CC31, which has been reported to be isolated more frequently from food than from humans. CC31 is not considered to be a hypervirulent clonal complex, as corroborated by its low invasive ability. Cases of human listeriosis caused by strains of this CC may be justified by the compromised immune system of the host (Schiavano et al., 2022). In contrast, the clinical strain tested from CC1, considered to be a common hypervirulent CC, showed an unexpectedly reduced invasion although a high level of adhesion was detected. Regarding the food isolates, the two strains with higher invasiveness belong to CC1 and CC7, which are usually associated with human cases but have also been reported in food. CC1 is highly associated with dairy and cattle products while CC7 has already been described as an intermediate CC (Lüth et al., 2020a). As expected, strains from CC9 and CC121 showed a low invasion ability. As both hypo-and hypervirulent CCs with a reduced invasion capacity were found in clinical cases, we can conclude that the state of the host immune system is very important since it may facilitate the occurrence of listeriosis. In addition, hypervirulent CCs can be found in foods, emphasizing their threat for individuals (Schiavano et al., 2022).

Wagner and his colleagues used Caco-2 cells to evaluate the invasion capacity of strains from thirteen different CCs. Strains from three CCs (CC5, CC9, and CC14) were not able to invade these cells, with only CC14 strains encoding the complete functional *inlA* gene. Strains from CC403 and CC415 were the most invasive, while strains from CC3, CC8 and CC121 were significantly less invasive. Among strains of these CCs with attenuated invasion, CC14 strains comprised all major virulent factors but still showed inefficiency at invading Caco-2 cells. Therefore, invasion and intracellular dissemination assays in Caco-2 and HEPG2 cells, both with an epithelial-like morphology, were performed with two strains of this CC. In Caco-2 cells, both CC14 strains showed a significantly reduced invasion capacity when compared to EGDe. However, only one CC14 strain showed a significantly lower invasion in HEPG2 cells. Conversely, intracellular multiplication in Caco-2 cells was significantly increased for both CC14 strains, while only one CC14 strain showed a greater intracellular spread in HEPG2 cells compared to EGDe (Wagner et al., 2022). Therefore, the virulence result of CC14 may be confusing when compared with previous findings, such as those in section 4.1.2., where it was characterized as a hypervirulent CC (Cardenas-Alvarez et al., 2019). These differences could not only be related to methodology used in the *G. mellonella* infection assays, where *L. monocytogenes* was injected directly into the hemolymph, bypassing the intestinal barrier, and thus not utilising the *inlA* and *inlB* genes, but also two distinct models (*in vitro* and *in vivo*) were used, highlighting the fact that different complexity could bring variable outcomes. Additionally, in Wagner's study although the *inlA* and *inlB* genes were present in the genetic profile of selected CC14 strains, their expression was significantly reduced compared to *L. monocytogenes* EGDe. This reduction in expression could possibly be explained by a point mutation in the promoter region (Wagner et al., 2022). These findings of Wagner et al. (2022), point up the drawback of using solely WGS data to define virulence potentials, since presence of a virulence gene does not necessarily lead to gene expression. Besides the different approaches used, these models showed some similarities regarding the high capacity of CC14 strains to spread within the selected models.

More recently, researchers investigated the 2019 outbreak in Andalusian, Spain, caused by a strain of CC388. Adhesion and invasion *in vitro* assays were performed on A549 cell line to compare the virulence potential of this outbreak strain with strains belonging to CC1, CC4 and a reference strain from CC2 (ATCC 19115). They observed a higher adhesion of the CC388 strain compared to CC1 and the reference strains, and lower adhesion compared to CC4 strain, although no significant differences were reported. A similar pattern was observed in invasion assays, with significant differences between strains of CC1, ATCC 19115 and CC388 versus CC4. It was therefore concluded that the CC388 strain had an equal or greater virulence

potential compared to other strains from hypervirulent CCs (Domínguez et al., 2023).

Concluding, 2D monolayer models have significantly advanced *L. monocytogenes* research, providing insight into key virulence properties and host-pathogen interactions. However, variations in results between different models require careful analysis. Therefore, their use must be complemented by molecular biology approaches to elucidate any unexpected phenotypic differences found by *in vitro* methods.

4.2.2 Survival in macrophages

Macrophages play a crucial role in the innate immune response, using their skilled phagocytic activity to fight infection. They recognise pathogens through pattern recognition receptors (PRRs) that bind to microbial-associated molecular patterns (MAMPs) (Kumar, 2020) such as DNA, RNA, lipopolysaccharides, and lipoproteins, thereby activating the host immune response (Fitzgerald and Kagan, 2020). This interaction triggers signalling pathways that culminate in the secretion of cytokines and the process of phagocytosis. Once engulfed by macrophages, pathogens are entrapped in acidic phagosomes where antimicrobial molecules can be found (Levin et al., 2016). However, certain microbes have evolved mechanisms to evade the host's immune defences and proliferate intracellularly, rendering macrophages ineffective in protecting against such pathogens (Galli and Saleh, 2021).

Listeria monocytogenes is able to survive within macrophages, as demonstrated by Tilney and Portnoy in 1989, who elucidated its mechanism of infection within these immune cells (Tilney and Portnoy, 1989). Considering this, different macrophage cell lines have been used to simulate the host barriers following bacterial intestinal invasion (Liu et al., 2007). Besides the employment of epithelial-like cells (Caco-2 and HEPG2) to evaluate *L. monocytogenes* virulence potential, Wagner et al. (2022) also used the human macrophage-like THP-1 cell line for this purpose. As CC14 strains were unable to invade Caco-2 cells, despite the presence of all key virulence genes, two CC14 isolates were used to invade and multiply intracellularly within macrophage cells. Although no significant differences in invasion capacity were observed between two CC14 strains and EGDe, intracellular multiplication was significantly increased in THP1 for both CC14 strains (Wagner et al., 2022). In 2016, Dreyer et al. conducted a study to investigate putative *L. monocytogenes* strain-associated virulence in isolates from the farm environment and diseased animals, focusing on rhombencephalitis cases where ST1 (CC1) was overrepresented. The *in vitro* assays were conducted on a bovine macrophage cell line (BoMac) and it was observed that STs associated with encephalitic infections (ST1, ST4 (CC4) and ST412 (CC412 – lineage II)) were able to invade and replicate more efficiently than those from the farm environment. Additionally, none of the isolates presented truncated InlA, which is commonly associated with virulence attenuation. Thus, although ST412 isolates from lineage II accounted for only 7% of rhombencephalitis cases (which is not a statistically significant association between ST and clinical outcome), they presented an increased virulence potential, highlighting the fact that these clinical-associated characteristics are not exclusive to lineage I isolates, raising awareness of the potential risk other CCs'. In addition, the *inlA* gene is not the only biomarker for differential virulence (Dreyer et al., 2016).

Another study conducted in 2017 aimed to test the relevance of certain virulence genes (*inlJ*1, *inlF* and *lls*) in the hypervirulence capacity of CC1 strains. The *L. monocytogenes* CC1 parental strain and its respective deletion gene mutants were compared to the EGDe (CC9) strain in different cell culture models, including macrophages. The BoMac cell line was used to mimic the intracellular phagosome environment. Despite both strains infecting all cell models, the CC1 isolate exhibited higher invasiveness than EGDe in some cell lines. For example, invasion into BoMac cells was 2.2 times higher for the strain from the hypervirulent CC. Moreover, the CC1 strain showed a significantly greater number of infection foci in BoMac cells, indicating an enhanced capacity to spread intercellularly and corroborating its stronger internalization phenotype. However, the intracellular multiplication in all cell lines was not significantly different between the two strains. *Listeria monocytogenes* exhibits a cell-specific interaction, as evidenced by the differential infection capacity observed between these two strains in specific cell types, including macrophages (Rupp et al., 2017).

In a more recent study, macrophages were used for a different comparative analysis of CCs beyond those previously mentioned, using various cell models for investigating invasion capacity, intracellular spread, and multiplication associated with potential PMSC mutations in InlA. Chalenko et al. (2022) found that InlB could modify the invasion and proliferation capacity of *L. monocytogenes* within macrophages. Consequently, they proposed to investigate whether the interaction between InlB and cell receptors would affect the intracellular infection cycle of the pathogen in these immune cells. Interestingly, this study explored the phylogenetically determined diversity of InlB to understand its impact on pathogen-cell interactions. The first step was to investigate the effective interaction between different InlB isoforms found in lineage I and II of *L. monocytogenes* strains and their two target receptors, using three distinct receptor-binding domains of InlB (idInlB) – idInlB$_{CC1}$, idInlB$_{CC7}$ and idInlB$_{CC9}$ – representing different virulence potentials. The study of the interaction of idInlB-human receptors (c-Met and gC1qR) was possible by the measurement of dissociation constants using Microscale Thermoforesis technology. The results showed that the interaction between idInlB variants and human receptors differs in terms of binding strength. idInlB$_{cc1}$ showed stronger binding to C-Met receptor compared to idInlB$_{cc7}$ and idInlB$_{cc9}$, while idInlB$_{cc9}$ presented weaker binding to the gC1qR receptor, with no significance differences observed between idInlB$_{CC1}$ and idInlB$_{CC7}$ variants. Based on the EGDeΔinlB strain (lacking the *inlB* gene), isogenic *L. monocytogenes* strains were also constructed, namely *LmInlBCC1*, *LmInlBCC7* and *LmInlBCC9*. These strains harboured full-length internalin B isoforms that differed only in the idInlB domain. When human M1 macrophages were infected with these strains, different multiplication capacities were observed, with *LmInlBCC1* showing a significantly higher proliferation capacity compared to the other. However, no significant differences were observed regarding the cell uptake. Altogether, these results suggest that phylogenetic differences in InlB affect the ability of *L. monocytogenes* to interfere with macrophage activity. In particular, it was suggested that InlB$_{CC1}$ efficiently overcomes immune barriers of these cells, which is consistent with the high occurrence of CC1 observed in epidemiological data (Chalenko et al., 2023).

4.2.3 Organoids

Despite the unquestionable use of 2D models in studying microbe-host interactions, these models are limited in mimicking real features, such as peristaltic movements, transitions between different intestinal cells, interactions with the intestinal microbiota, and their inability to be maintained for long periods (Taebnia et al., 2023). These gaps in accurately reproducing the function and structure of the human intestinal epithelium, limit the value of 2D models. Therefore, researchers have developed more comprehensive and complex models, that do not replace monolayer models, but rather complement them by incorporating physiological components or simulating infectious disease scenarios that are difficult to assess using simpler culture methods (Taebnia et al., 2023). In the 21st century, steam cells started to be cultivated to generate organoids, which have effectively bridged the gap between traditional 2D monolayer cultures and *ex vivo* models (Han et al., 2021; Sato et al., 2009; Taebnia et al., 2023).

In 2014, an important finding allowed the development of a reproducible method known as directed differentiation, which enables the specialization of leucine-rich repeat containing G protein-coupled receptor 5 (LGR5+) stem cells into various cell types, including goblet cells, enterocytes, stem cells, Paneth cells and enteroendocrine cells (Yin et al., 2014). These findings hold great promise for studying host-pathogen interactions and for investigating the responses and properties of specific cell types, such as the barrier role. Directed differentiation cannot be overstated in light of previous research showing that *L. monocytogenes* can surpass the intestinal barrier not only through enterocytes but also through M cells and goblet cells (Corr et al., 2006; Nikitas et al., 2011). Currently, organoids can be derived from cells of different species and consists of either differentiated cells, stem cells or a combination of thereof (Davies, 2018). The organoids have their apical side facing the lumen (central position), while basolateral crypt regions are directed to the outside (budding structure), mimicking the real intestinal epithelium. This reversed polarity is a challenge for microbiological research, as it is difficult to access to the lumen intestinal organoids (Huang et al., 2021). To overcome this obstacle, microinjection, mechanical dissociation techniques or "apical-out organoids" can be used (Co et al., 2019; Huang et al., 2021; Karve et al., 2017).

Many studies have used organoids to investigate host-pathogen interactions involving various microorganisms, including *L. monocytogenes* (Co et al., 2019; Huang et al., 2021; Kim et al., 2021; Roodsant et al., 2020; Zhou et al., 2022a,c). In the research of listeriosis, different types of intestinal (i.e., fetal human intestinal organoids, adult human intestinal basal-out and apical-out organoids, adult and young murine small intestinal organoids) and brain (i.e., organotypic brain slices) organoids have been used (Co et al., 2019; Guldimann et al., 2012; Hentschel et al., 2021; Huang et al., 2021; Kim et al., 2021). However, to our knowledge the use of placental organoids in the study of *L. monocytogenes* have not yet been reported (Yan et al., 2023).

Additionally, presently, the comparative analysis of different virulent *L. monocytogenes* strains using organoids has only been performed by Zhou and colleagues. However, the main objective of the authors was the study of protein changes in the host epithelium. Organoids were infected with two different strains – a "virulent strain" (serotype 1/2a) and a "low virulent strain" (serotype 4a) – and quantitative proteomic analysis of the infected mice organoids was performed (Zhou et al., 2022b). In general, it was shown that both strains were able to reduce the host's energy metabolism, stimulate the host's immune response and increase the expression of proteins related to adhesion and invasion, as expected. Although some differences were found between the two strains, while the virulent strain significantly activated the ferroptosis pathway – known as a cell death pathway – the attenuated strain exhibited a higher activation of the complement system, which has a crucial function in innate immune responses. Notably, both strains down-regulated nucleotide-binding oligomerization domain 2 (*NOD2*), a receptor in the NOD-like receptor signalling pathway, which is crucial for innate immune responses and can recognise pathogens through muramyl dipeptide (MDP) (Liu et al., 2023; Zhou et al., 2022b). This down regulation could potentially hamper the multiplication and protection of intestinal stem cells. So, it was expected that organoids infected with *L. monocytogenes* would show upregulation of *NOD2*. However, another study showed that germ-free mice expressed reduced levels of *NOD2*, which subsequently increased when gut commensals were added. This observation could potentially explain the reduced expression of *NOD2* in organoids as they lack gut-associated bacteria (Petnicki-Ocwieja et al., 2009). Overall, it was concluded that the immune activity and biological functions were identical in the two different strains, although some differential expression of different proteins within the pathway were observe (Zhou et al., 2022b). Considering this, the use of organoids for *L. monocytogenes* studies have been reported. However, to the best of our knowledge, no article on CC-associated virulence has been published. Thus, further research on the use of organoids as infection models for this pathogen is needed to explore their viability for comparative analysis of differentially virulent strains.

4.3 Molecular approaches to study virulence

The success of polymerase chain reaction (PCR) is largely due to its capacity to amplify minimal amounts of genetic material, to millions of copies in a very short time (Farrell, 2010; Mullis et al., 1986). Technical improvements have led to the quantification of gene expression through techniques such as quantitative reverse transcriptase PCR (qRT-PCR), which enables the detection and quantification of RNA products (Farrell, 2010; Zhu et al., 2020). Although qRT-PCR is considered the gold-standard for mRNA quantification, some drawbacks limit its use. Among these we highlight the additional reverse transcription (RT) step, which can introduce contamination and inhibitions, common issues in the quantification of the actual cDNA present in the sample. In addition, relative quantification requires well established reference genes that are not affected by experimental conditions, and inadequate optimization of primers and annealing temperatures can lead to non-specific sequence targeting. To address these limitations, other molecular approaches such as digital PCR (dPCR) have been developed, which allow absolute quantification without the need for reference genes and calibration curves and with a reduced probability of contamination, thus improving interlaboratory comparability (Baettig et al., 2023; Cao et al., 2020; Lei et al., 2021;

Witte et al., 2016). The use of dPCR for gene expression analysis increasing, although its application is primarily limited to the detection and quantification of *L. monocytogenes* cells and biofilms (Chen et al., 2017; Grudlewska-Buda et al., 2020; Ricchi et al., 2017; Witte et al., 2016). Gene expression profiling techniques have greatly improved our understanding of how pathogens' modulate gene transcription after interacting after interacting with the host environment, strategically recruiting their genome during the infection life cycle. Consequently, genes that exhibit differential expression during infections have captured the attention of researchers, providing insight into which virulence genes are essential for microbial pathogenicity (Shelburne and Musser, 2004).

To date, no studies using dPCR in virulence potential analysis among CCs have been reported. RT-qPCR technology is the only that has been used to study how *L. monocytogenes* strains from distinct CCs differentially express stress response genes in diverse contexts (da Silva et al., 2021; Guerreiro et al., 2022; Wambui et al., 2020; Wu et al., 2022). However, only a few research papers reporting the use of this technique to analyse differential gene expression of virulence genes associated with CCs have been published (Rupp et al., 2017; Wagner et al., 2022). Rupp et al. (2017), aimed to understand the hypervirulence of CC1, hypothesizing that it could be due to additional virulence features or genetic variation within previously studied virulence genes. They focused on three specific genes – alleles of *inlJ*1 and *inlF* and *lls* gene – two of which show some differences from lineage II strains, while the last is commonly found in lineage I CCs. Therefore, a strain from CC1 and EGDe strain (CC9) were used for intracellular (Caco-2 and BoMac cell lines) and extracellular (BHI broth) infection assays, where the gene expression of the CC1 strain was further analysed. These three virulence genes were expressed in the CC1 strain under both conditions, but the resulting PCR bands were less intense than the control genes (such as *actA* and *rrs* (16S)). However, no comparative analysis of gene expression between these two strains was explored. This study concluded that the CC1 strain was able to invade cells more effectively and exhibited an enhanced intracellular spread compared to EGDe. Nevertheless, the selected virulence genes were not found to correlate with these phenotypes, despite their strong association with the CC1 strains (Rupp et al., 2017).

Wagner and colleagues worked on the genotypic and phenotypic characterization of *L. monocytogenes* strains from the meat and salmon processing industry in Norway (Wagner et al., 2022). *In vitro* assays in Caco-2 cells and WGS analysis showed that CC14 strains lacked invasion capacity while carrying the full-length *inlA* gene. This interesting result led to a further analysis of the invasion and internalization capacity of CC14 strains using different cell lines (Caco-2, HEPG2 and THP1 cells) and the subsequent gene transcription analysis. EGDe was used as a reference. Under the conditions of Caco-2 cells infection, there were significant differences in gene expression between CC14 strains and EGDe – the expression of *inlA* and *inlB* genes was decreased in CC14 isolates, the other virulence genes (*actA*, *hly* and *prfA*) were not differentially expressed when compared to the EGDe strain. qRT-PCR was also used to study gene expression after the reconstruction of *inlA* in isolates from CC9 and CC121 that carried PMSCs mutations. Under Caco-2 cells growth conditions, the researchers observed no significant differences in the expression of *inlA*, *inlB* and *prfA* between the wildtype (WT) strains and their respective mutants. However, significant differences in the *inlA* expression were observed in both CC9 WT and one of its mutants compared to EGDe. The CC121 *inlA* reconstructed mutants showed no significant differences in gene expression when compared to EGDe, indicating successful gene reconstruction (Wagner et al., 2022). This study highlights the need for analysis of gene expression, since strains from CC14, although harbouring the full-length *inlA* gene, showed reduced expression of this virulence gene. We can conclude that the qRT-PCR technique is an essential tool to obtain further information about the virulence potential of *L. monocytogenes* strains.

5 Discussion

Characterization of the virulence potential within *L. monocytogenes* strains is essential for effective risk assessment and to reduce the human and economic losses associated with listeriosis. Multi-locus sequence typing, combined with epidemiological data, has facilitated the identification of more or less virulent CCs, helping researchers to refine their understanding of the infection patterns of this pathogen. However, despite advances in CC characterization, the specific virulence markers that confer distinct disease-causing capacities remain poorly understood.

This review highlights the limited use of different infection models to study the virulence potential of *L. monocytogenes* CC. While some models, such as *G. mellonella* larvae, have not been extensively studied, results suggest variability in results depending on the model used. Some studies successfully distinguish hyper-and hypovirulent CCs, while others show inconsistent results, suggesting a strain-dependent characterization of infection risk within CCs. The strain-dependent nature of *L. monocytogenes* virulence potential, together with highly variable host susceptibility and evidence that virulence may be influenced by the food matrix, limits the usefulness of subtypes. Grouping them into Clonal Complexes (CCs) may mask relevant differences in their pathogenicity. Additionally, we highlight the fact that a standard infection model for studying the virulence potential of *L. monocytogenes* CCs has not yet been established. This lack of standardization difficult comparisons between dies, as variations in experimental conditions, infection doses and techniques may lead to different virulence outcomes. Despite these challenges, the use of different approaches to validate previous findings is recommended, and improvements to existing models, mainly 3D systems, are ongoing.

The presence or absence of virulence genes alone is not sufficient to determine pathogenicity, highlighting the multifactorial nature of listeriosis. Consequently, the achievement of a zero risk infection remains elusive and requires a comprehensive understanding of the host immunity and pathogen virulence machinery. Despite ongoing efforts, a reliable virulence biomarker capable of differentiating attenuated strains has yet to be identified, underscoring the importance of continued research and refinement of infection models to advance our understanding and control of listeriosis. To conclude, we would like to highlight a recent study that used artificial intelligence to predict the virulence potential of *L. monocytogenes* at the subspecies level using WGS datasets. The use of the pan-genome showed the best predictive results, pointing up the possible value of pan-genome related genes in virulence. Although the authors acknowledge some of the limitations

of their models, such as the difficulty of generalizing data based on WGS from only three countries, which rises concerns about the application of these predictive machine learning models in other regions (Gmeiner et al., 2024), these findings show that innovative technologies are being explored for future risk assessment. Additionally, besides the limitations of the current use of subtype classification for characterizing *L. monocytogenes* virulence, their use cannot be ruled out as more is learned about risk variability. In the future, standardized risk assessment models may serve as valuable tools for the food industry in risk management upon *L. monocytogenes* contamination.

Author contributions

MS: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. RM: Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. VF: Conceptualization, Writing – review & editing, Visualization, Supervision. PT: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition.

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Conflict of interest

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

Publisher's note

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Characterization of *Listeria monocytogenes* strains isolated from soil under organic carrot farming

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Introduction: *Listeria monocytogenes* are Gram-positive, non-spore-producing rods that are the etiological agent of listeriosis. *L. monocytogenes* is isolated from soil, water, sewage, rotting vegetation, and the main source of these rods for humans is food (fish, unpasteurized dairy products, or raw fruits and vegetables). In recent years, there has been an increase in consumer interest in healthy food, especially organically grown. The use of natural fertilizers during organic farming can be a source of pathogens, including *L. monocytogenes* in the soil and finally in vegetables and fruits. The aim of this study was to assess the prevalence of *L. monocytogenes* in soil samples from organic carrot crops (Poland) and to characterize the tested strains. Microbial contamination of the soil has a direct impact on the safety of the crops grown on it. This is an important aspect in organic farming, where products are chosen as a healthier option and consumed by children and the elderly.

Methods: The isolates were subjected to genetic similarity assessment (PFGE method), and the tested strains were evaluated for antibiotic susceptibility (disc-diffusion method), invasiveness (HT-29 line human colon cancer cell line), coaggregation with *Salmonella* Enteritidis, biofilm-forming ability and the effect of disinfectants on the biofilm.

Results: Twenty-seven isolates of *L. monocytogenes* were isolated from 250 soil samples, 10 of which were genetically different. 80% of the tested strains were sensitive to the tested antibiotics. Antibiotic resistance was demonstrated in two strains (strain 11 – resistant to penicillin and cotrimoxazole, strain 22 – resistant to penicillin). The highest invasiveness against HT-29 cells at 23.2% was shown for strain 11. However, invasiveness of less than 1% was demonstrated for three strains, and strain 13 showed no ability to invade HT-29 human colon cancer cells. The level of coaggregation between the tested strains and *S.* Enteritidis ranged from 22.2 to 39.1%. The number of biofilm-isolated rods from the stainless steel surface was 6.37 to 7.10 log colony-forming unit (CFU)/cm^2, while on polypropylene it was from 6.75 to 8.06 log CFU/cm^2. The effectiveness of the disinfectants used depended on the duration of action and the concentration of the disinfectant. Chlorosol was shown to be the disinfectant causing stronger biofilm eradication on each of the tested surfaces. It has been shown that soils and thus food from organic farming can be a source of *L. monocytogenes*. These rods can vary in phenotypic characteristics and virulence levels.

Discussion: The research conducted allows to draw attention to the occurrence of pathogens, including *L. monocytogenes* in crops from organic farming. In addition,

the results presented can help to introduce standards regulating the safety of organic farming, taking into account the occurrence of antibiotic-resistant or highly invasive strains, thus maintaining food safety.

KEYWORDS

Listeria monocytogenes, organic farming, soil, biofilm, metabolic rate, invasiveness, antibiotic resistance

1 Introduction

Agriculture, is a fundamental source of food products, essential for the entire society. In recent years, there has been a noticeable increase in the promotion of organic farming, due to its numerous environmental benefits, as well as the benefits of future consumers making an informed choice of healthy food products (Scialabba and Hattam, 2002). The principle of organic farming is to refrain from using agricultural, veterinary and food chemicals in the food production process (Dumontet et al., 2017). What results is efficient production that combines practices that promote environmental protection and preserve access to limited natural resources (Dumontet et al., 2017).

Despite the many benefits of organic farming, it is important to remember the food safety risks of using natural fertilizers. Contamination of vegetables can occur during cultivation, harvesting or distribution (Mandrell, 2009). An important element of organic farming is soil. According to the Regulation of the European Parliament and of the Council (EU) 2018/848 (2018), soil fertility and biological activity are maintained and increased, mainly through the use of manure, or organic matter. In manure or slurry, under the right conditions, pathogenic bacteria can survive for several weeks or even months and then enter the soil, water or crops causing contamination (Alegbeleye et al., 2018). It has been shown that the greater the contact of plants with contaminated soil, the greater the degree of contamination, including microbial. Fertilizers have been shown to be reservoirs of enteric pathogens that can contaminate crops, fruits and vegetables (Jiang and Shepherd, 2009). A higher risk of contamination applies to vegetables and fruits in direct contact with the soil, such as root and leafy vegetables (e.g., carrots) (Islam et al., 2004). Another important source of microbial contamination of the soil environment is water. Agriculture mostly uses ground and surface water, into which biological agents can penetrate under certain conditions. The main risks are spills from reservoirs or manure storage sites, livestock and wildlife feces, or water runoff from contaminated fields (Alegbeleye et al., 2018). Numerous bacterial pathogens are well adapted to survive and multiply in both soil and water. These microorganisms usually form a biofilm that adheres to the plant surface, or migrate through the root system to other plant parts (Ryser et al., 2009; Yaron, 2014). The process of washing fruits and vegetables only removes surface contaminants, without removing the biofilm crust, or pathogens present in the inner tissues of the plants (Vogeleer et al., 2014). Numerous reports on organically grown vegetables (Heaton and Jones, 2008; Maffei et al., 2013; Tango et al., 2014), have shown that consuming food products from such farming does not increase the incidence of foodborne illness. Therefore, the issue of microbiological safety of organic food is constantly questioned (Maffei et al., 2016). However, it has been shown that raw vegetables, including those from organic farming, can be contaminated by pathogens such as, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli*, *Campylobacter* spp. (Golberg et al., 2011; Kljujev et al., 2018).

One of the more significant pathogen risks associated with organic farming is *L. monocytogenes*. *L. monocytogenes* are Gram-positive, non-spore-producing rods characterized by adaptation to variable and adverse environmental conditions (Wiktorczyk-Kapischke et al., 2023). The main source of *L. monocytogenes* for humans is food, mainly products from unpasteurized milk, meat and meat products, fish and fishery products, and raw fruits and vegetables (European Food Safety Authority and European Centre for Disease Prevention and Control, 2023). It should be emphasized that listeriosis has a high mortality rate, at around 30%. The most vulnerable to infection are the elderly, pregnant women, newborns, or immunocompromised people (World Health Organization, 2018). The source of contamination of vegetable products by *L. monocytogenes* can be soil, manure and water (Kljujev et al., 2018). Some studies have shown that *L. monocytogenes*, can survive on the surface of a damaged seed coat during plant germination. This implies the possibility of *L. monocytogenes* contamination of the entire plant (Gorski et al., 2004). A key aspect affecting the problems of eliminating *L. monocytogenes* from raw fruits and vegetables is the ability of these rods to form a biofilm (Oliveira et al., 2010). According to Botticella et al. (2013), biofilm formation allows *L. monocytogenes* to persist for long periods of time in the food processing environment, and therefore is a source of recurrent contamination and poses a food safety risk. Due to the increasing number of patients diagnosed with listeriosis and the identification of multidrug-resistant strains, it is advisable to assess antimicrobial resistance among *L. monocytogenes* isolates from food products (European Food Safety Authority and European Centre for Disease Prevention and Control, 2023). Thus, it is important to assess the occurrence of *L. monocytogenes* in soil from organic crops in order to assess food safety risks.

The aim of this study was to evaluate the occurrence of *L. monocytogenes* in soil samples from organic carrot cultivation (Poland) and to characterize selected features of the obtained strains determining their virulence. The above objective is particularly significant because microbial contamination of soil directly affects crop safety, especially in organic farming, where produce is often selected for its health benefits and consumed by vulnerable groups like children and the elderly.

2 Materials and methods

2.1 Material

2.1.1 Soil sampling and isolation of *Listeria monocytogenes*

The material for the study consisted of 250 soil samples taken from 5 fields (Poland) where carrots were grown organically. The

cultivation was carried out on light sandy loam soils classified as quality class IVa. The preceding crops were cucumbers or onions. Organic farming practices had been implemented on these fields for 5 years. The growing season lasted approximately 220 days. Soil samples were taken from sites in the immediate vicinity of the growing carrots. Fifty samples of 200 g per field were taken.

After delivery to the laboratory, samples were shaken in sterile buffered saline (PBS, Sigma-Aldrich) for 24 h (300 rpm, room temperature) and then sonicated for 15 min (Ultrasonic DU-4, Nickel-Electro Ltd., United Kingdom).

Then, 25 mL of the resulting suspension was transferred to 225 mL of half-Fraser broth (Oxoid) and subjected to pregrowth for *L. monocytogenes*. After 24 h incubation (30°C), 1 mL of the culture was transferred to 9 mL of Fraser broth (Oxoid) and incubated (37°C, 48 h). After the secondary multiplication step, reduction culture was performed on medium according to Ottaviani and Agosti (ALOA) (Oxoid) and incubated (24 h, 37°C). The grown colonies, typical of *L. monocytogenes*, were screened on Columbia Agar with 5% sheep blood (Biomerieux) and incubated (37°C, 24 h). The grown colonies were used for further studies.

2.2 Methods

2.2.1 Identification of *Listeria monocytogenes* strains using the PCR method

Species identification of the strains was carried out by PCR using previously isolated DNA. DNA isolation was performed using the Genomic Mini kit (A&A Biotechnology) according to the manufacturer's procedure. The primer pair L1 (5'-CAG CAG CCG CGG TAA TAC-3′) and L2 (5'-CTC CAT AAA GGT GAC CCT-3′) (product size: 938 bp) (Border et al., 1990), designed based on the 16S rRNA sequence allowed the evaluation of the affiliation of the tested strains to the genus *Listeria*, while the pair LM1 (5'-CCT AAG ACG CCA ATC GAA-3′) and LM2 (5′-AAG CAC TTG CAA CTG CTC-3′) (product size: 750 bp) (Bansal, 1996), designed based on the sequence of the gene encoding listeriolysin O (LLO) allowed the identification of isolates to the species *L. monocytogenes*.

The standardized PCR protocol for 25 µL reaction mixture included 1 × PCR buffer (Promega), 2 mM $MgCl_2$ (ABO), 1.25 mmol dNTPs (Promega), 0.5 µM of each primer (Oligo.pl), 1 unit of Taq DNA polymerase (Promega) and ultrapure water. DNA isolated from *L. monocytogenes* ATCC 19111 strain was the control. The PCR program was set as follows: initial denaturation 94°C/2 min; 30 cycles of denaturation 94°C/30 s, annealing 50°C/30 s and duration 72°C/1 min; extension 72°C/1 min.

2.2.2 Assessment of genetic similarity of isolates using PFGE technique

The genetic similarity of the isolates was assessed using pulsed-field gel electrophoresis (PFGE), a procedure carried out in accordance with PulseNet recommendations (PulseNet USA, 2013). Bacterial suspensions were prepared in TE buffer (10 mM Tris–HCl, 100 mM EDTA) (Novazyme) with a MacFarland density of 4.0. Then, lysozyme (10 mg/mL, EurX) and proteinase K (20 mg/mL, Thermo Fisher Scientific) were added to the suspension and incubated (55°C, 40 min). Blocs were prepared using 1.0% agarose (Certified Megabase) (Bio-Rad). After solidification, the blocks were incubated (54°C, 2 h) in lysis buffer (50 mM Tris–HCl, 50 mM EDTA, 1.0% lauryl sarcosyl (Sigma-Aldrich), 0.15 mg/mL proteinase K). Then, the blocks were washed twice in ultrapure water and four times in TE buffer. Pre-restriction was carried out at 30°C for 10 min. DNA restriction (30°C, 7 h) was carried out in buffer consisting of: *ApaI* enzyme (10 U/µl) (Thermo Fisher Scientific), Tango buffer (Thermo Fisher Scientific), water. Electrophoretic separation was carried out in a 1.0% agarose gel (Certified Megabase) (Bio-Rad) in a CHEF Mapper apparatus (Bio-Rad) using the following electrophoresis conditions: initial and final pulse duration: 4–40 s, voltage: 6 V/cm, pulse angle: 120°, temperature: 14°C. Electrophoresis was carried out for 17 h. Image visualization was performed using the GelDoc XR system (Bio-Rad).

To determine the degree of genetic similarity between the strains studied, a phylogenetic dendrogram was plotted using CLIQS 1D Pro software (TotalLab). Clustering analysis was performed using hierarchical clustering using the Unweighted Pair Group Method of Aritmetic Means (UPGMA) technique with Dice coefficient.

2.2.3 Assessment of antibiotic susceptibility

The susceptibility of *L. monocytogenes* strains to antibiotics was assessed using the disc-diffusion method on Mueller-Hinton Agar with 5.0% horse blood and 20 mg/L β-NAD (MHF) (bioMérieux).

The tested strains were cultured from freezing onto CAB medium. After 20 h of incubation, a suspension of bacteria in 0.9% NaCl (Avantor) at a density of 0.5 McFarland was prepared. Inoculum was spread on MHF medium, then antibiotic discs [penicillin (1 U), ampicillin (2 µg), meropenem (10 µg), erythromycin (15 µg), trimethoprim-sulfamethoxazole (1.25–23.75 µg)] (Argenta) were applied. The performed antibiograms were incubated at 35°C for 20 h. After the incubation period, zones of growth inhibition around the discs were measured and the results were interpreted according to the recommendations of EUCAST v. 12.0 (European Committee on Antimicrobial Susceptibility Testing, n.d).

2.2.4 Evaluation of the invasiveness of *Listeria monocytogenes*—plaque forming assay test

Single colonies were transferred to 5 mL of Brain Heart Infusion (BHI) broth and incubated in a thermoblock (TDB-100, Biosan) at 37°C (230 rpm, 6 h). In the next step, 5 µL of the bacterial suspension was transferred to 5 mL of BHI broth and incubated 18 h until an OD_{600} of 2.4–2.6 was obtained (measured with a DU 8800D spectrophotometer). Multiplied bacteria, at 5–6 log CFU, were used to infect HT-29 human colon cancer cell line.

HT-29 cells were seeded into 6-well polystyrene culture plates (Genoplast) and incubated until approximately 90% confluence in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich), formulated with: 10% FBS (fetal bovine serum) (Gibco), 2 mM glutamine, and 100 IU/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich). Then, 24 h before the infection was performed, the medium was changed to DMEM containing no antibiotics. A suspension of bacteria in BHI at 5–6 log CFU was added to HT-29 cell cultures and incubated for 2 h (37°C, 5% CO_2). The wells were then washed twice with sterile PBS solution (Sigma-Aldrich), the medium was changed to DMEM containing 100 µg/mL gentamicin (Sigma-Aldrich) and incubated for 1.5 h (37°C, 5% CO_2). The gentamicin medium was removed, and medium containing 10 µg/mL gentamicin and 1.0% low-melting point agarose (Prona, Gdansk) was added to the wells. After 48 h, the number of plaques was determined. Bacterial invasiveness was expressed as a quotient of the number of plaques, expressing the number of bacteria that penetrated into HT-29 cells, and the number of bacteria entering the wells. Invasiveness was expressed as a percentage.

2.2.5 Evaluation of the metabolic rate of *Listeria monocytogenes* strains

From the grown colonies of each *L. monocytogenes* strain, a 0.5 McF suspension was prepared in sterile PBS solution (Sigma-Aldrich). The suspensions were then diluted 100-fold in tryptose-soy broth (TSB) (Becton Dickinson).

A set of two plates was prepared: (1) containing tetrazolium salt (MTT) (Sigma-Aldrich); (2) without it. Into the wells of the multiwell plates (set with MTT), 80 μL of TSB was introduced, followed by the addition of 20 μL of MTT solution (5 ng/mL) and 100 μL of suspension (3 replicates). In the set-up without MTT, plates contained 100 μL of TSB and 100 μL of suspension of each strain (3 replicates). The negative control was 200 μL of sterile TSB (3 replicates).

The plates were incubated at 37°C in a humid chamber. After a predetermined incubation time (0, 1, 2, 3, 4, 5, 7, and 24 h), absorbance was measured using a Synergy HT (BIO-TEK) multi-detector microplate reader at 570 nm. After the designated incubation time, a solution of acidic isopropanol (isopropanol (Avantor) + 5% (v/v) 1 M HCl (Avantor)) with a volume of 200 μL and a concentration of 0.04 mol/dm3 was added to each well of the kit with MTT to dissolve the precipitated formazone.

Absorbance of samples without the addition of MTT (to determine the multiplication of bacteria in suspension in TSB) was performed at 595 nm. No acidic isopanol solution was added to the plates and the same plates were further incubated after the measurement.

Immediately before the absorbance measurement, the plates were shaken for 15 min (300 rpm, room temperature). To determine the metabolic activity coefficient (MAC), the metabolic activity values (MAV) of a given strain at a specific measurement date were calculated according to the formula:

$$MAV = AMTT_t - Az_t.$$

where:

MAV, metabolic activity of a given strain from the test or control group after a specified incubation time t

A_{MTTt}, absorbance value measured after a specified incubation time t of a given strain from the test or control group in a sample containing a bacterial suspension with MTT

A_{zt}, absorbance value measured after a specified incubation time t of a given strain of the test and control group in a sample containing a bacterial suspension without MTT

Based on the metabolic activity, the metabolic activity coefficient of the strain was calculated, according to the formula:

$$MAF = \frac{\sum AM_t}{n}$$

where:

MAF, metabolic activity coefficient

$\sum$AMt, sum of metabolic activity values from individual times (after 0, 1, 2, 3, 4, 5, 7 and 24 h of incubation) of a given strain from the control or test group

n, number of absorbance measurements made – 8

The value of the metabolic rate allowed us to compare the metabolic rate of the different *L. monocytogenes* strains tested.

2.2.6 Assessment of coaggregation ability between *Listeria monocytogenes* and *Salmonella* Enteritidis

In this stage of the study, in addition to the obtained strains of *L. monocytogenes*, a strain of *S.* Enteritidis isolated from poultry meat was used. Based on 24 h cultures of *L. monocytogenes* and *S.* Enteritidis strains on CAB medium, suspensions of the tested isolates were made at an optical density of 0.7 McF in reaction buffer (1.0 mM Tris–HCl (Sigma Aldrich), 0.1 mM $MgCl_2$ (Avantor), 0.1 mM $CaCl_2$ (Avantor), 0.15 M NaCl (Avantor)) (Kinder and Holt, 1994). A suspension of *L. monocytogenes* test isolates (1 mL) was combined with a suspension of *S.* Enteritidis (1 mL) and incubated at 37°C for 2 h. At the same time, an autoaggregation test was performed, in which 1 mL of *L. monocytogenes* suspension and 1 mL of *S.* Enteritidis suspension in reaction buffer were incubated (2 h, 37°C) in separate tubes. After the incubation period, the suspensions were subjected to centrifugation (7 × g, 2 min.), and the supernatant (0.6 mL) was collected and analyzed spectrophotometrically (Beckman DU-640 spectrophotometer) at 650 nm (Kinder and Holt, 1994). The assessment of coaggregation levels for species X (*L. monocytogenes*) and Y (*S.* Enteritidis) was calculated according to the formula:

$$\%\text{coaggregation} = \left[\left(\left[\{A650X + A650Y\} / 2\right] - A650\left(X + Y\right)\right) / \{A650X + A650Y\} / 2\right] \text{x } 100$$

where:

% coaggregation, the level of coaggregation between strains

A_{650}, absorbance value at 650 nm

X, autoaggregation level of strain X (*L. monocytogenes*)

Y, autoaggregation level of strain Y (*S.* Enteritidis)

X + Y, coaggregation level of strain X (*L. monocytogenes*) and Y (*S.* Enteritidis) (Kinder and Holt, 1994)

2.2.7 Assessment of biofilm formation

The ability to form biofilm on sterile polypropylene and AISI 304 stainless steel fragments (1cm² area) (washed and sterilized) was evaluated. Test strains were cultured on CAB medium and, after incubation (24 h, 37°C), 3 mL suspensions of test strains at a density of 0.5 McF in BHI medium were prepared. Sterile polypropylene or stainless steel fragments were placed in the suspensions and incubated (24 h, 37°C). After this time, the stainless steel or polypropylene fragments were rinsed in sterile PBS solution and transferred to sterile BHI medium. The procedure was repeated 2 times. Then, the surfaces were rinsed twice in PBS solution and sonicated in an Ultrasonic DU-4 sonicator (Nickel-Electro) for 20 min. After sonication, a series of decimal dilutions were made in PBS solution to a dilution of 10^{-6} and cultures at 0.1 mL per CAB medium. The plates were incubated for 24 h at 37°C. After incubation, the grown colonies were counted, and the result was reported as the number of colony-forming units (CFU) per cm² of test area (CFU/cm²).

2.2.8 Effectiveness of disinfectants on biofilm formed *Listeria monocytogenes*

The biofilm produced on stainless steel and polypropylene was used to evaluate the effectiveness of two disinfectants:

1 Alusol (an aqueous solution of phosphoric acid, hydrochloric acid and non-ionic surfactants) (Radex)
2 Chlorosol (an aqueous solution of sodium hypochlorite and stabilizing substances) (Radex)

Working solutions were prepared on the basis of sterile hard water (Polish Standard PNEN-1276, 2010) at concentrations of: 0.1, 0.5 and 1.0%. Test surfaces with biofilm formation were immersed in the respective concentrations of disinfectant solutions for 1, 5 and 15 min. After this time, the samples were shaken for 2 min (400 rpm) in a neutralizer (tween 80 (Sigma-Aldrich) - 10.0 g; lecithin (Sigma-Aldrich) - 1.0 g; histidine L (Sigma-Aldrich) - 0.5 g; $Na_2S_2O_3$ (Avantor) - 2.5 g; water - 1000 mL) and sonicated in a sonicator for 10 min. After sonication, a series of decimal dilutions were made (up to 10^{-6} in PBS) and cultured at 0.1 mL per CAB medium. Plates were incubated for 24 h at 37°C. After incubation, the grown microbial colonies were counted and reported as CFU/cm^2 of the area tested.

The negative control consisted of stainless steel and polypropylene fragments incubated in sterile BHI solution under the same conditions.

2.2.9 LIVE/DEAD fluorescence staining

For the tested *L. monocytogenes* strains, stained slides were prepared using the LIVE/DEAD Bac Light Bacterial Viability Kit (Thermo Fisher Scientific). In these preparations, the proportion of live and dead *L. monocytogenes* cells in a single layer of biofilm formed on the surface of stainless steel and polypropylene, treated and untreated with disinfectants, was determined under a fluorescence microscope (Nikon Eclipse Ci, magnification 1,000x).

Samples were stained with the LIVE/DEAD Bac Light Bacterial Viability Kit according to the manufacturer's instructions and incubated for 15 min at 37°C without light.

2.2.10 Statistical analysis

The results for biofilm formation on the tested surfaces and the effectiveness of the disinfectants used were converted to logarithms (log CFU). When evaluating the effectiveness of disinfectants, logarithmic decreases in the number of bacteria were calculated.

Means were calculated for the results obtained. Based on Statistica (TIBCO Software Inc., Palo Alto, CA, USA) software checked for the occurrence of significant differences in the strength of biofilm formation depending on the strain of *L. monocytogenes* and the tested surface. The existence of statistically significant differences between decreases in the number of bacteria recovered from the biofilm under disinfection depending on the disinfectant used, its concentration and duration of action, and the type of surface was also checked. A multivariate analysis of variance ANOVA was performed in both cases and Tukey's post-hoc test was used with a significance level of 0.05.

3 Results

3.1 Prevalence of *Listeria monocytogenes* in collected soil samples

The percentage of positive soil samples was 10.8% (Table 1). Twenty-seven isolates of *L. monocytogenes* were isolated (Table 1). Isolates of the tested rods were not obtained from samples from P2 and P5 fields. By multiplex PCR, it was confirmed that all isolates used in the study belonged to the *L. monocytogenes* species.

3.2 Evaluation of genetic similarity of tested isolates

The isolates of *L. monocytogenes* were classified into two main monophyletic branches (Figure 1). Branch I included 3 isolates from field P1 and P3, and branch II included 24 isolates from all fields with positive samples. Five groups comprising genetically identical isolates were shown. The first group included isolates: 1 and 2 from field P1, the second group included isolates 3–10 from field P1, the third group included isolates 11–12 from field P3, the fourth group included isolates 13–16 from field P3 and the fifth group included isolates 22–27 from field P4. The largest number of isolates ($n = 11$), and among them the largest number of genetically different strains ($n = 5$) were found in samples originating from field P4.

From the pool of 27 isolates, 10 genetically different strains of *L. monocytogenes* were isolated and used in further studies.

3.3 Assessment of antibiotic susceptibility of tested strains of *Listeria monocytogenes*

The susceptibility of the tested strains to five antibiotics (penicillin, ampicillin, meropenem, erythromycin, trimethoprim-sulfamethoxazole) was evaluated. It was found that 8 (80.0%) strains were susceptible to all antibiotics tested. One strain (10.0%) was resistant to penicillin and cotrimoxazole, and one (10.0%) strain was resistant to penicillin, with preserved susceptibility to other antibiotics.

TABLE 1 Number of *L. monocytogenes* isolates obtained from the samples tested.

Voivodeship	Field	Number of samples	Number of isolates (percentage of positive samples)	Isolate number
Greater Poland (Poland)	P1	50	10 (20.0%)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
	P2	50	0 (0.0%)	---
Kuyavia-Pomerania (Poland)	P3	50	11 (22.0%)	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21
	P4	50	6 (12.0%)	22, 23, 24, 25, 26, 27
	P5	50	0 (0.0%)	---
Total		250	27 (10.8%)	---

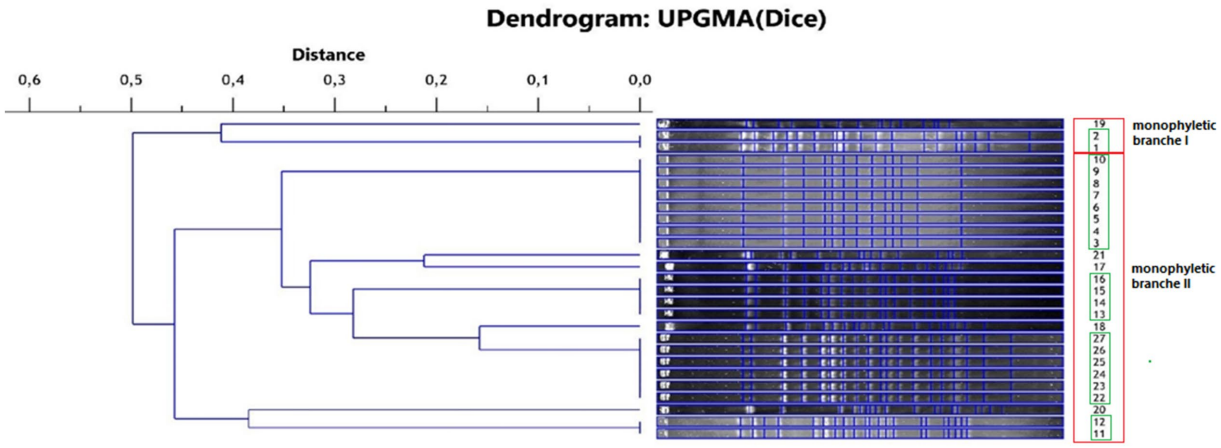

FIGURE 1
Phylogenetic dendrogram of the genetic similarity of the tested *L. monocytogenes* isolates (red frame—monophyletic branch, green frame—isolates representing the same strain).

3.4 Evaluation of the invasiveness of the tested strains of *Listeria monocytogenes*

The ability of *L. monocytogenes* to adhere and invade is directly related to the virulence level of this pathogen (Wałecka-Zacharska et al., 2013). Invasive form of listeriosis (especially among high-risk groups) is associated with a high mortality rate (World Health Organization, 2018). It was shown that the highest (23.22%) invasiveness was characterized by strain 11 and the lowest (0.38%) by strain 3 (Table 2). Strain 13 was not found to have the ability to invade HT-29 human colon cancer cells. Low invasiveness of less than 1% was characterized by three strains (3, 17 and 21), and high invasiveness of 17.77% was also shown for strain 22 (Table 2).

TABLE 2 Invasiveness of the tested strains of *L. monocytogenes*.

Strain number	Invasiveness [%]
1	3.09 ± 1.14
3	0.38 ± 0.12
11	23.22 ± 8.83
13	n.f.*
17	0.58 ± 0.10
18	3.11 ± 1.07
19	1.66 ± 0.31
20	1.46 ± 0.55
21	0.89 ± 0.06
22	17.77 ± 3.38

n.f.*, not found.

3.5 Evaluation of the metabolic rate of *Listeria monocytogenes* strains

The metabolic activity of the tested strains was examined using MTT. For the tested strains of *L. monocytogenes*, changes in metabolic activity were observed as the set time increased (Table 3). There was an increase in the absorbance of strains with and without MTT after the specified incubation time. The coefficient of metabolic activity in the test group (MTT) ranged from 0.748 for strain 17 to 1.117 for strain 19 (Table 3).

3.6 Evaluation of the ability to coaggregate between *Listeria monocytogenes* and *Salmonella* Enteritidis

Coaggregation is the process of reversible accumulation of bacterial cells of two different bacterial strains. Coaggregation plays an important role during surface colonization (including in processing plants or vegetable surfaces) and biofilm formation (Kinder and Holt, 1994). It was shown that the level of coaggregation between tested *L. monocytogenes* strains and *S.* Enteritidis ranged from 22.2 to 39.1% (Table 4). The highest (39.1%) level of coaggregation was found between *S.* Enteritidis and strain 21 (Table 4). In contrast, the lowest degree of coaggregation, at 22.2%, was shown between *S.* Enteritidis and strain no. 17 (Table 4).

3.7 Evaluation of biofilm formation on tested surfaces

One of the main causes of food contamination by *L. monocytogenes* is its ability to form a biofilm and survive in adverse environmental conditions. The multi-layered structure of the biofilm makes it difficult for biocides to reach the deeper layers of the biofilm. In this study, we evaluated the biofilm-forming ability of *L. monocytogenes* on the surface of stainless steel and polypropylene (Doijad et al., 2015). The number of *L. monocytogenes* rods reisolated from the biofilm formed on the steel surface ranged from 6.37 to 7.10 log CFU/cm^2 and for most strains was higher than on the polypropylene. The differences found were not statistically significant (Table 5). Strains 17, 19, and 20 formed biofilm more strongly on the surface of polypropylene than on

TABLE 3 The metabolic rate of the tested *L. monocytogenes* strains and the activity coefficient.

	MTT (absorbance 570 nm)										
	Strain number										
Time [h]	1	3	11	13	17	18	19	20	21	22	K(−)
0	0.200	0.213	0.199	0.192	0.163	0.201	0.225	0.194	0.173	0.186	0.161
1	0.461	0.529	0.493	0.479	0.516	0.399	0.614	0.357	0.617	0.351	0.184
2	0.801	0.826	0.801	0.907	0.651	0.701	0.896	0.768	0.749	0.715	0.176
3	1.156	1.185	1.123	1.052	0.867	1.006	1.201	1.191	0.967	1.154	0.169
4	1.199	1.294	1.369	1.170	1.115	1.131	1.400	1.412	1.238	1.312	0.203
5	1.492	1.732	1.701	1.599	1.434	1.502	1.867	1.526	1.591	1.720	0.129
7	2.137	2.287	2.215	2.001	1.701	1.806	2.315	1.973	1.909	1.962	0.191
24	2.788	2.951	2.873	2.618	2.104	2.241	3.019	2.259	2.296	2.206	0.188

	Without MTT (absorbance 595 nm)										
	Strain number										
Time [h]	1	3	11	13	17	18	19	20	21	22	K(−)
0	0.095	0.100	0.092	0.090	0.080	0.087	0.099	0.087	0.076	0.091	0.06
1	0.106	0.132	0.120	0.099	0.116	0.110	0.123	0.102	0.146	0.115	0.077
2	0.119	0.135	0.128	0.120	0.101	0.132	0.152	0.182	0.198	0.129	0.074
3	0.139	0.176	0.139	0.147	0.126	0.157	0.175	0.199	0.214	0.164	0.07
4	0.183	0.192	0.176	0.150	0.167	0.192	0.201	0.203	0.227	0.183	0.086
5	0.216	0.226	0.227	0.169	0.160	0.195	0.234	0.221	0.264	0.247	0.091
7	0.385	0.397	0.352	0.351	0.279	0.325	0.437	0.404	0.401	0.301	0.101
24	0.671	0.732	0.687	0.630	0.459	0.519	0.766	0.552	0.559	0.482	0.114

	Activity factor										
Strain number	1	3	11	13	17	18	19	20	21	22	
	0.900	1.070	1.058	0.992	0.748	0.934	1.117	0.894	0.949	0.840	

MTT, tetrazolium salt.

TABLE 4 Coaggregation coefficient calculated for the tested strains of *L. monocytogenes*.

Strain number	Mix	Individually	Coaggregation [%]
1	0.031	0.039	35.4
3	0.039	0.051	27.8
11	0.038	0.044	24.8
13	0.036	0.056	36.3
17	0.042	0.051	22.2
18	0.036	0.052	33.9
19	0.038	0.052	30.3
20	0.031	0.036	33.3
21	0.039	0.071	39.1
22	0.038	0.056	32.7

steel, with the differences not statistically significant (Table 5). The recovery of bacteria from the biofilm on the polypropylene surface ranged from 6.75 to 8.06 log CFU/cm^2 (Table 5).

3.8 Evaluation of the efficacy of tested disinfectants against *Listeria monocytogenes* biofilm

Listeria biofilms contribute to secondary food contamination, posing a threat to public health (Singh et al., 2017). The biofilm structure protects the deeper layers of bacterial cells from disinfectants so it is important to evaluate the effectiveness of available agents. It was shown that both disinfectants tested, regardless of concentration and duration of action, caused a decrease in the number of reisolated bacteria for all strains of *L. monocytogenes* included in the study. The antibiofilm effect depended on the type of agent, its concentration, duration of action and the type of biofilm-covered surface (Figure 2).

3.9 Comparison of the effectiveness of disinfectants

Chlorosol was shown to be the disinfectant causing stronger biofilm eradication on each of the tested surfaces. The decreases in the number of bacteria recovered from the biofilm on steel after its use

TABLE 5 Intensity of biofilm formation by *L. monocytogenes* strains.

Strain	Stainless steel AISI 304 [log CFU/cm^2]	Polypropylene [log CFU/cm^2]	*p*-value
1	6.90 (±0.214)^{a,b}	7.42 (±0.262)^{a,b}	0.99
3	6.92 (±0.503)^{a,b}	6.99 (±0.587)^{a,b}	1.00
11	7.01 (±0.708)^{a,b}	7.39 (±0.322)^{a,b}	0.98
13	6.37 (±0.111)^{a}	7.00 (±0.178)^{a,b}	0.95
17	6.85 (±0.831)^{a,b}	6.74 (±0.588)^{a,b}	1.00
18	6.81 (±0.339)^{a,b}	7.22 (±0.129)^{a,b}	0.97
19	7.10 (±0.081)^{a,b}	7.02 (±0.252)^{a,b}	1.00
20	7.05 (±0.297)^{a,b}	7.04 (±0.693)^{a,b}	1.00
21	7.00 (±0.189)^{a,b}	7.14 (±0.755)^{a,b}	0.99
22	7.08 (±0.521)^{a,b}	7.81 (±0.371)^{b}	0.89

^{a,b,c}Values marked with different letters differ statistically significant.

ranged from 3.38 to 6.93 log CFU/cm^2, and on polypropylene from 3.08 to 7.25 log CFU/cm^2, and in many cases were equivalent to reducing the number of cells in the biofilm to below the method's detection threshold (Figure 2). For Alusol, the recorded decreases on steel ranged from 0.10 to 4.11 log CFU/cm^2, and on polypropylene from 0.38 to 2.43 log CFU/cm^2 (Figure 2). At a given concentration, duration of action and on the same surface, the decreases in bacterial counts obtained under Chlorosol were statistically significantly higher than those found after application of Alusol (Figure 2).

It was shown that the use of disinfectants at a higher concentration for a time of 1 min produced an effect comparable to their use at a lower concentration with a contact time of 15 min. The exception was the effect of 0.5% Chlorosol on the biofilm formed on polypropylene (Figure 2).

3.10 Visual evaluation of the antibiofilm efficacy of the disinfectants used using fluorescence microscopy

Microscopic images obtained after using the LIVE/DEAD kit confirmed the results of microbiological analyses regarding the effectiveness of the tested disinfectants. It was shown that increasing the concentration of the test agent and extending the duration of its action resulted in an increase in the proportion of dead *L. monocytogenes* cells stained red in the preparation. An example visualization of the effectiveness of Chlorosol against *L. monocytogenes* biofilm on steel is shown in Figure 3.

4 Discussion

The intensive development of organic farming generates more and more profits, which gives small and local farmers a chance to develop (Seufert et al., 2017). Among the many legal regulations and principles on which the system of organic farming is based, an important issue is the proper fertilization of crops, in which the use of artificial plant protection products is prohibited, and only fertilizers of natural origin are used. This raises the question of the safety of such fertilizers and the subsequent harvested crops, especially in the context of microbiological risks. The presence of pathogens in the soil and, consequently, in yields from organic crops poses a threat to public health security. Thus, it seems a necessity to evaluate soils and characterize pathogens isolated from the soil. Most food products from organic farming are vegetables and fruits that do not need to be subjected to prior heat treatment. This involves the risk of transmission of many pathogens, including *L. monocytogenes* (Kljujev et al., 2018). In addition, it has been shown (Weller et al., 2016; Harrand et al., 2020) that *L. monocytogenes* can survive at detectable levels in the soil for 128 days after manure application and can potentially contaminate products through soil cultivation or contaminated water. Our own study assessed soil samples collected in southwestern Poland from organic carrot farming. In addition, we evaluated the isolated strains for selected characteristics that affect their virulence. The presence of *L. monocytogenes* rods was found in 27 (10.8%) of the collected soil samples. Genetic similarity analysis (PFGE, "gold standard") showed 10 genetically different strains that were characterized. Commission Regulation (EU) 2024/2895 of November 20, 2024 on food safety for *L. monocytogenes* sets standards for the pathogen. As of July 2026, a new food safety criterion is to be in effect that *L. monocytogenes* cannot be detected in a 25 g product, including in food marketed during its shelf life (Commission Regulation (EU) 2024/2895, 2024). Our results indicate the presence of *L. monocytogenes* in soil samples, which could result in contamination of carrots and thus pose a risk to future consumers. A microbiological analysis of lettuce, radishes, carrots and beets in Poland was carried out by Szczech et al. (2018), who showed that the number of mesophilic bacteria, yeasts and molds was comparable in the case of radishes and carrots, both in the organic farming system and conventional ones (total number of mesophilic bacteria at the level of 7.0 log10 CFU × g^{-1} and 6.6 log10 CFU × g^{-1}, for radishes and carrots, respectively). In turn, Szymczak et al. (2014) showed that 5.0% of organic parsnips tested were contaminated with *L. monocytogenes*. Kljujev et al. (2018) showed that 25.58% of vegetable samples from conventional farming were contaminated with *Listeria* bacteria, while only one of 43 tested samples taken from the field and greenhouse was positive for *L. monocytogenes* (carrot). The researchers also showed that the highest number of *L. monocytogenes* cells was located in the inner layers of the carrot root (10^5 cells/mm^3 of dry root) (Kljujev et al., 2018). Our results, as well as data from other researchers, indicate that *L. monocytogenes* is present in both soil and vegetables, which poses a risk to future consumers. The high level of microbial contamination of vegetables indicates their potential spoilage risk and poor quality. According to the literature, leafy vegetables are considered those with the highest potential for microbial risk (Berger et al., 2010). To reduce the risk of foodborne illnesses, it is important to follow proper food safety practices when growing, processing and preparing food.

Due to the systematically increasing number of patients diagnosed with listeriosis and the identification of multidrug-resistant strains, it is advisable to assess antimicrobial resistance among *L. monocytogenes* isolates from food products (European Food Safety Authority and European Centre for Disease Prevention and Control, 2023). In this study, the antibiotic sensitivity of *L. monocytogenes* strains was also assessed. It was shown that 8 (80.0%) strains were sensitive to all tested antibiotics. Our study showed that strain 11 was resistant to penicillin and co-trimoxazole, and strain 22 was resistant to penicillin. Penicillin resistance among 66.7% of tested strains isolated from samples of milk (and milk products), meat (beef and poultry), and fish was

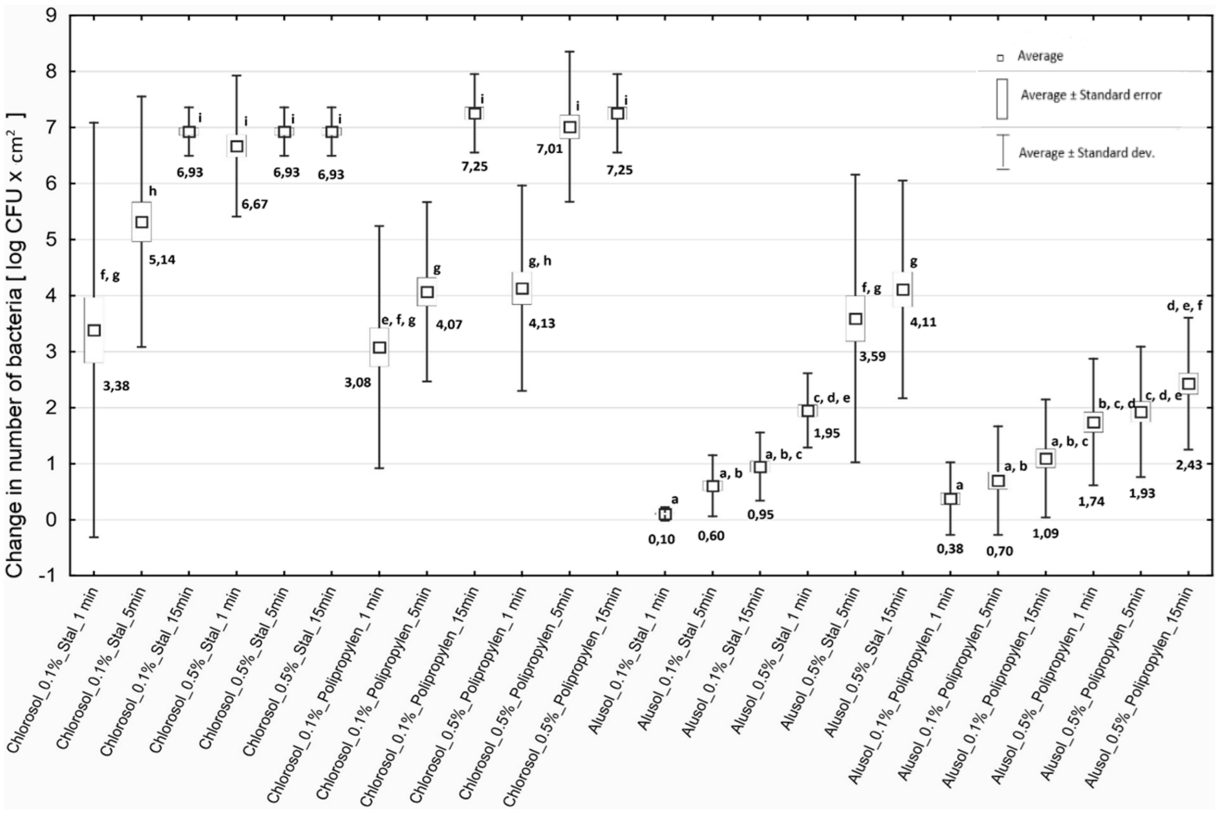

FIGURE 2
Decreases in bacterial counts depending on the experimental variant used. Steel - stainless steel; Polypropylene—polypropylene.

demonstrated by Khan et al. (2014). Also, Jamali et al. (2015) observed resistance to penicillin among 83.7% of strains, and to co-trimoxazole in 88.4% of isolates *L. monocytogenes* from food. These data indicate the need to assess antibiotic susceptibility, especially among *L. monocytogenes* strains isolated from food.

Another aspect addressed in the study was the level of invasiveness of *L. monocytogenes* strains toward HT-29 human colon cancer cells. It was shown that the invasiveness ranged from 0.38% (strain 3) to 23.22% (strain 22). Strain 13 did not have the ability to invade cells of the HT-29 line. In turn, Kuda et al. (2015) showed that the level of adhesion of *L. monocytogenes* to intact cells of the HT-29-Luc line was at the level of 6.7 and 6.1 log CFU/well. Jaradat and Bhunia (2003) showed that all strains had the ability to attach to Caco-2 cells, and the level of invasiveness ranged from 1.8 to 31.4%. Similarly, Van Langendonck et al. (1998) observed that all clinical isolates of *L. monocytogenes* were able to invade Caco-2 cells, although the invasiveness level ranged from 0.6 to 23.0%. In turn, Wiktorczyk-Kapischke et al. (2022) showed that the invasiveness of persistent *L. monocytogenes* strains ranged from 1.07 to 11.21%. Both our results and those of other researchers (Kuda et al., 2015; Wiktorczyk-Kapischke et al., 2022) show that *L. monocytogenes* strains are characterized by varied invasiveness. The ability of *L. monocytogenes* to adhere, invade, and grow in intestinal cells is directly related to the virulence of the pathogen (Wałecka-Zacharska et al., 2013), therefore the assessment of this parameter is important for the characterization of these bacteria.

In this study, the metabolic rate of the tested *L. monocytogenes* strains was also assessed based on MTT reduction. Changes in the metabolic activity of the strains were demonstrated, which was correlated with the duration of the experiment. The metabolic activity factor for the tested strains ranged from 0.748 to 1.117. The MTT test is a popular and frequently used tool in estimating the metabolic activity of living cells (Grela et al., 2018). Slama et al. (2012) showed the highest levels of metabolic activity (MTT reduction) were observed for cells subjected to cold stress over a period of 2 years (Slama et al., 2012).

The level of coaggregation between the tested *L. monocytogenes* strains and *S.* Enteritidis rods ranged from 22.2 to 39.1%. In turn, Skowron et al. (2019) showed that the coaggregation level between *L. monocytogenes* and *S.* Enteritidis ranged from 16.5 to 36.3%. Janković et al. (2012) assessed the ability to coaggregate three potential probiotic strains (*Lactobacillus plantarum*) with three pathogens, i.e., *Salomenella* Typhimurium, *L. monocytogenes* strain EGD and *E. coli* (EHEC). The authors of the study showed that all lactobacilli coagulated with the selected food-borne pathogens tested. The level of coaggregation between *L. plantarum* and *L. monocytogenes* ranged from 6.5 to 39.7%. In our study, the lowest coaggregation value between *L. monocytogenes* and *S.* Enteritidis was 22.2%. Gómez et al. (2016) showed that the highest level of coaggregation between *L. monocytogenes* and *Lactobacillus curvatus* was 69.0%, while the lowest level of coaggregation (53.4%) was recorded between *L. monocytogenes* and *Weissella viridescens*. The use of the coaggregation test is a reliable method for assessing the close Gram

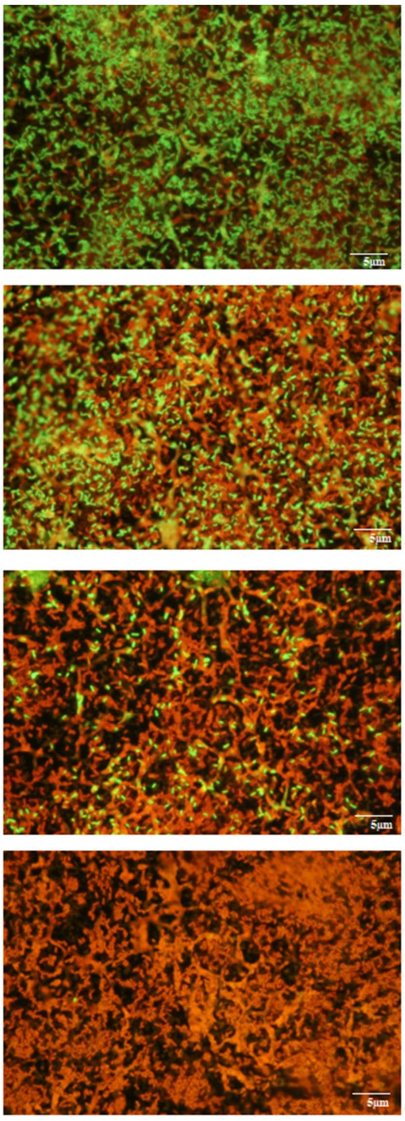

FIGURE 3
Proportion of living and dead cells in the biofilm layer (CH, chlorosol).

et al., 2007interaction between lactic acid rods and pathogenic bacteria, especially those responsible for poisoning and infections in the gastrointestinal tract (Collado et al., 2007; Soleimani, 2010).

The ability to form a biofilm determines the presence of *L. monocytogenes* on various surfaces, including vegetables. The biofilm structure is more difficult to remove and thus may not be removed during the vegetable and fruit washing process, posing a risk to public health protection (Botticella et al., 2013). An important aspect of the research was the assessment of the ability of *L. monocytogenes* strains to form a biofilm on the surface of stainless steel and polypropylene. It was shown that most *L. monocytogenes* strains formed a more durable biofilm on the surface of polypropylene than on stainless steel. In turn, Reis-Teixeira et al. (2017) showed that the number of cells adhering to the surface of stainless steel and glass after 3 h of incubation was at the level of 10^5–10^6 CFU/cm^2 and 10^6–10^8 CFU/cm^2 after 24 h. No further an increase in the number of cells in the biofilm structure, despite extending the incubation to 192 h (Reis-Teixeira et al., 2017). However, de Oliveira et al. (2010) showed that *L. monocytogenes* adheres to stainless steel coupons, and the number of cells in the biofilm structure is 4.89 log CFU/cm^2 after 3 h of incubation. Many studies confirmed the ability *L. monocytogenes* do rapidly adhere to stainless steel surface (Briandet et al., 1999; Ronner and Wong, 1993; Poimenidou et al., 2016). However, these rods do not tend to form thick biofilms composed of several layers (9 to 12 log CFU x cm^{-2}), but rather adhere to surfaces at levels of 4–6 log CFU x cm^{-2} (Gram et al., 2007). However, Poimenidou et al. (2016) found that the type of surface significantly influenced the formation of biofilm by *L. monocytogenes*. Poimenidou et al. (2016) showed that the average cell population in the biofilm structure on polystyrene (5.6 log CFU × cm^{-2}) was higher than on stainless steel (4.7 log CFU × cm^{-2}), which is consistent with our results.

Currently, the resistance of *L. monocytogenes* to disinfectants is the subject of numerous concerns in the context of the food industry and public health. In this study, the biofilm formed by the tested *L. monocytogenes* rods on the surface of stainless steel and polypropylene was treated with specific disinfectants. Both of these agents were effective against *L. monocytogenes* in the biofilm structure, however, their antibiofilm effect was correlated with the type of surface, concentration and type of preparation, and contact time. Higher effectiveness was demonstrated in the case of Chlorosol. During the microscopic assessment of the effect of disinfectants on the formed biofilm, it was shown that increasing the concentration of the tested agent and extending the duration of its action resulted in an increase in the share of dead *L. monocytogenes* cells. In turn, El-Kest and Marth (1988) showed that a solution of 1 mg/mL of free chlorine within 10 min reduced the number of *L. monocytogenes* in 48-h cultures by a factor of 4.27. Bremer et al. (2002) showed a significantly higher percentage of *L. monocytogenes* cells eliminated in the biofilm structure on stainless steel coupons compared to cells on polyvinyl surfaces. Also Pan et al. (2006) showed greater tolerance to the treatment with chlorine-based agents toward the biofilm formed on the Teflon surface compared to the stainless steel surface. Moreover, Folsom et al. (2006) observed that the adaptation of planktonic cells and their subsequent growth on stainless steel makes biofilms more resistant to the action of chlorine-based agents, regardless of the subtype, biofilm cell density and its morphology. In our own study, a disinfectant whose active substance was an aqueous solution of phosphoric and hydrochloric acid (Alusol) was less effective than sodium hypochlorite. In turn, Skoworn et al. (2018) showed that the most effective disinfectant against *L. monocytogenes* was peracetic acid and hydrogen peroxide (decrease in the number of bacteria at the level of 5.10–6.62 log CFU × cm^{-2} and 5.70–7.39 log CFU × cm-^{2}, after 1 and 5 min exposure, respectively). However, Beltrame et al. (2015) showed that treatment with peracetic acid and sodium hypochlorite effectively eliminates *L. monocytogenes* from a polyethylene cutting board used in a food processing plant. This study demonstrated that sodium hypochlorite effectively eliminates *L. monocytogenes* in the form of biofilm from the tested surfaces, especially stainless steel. The opposite result was reported by Krysiński et al. (1992), who showed the lowest antimicrobial activity of a chlorine-based disinfectant. In turn, Chen et al. (2015) found that peracetic acid and sodium hypochlorite were ineffective against the tested microorganisms (*L. monocytogenes*, *S.* typhimurium, *E. coli*) in the biofilm structure on the surface of

stainless steel. Data on effective methods for eradicating the *L. monocytogenes* biofilm are crucial to maintaining food safety, especially within food processing plants.

The conducted study had some limitations. Soil samples were collected from only two provinces in Poland, which is a small number of samples in relation to epidemiological studies. The number of genetically different *L. monocytogenes* strains examined was only 10. Therefore, further studies covering a larger range of samples and geographical area are recommended. An important aspect that should also be investigated is vegetables and their microbiological state during storage, as a direct factor affecting consumer safety.

5 Conclusion

Organic farming is a sector that is developing very dynamically all over the world, but the most dynamically developing organic farming are located in European countries. Although organic farming offers many benefits, it is crucial to highlight the food safety risks, particularly the presence of pathogens in the soil. This work indicates that organic food may be a source of pathogenic microorganisms, such as *L. monocytogenes*, the presence of which was found in 10.8% of soil samples from fields where organic carrots were grown. The work broadly characterized these strains and demonstrated their resistance to antibiotics, invasiveness toward the HT-29 cell line and the ability to coaggregate and form a biofilm. The presence of *L. monocytogenes* strains with high levels of virulence isolated from soil may pose risks to future consumers and public health associated with outbreaks and therapeutic difficulties for antibiotic-resistant strains. The conducted research may help in the future to introduce standards regulating the safety of organic farming. An important aspect would be to evaluate natural fertilizers for the presence of pathogens to avoid their penetration into the soil.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MN: Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft. ZP: Conceptualization, Writing – review & editing, Project administration, Resources, Validation. NW-K: Investigation, Visualization, Writing – original draft. KG-B: Data curation, Resources, Writing – original draft. AB: Visualization, Writing – original draft. JS-K: Data curation, Writing – original draft. EW-Z: Writing – review & editing. MH-K: Writing original draft. KS: Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

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

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Early detection and population dynamics of *Listeria monocytogenes* in naturally contaminated drains from a meat processing plant

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Listeria monocytogenes, a significant foodborne pathogen, often contaminates ready-to-eat foods through cross-contamination in food processing environments, and floor drains represent one of the most common sites of persistence. Subtyping of *L. monocytogenes* from food processing plants for the purpose of source tracking is usually performed on a single colony obtained after selective enrichment. This study investigates the temporal variation and population dynamics of *L. monocytogenes* in drains, focusing on the diversity of *L. monocytogenes* and the impact of the resident microbiota. Six different drains in a meat processing plant were each sampled four times over a period of 8 weeks and subjected to two-step selective enrichment in Half Fraser and Full Fraser broths. The clonal complexes (CCs) of at least 20 individual *L. monocytogenes* isolates from each positive sample (460 isolates in total) were determined using either the Geno*Listeria* Multiplex qPCR assay or whole genome sequencing (WGS). The microbiota in drains and enrichment cultures was analyzed by 16S rRNA gene amplicon sequencing and metagenomic or quasimetagenomic sequencing. *L. monocytogenes* was detected in the majority of samples and four different CCs were identified – CC9, CC11 (ST451), CC121 and CC8 – with up to three CCs in the same sample and with different CCs dominating in different drains. The same clones of CC9, CC11, and CC121 had persisted in the facility for 3–5 years. The composition of the drain microbiota remained relatively stable over time, with *Pseudomonas*, *Acinetobacter*, *Janthinobacterium*, *Chryseobacterium*, *Staphylococcus*, and *Sphingomonas* as the most commonly identified genera. There were no apparent differences in the microbial genera present in *L. monocytogenes* positive and negative drains or samples. The study highlights the use of techniques such as qPCR and quasimetagenomics for monitoring and controlling the risk of *L. monocytogenes* contamination in processing environments.

KEYWORDS

persistence, quasimetagenomics, metagenomics, microbiome, microbiota, subtyping, strain-level typing, food processing environment

1 Introduction

Listeria monocytogenes, the causative agent of listeriosis, is one of the most concerning foodborne human bacterial pathogens. Although this pathogen is widely found in the environment, major incidences of listeriosis are caused by consumption of contaminated ready-to-eat (RTE) food. Contamination of the final product is usually due to cross-contamination from the food processing environment (Vaìzquez-Boland et al., 2001; Jordan et al., 2018). It is well-known that *L. monocytogenes* can colonize niches such as equipment, floors, and drains in a food processing plant over extended time-periods (a situation known as persistence), and lead to recurring contamination of raw materials and products (Møretrø and Langsrud, 2004; Ferreira et al., 2014). Drains are the most common sites of persistence of *L. monocytogenes* in meat processing plants (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2024). Effective control of *L. monocytogenes* requires a seek-and-destroy strategy, which involves sampling of raw materials and food processing surfaces to identify targets for corrective actions (Food Safety and Inspection Service [FSIS], 2014; Malley et al., 2015). It is essential to distinguish between sporadic occurrences and persistent contamination of *L. monocytogenes*, as these require different eradication strategies. To detect persistence, the sampling and analysis methods must be capable of identifying *L. monocytogenes*, even at low levels, each time the same area is sampled. This typically involves sampling a large area with sufficient mechanical force to detach biofilm followed by selective enrichment. Ideally, recurrent *L. monocytogenes* should be subtyped to confirm the presence of the same clone over time. Consistent results may be challenging to obtain if a sample point contains multiple clones, as usually only one isolate from each sampling point is subtyped.

The resident microbiota can influence the growth, attachment, and biofilm formation of *L. monocytogenes* (Fox et al., 2014; Fagerlund et al., 2021). A proactive control strategy would be to identify and eradicate bacterial communities that promote *L. monocytogenes* attachment and biofilm production. Potential indicator bacterial taxa associated with higher abundance or detection of *L. monocytogenes* have recently been identified from fruit packing facilities (*Pseudomonas*, *Stenotrophomonas*, and *Microbacterium*; Rolon et al., 2023), from a frozen vegetable processing facility (*Enterobacter*, *Serratia*, and *Carnobacterium*; Pracser et al., 2024), from meat samples from a meat processing facility (*Pseudomonas*, *Acinetobacter*, and *Janthinobacterium*; Zwirzitz et al., 2021), and from fabrication and processing rooms of a small meat processing facility (*Acinetobacter*, *Chryseobacterium*, and *Psychrobacter*; Belk et al., 2022). Other studies have identified bacterial taxa that were negatively associated with *L. monocytogenes* occurrence or growth, e.g., from meat conveyor surfaces in a swine slaughterhouse (*Herminiimonas*, *Bryobacter*, *Caulobacter*, *Sphingomonas*, and *Mycobacterium*; Cherifi et al., 2022) and from mushroom processing environments (*Enterobacteriaceae* and *Lactococcus*; Lake et al., 2023). Other studies report no detected associations in this regard (Diaz et al., 2025), and so far, there does not seem to be a clear consensus on which taxa are associated with the presence or absence of *L. monocytogenes* in food industry environments.

It is well-known that certain genotypes of *L. monocytogenes* are more commonly associated with food processing environments and that different subtypes may have different levels of virulence and consequently food safety risk (Maury et al., 2016; Food and Agriculture Organization [FAO] and World Health Organization [WHO], 2022). Subtyping is therefore essential for source tracking, implementation of effective control measures, and risk-based food safety management in the food industry. The currently employed protocols for detection of *L. monocytogenes* involve a two-step selective enrichment and subsequent confirmatory biochemical or molecular species identification (International Organization of Standardization [ISO], 2017). This step may then be followed by genetic subtyping, commonly of one single isolate per positive sample. The length of time from sampling to *L. monocytogenes* detection and identification of subtype is a huge barrier for timely implementation of proper corrective actions, e.g., identification and removal of reservoirs in food processing environments or contaminated products on the market. Thus, there is a need for rapid methods for both detection and subtyping of *L. monocytogenes*.

Prior to the introduction of MLST in 1998 (Maiden et al., 1998), bacteria were typically subtyped using classical immunological serotyping, which distinguishes bacteria within a species based on the detection of specific surface structures (O- and H-antigens). *L. monocytogenes* serotyping, developed in the late 1970s (Seeliger and Höhne, 1979), differentiated *L. monocytogenes* into 13 serotypes, four of which are common among isolates from food and clinical cases (1/2a, 1/2b, 1/2c, and 4b). After the first *L. monocytogenes* whole genome sequences became available in the early 2000's, a DNA-based molecular analysis, based on PCR amplification of six genes, was developed as a simpler method for distinguishing between different serotypes (Doumith et al., 2004; Kérouanton et al., 2010; Vitullo et al., 2013). The assay could differentiate five PCR-serogroups based on a pattern of presence or absence of specific marker genes or gene variants. For example, the gene *lmo1118* was found to only be present in *L. monocytogenes* of PCR-serogroup IIc (serotypes 1/2c and 3c).

The phylogenetic structure of the species *L. monocytogenes* is now known to consist of well-defined, tight clusters of closely related strains, which largely correspond to clonal clusters (CCs) defined by the classical seven-gene MLST (Chen et al., 2016; Moura et al., 2016). *L. monocytogenes* MLST sequence types (STs) are defined as the unique association of alleles from seven housekeeping genes, and a CC is formed by grouping STs that share alleles at six out of the seven loci (Ragon et al., 2008). Most recent studies employ whole genome sequencing (WGS) for determination of MLST CCs, as it has become cost-effective relative to the classical method employing PCR followed by Sanger sequencing. Furthermore, it simultaneously provides data enabling subsequent higher resolution comparison of isolates within the same CC, which is essential for determining whether two or more isolates are sufficiently related to indicate a common source (Pightling et al., 2018). However, both sequencing-based approaches are relatively time-consuming and expensive compared with PCR-based analyses, limiting their potential for analysis of larger datasets and routine surveillance in the food industry.

To address the need for faster and more convenient subtyping, several new analysis methods have recently been developed based on the same principle as PCR-serogrouping, but with more marker

genes (ranging from 13 to 31). These are more detailed attempts to correlate patterns of presence or absence of genetic markers with prevalent genetic populations of *L. monocytogenes* (i.e., CCs). Examples include GENE-UP Typer from bioMérieux[1], the Listeria PatternAlert assay from Rheonix[2], and the GenoListeria Multiplex assay developed by ANSES, which distinguishes between 30 CCs widely circulating in Europe (Félix, 2023; Félix et al., 2023). Although these approaches are faster than traditional MLST and WGS, they are similarly affected by the potential loss of diversity when selecting a single colony for analysis, as different subtypes of *L. monocytogenes* may be present in the same sample (Döpfer et al., 2008; Chen et al., 2020; Stessl et al., 2020; Wagner et al., 2021; Acciari et al., 2022).

Another potential reason for failure to detect all *L. monocytogenes* variants that may contaminate the product is enrichment bias, resulting in a change in the relative proportion of *L. monocytogenes* subtypes during the course of the selective enrichment. Previous work by our group and others have shown that *L. monocytogenes* subtypes have slightly different growth potential in Fraser broth (Wagner et al., 2021; Rosa Rodrigues de Souza et al., 2023). However, when *L. monocytogenes* strains were co-enriched with a mock community background microbiota composed of strains capable of growing in Fraser broth, the relative proportions of the different *L. monocytogenes* STs remained relatively consistent during selective enrichment (Wagner et al., 2021). In that study, we also demonstrated that quasimetagenomic sequencing (metagenomic shotgun sequencing of enrichment cultures; Ottesen et al., 2020) could be used for faster identification of *L. monocytogenes*. Additionally, Illumina sequencing enabled the prediction of the presence of co-occurring *L. monocytogenes* strains. Although methods exist for mapping of sequence read data to MLST databases (Zolfo et al., 2017; Clausen et al., 2018), extracting strain-level subtyping information from metagenomic data remains challenging, even though several software tools for strain-level identification from metagenomic data have been developed in recent years (Anyansi et al., 2020; Ghazi et al., 2022; Liao et al., 2023; Lindner et al., 2024).

In this study, the Geno*Listeria* Multiplex qPCR assay, WGS, 16S rRNA gene amplicon sequencing and metagenomic or quasimetagenomic sequencing was employed to examine the temporal variation and population dynamics of *L. monocytogenes* CCs and the co-occurring residential microbiota in naturally contaminated drains in a meat processing plant. A second objective was to assess the impact of the resident microbiota on *L. monocytogenes* presence in drains and during selective enrichment.

2 Materials and methods

2.1 Sampling and culture enrichment

Samples from six drains in a meat processing facility were collected using sampling cloths (swab cloths 32 × 40 cm, with 25 mL buffered peptone water with 10% neutralizing solution; SodiBox; cat no. 3040) on four occasions over a total period of 9 weeks. During the sampling period the facility, including the drains, was subjected to the cleaning and disinfection procedures routinely in use in the facility. After sampling, the cloths were stored at 4°C and analyzed within 2 h using the ISO 11290-1 method (International Organization of Standardization [ISO], 2017). Bags with sample cloths were added 100 mL Fraser broth base (Oxoid) and stomached for 1 min before the initial samples were collected (samples taken prior to enrichment). Half Fraser selective supplement (Oxoid) was then added to the remaining sample, before primary enrichment at 30°C for 24 h. Then, 100 µL culture was transferred to 10 mL Full Fraser broth (Oxoid) for secondary enrichment at 37°C for 24 h. Samples were withdrawn before start of enrichment, after 4 and 24 h enrichment in Half Fraser broth and after 24 h enrichment in Full Fraser broth. To determine cell counts, dilutions of the enrichment cultures were plated on *Listeria*-selective RAPID *L. mono* agar (RLM; Bio-Rad) and Standard Plate Count Agar (PCA; Oxoid). RLM plates were incubated at 37°C for 1–2 days, while the PCA plates were incubated for 3–5 days at 20°C. Plating on RLM was not performed for the drain samples prior to enrichment in the first week of sampling. For metagenomic analysis, samples were collected by withdrawing 4 mL culture (at the start and after 4 h) or 1 mL culture (after 24 h in Half and Full Fraser), centrifugation at 13,000 *g* for 5 min, washing once in 500 µL TE buffer, and then storage at −20°C.

Statistical differences for TVCs obtained from each swab cloth (prior to enrichment) was assessed using Minitab v.22 software and two-way analysis of variance (ANOVA) with drains and weeks as factors. TVCs were log transformed prior to statistical analysis to stabilize variance and normalize the data. The residuals met the assumptions of normality, homogeneity of variances, and independence. The tested null hypotheses were that there were no differences in TVCs between drains or weeks. After rejecting the null hypotheses ($p < 0.05$), Tukey's *post-hoc* test for pairwise comparisons was performed to compare differences between factors.

2.2 DNA extraction

For isolation of DNA from single isolates, single colonies were picked from RLM agar plates, inoculated in 5 mL Brain heart infusion (BHI) broth (Oxoid), and grown overnight at 37°C with shaking. The pellet from 800 µL culture was suspended in 500 µL of 2x Tris-EDTA buffer with 1.2% Triton X-100. Cells were lysed using lysing matrix B and a FastPrep instrument (both from MP Biomedicals), and genomic DNA was isolated using the DNeasy blood and tissue kit (Qiagen).

Isolation of DNA from samples from enrichment cultures was performed using DNeasy PowerLyzer Powersoil (Qiagen) according to the manufacturer's instructions; cells were lysed using a Precellys Evolution Instrument (Bertin Technologies) at 7,400 rpm for three rounds of 40 s.

2.3 Real-time qPCR for MLST subtyping

The Geno*Listeria* multiplex qPCR method (Félix, 2023; Félix et al., 2023) was performed as described with some modifications.

1 https://www.biomerieux.com/corp/en/our-offer/industry-products/gene-up-typer.html

2 https://rheonix.com/food-beverage-testing/listeria-patternalert-assay/

Simplex PCR reactions were performed using primers and probes for CC9 (PCR-serogroup IIc), CC11-ST451, CC121, CC199, and CC14-ST91-ST160-ST360. Isolates with no positive amplification in the simplex PCR reactions were analyzed using a *prs* and *plcA* multiplex qPCR to identify isolates as *Listeria spp.* and *L. monocytogenes*. Amplifications with Ct < 18 were considered positive. The remaining isolates were identified using WGS.

All probes were labeled with FAM/BHQ-1 except the *plcA* probe, which was labeled with VIC/BHQ-1. qPCR reactions were run in duplicates with 10 µL reaction volumes each containing 1 µL DNA, 5 µL 2x PerfeCTa qPCR ToughMix low ROX (Quantabio), and 0.3 µM each of probe, forward primer, and reverse primer. DNA concentrations were in the range 5–15 ng/µL. Amplification conditions with Fast Program on QuantStudio5 (BioTek) were: 95°C for 2 min, 40 cycles of 95°C 3 s and 60°C for 30 s.

2.4 WGS and genome assembly

Libraries for WGS were prepared using the Nextera XT DNA sample preparation kit (Illumina) and sequenced on a MiSeq platform with 300 bp paired-end reads. Raw reads were filtered on q15 and trimmed of adaptors before *de novo* genome assembly was performed using SPAdes v.3.13.0 (Bankevich et al., 2012) with the careful option and six *k*-mer sizes (21, 33, 55, 77, 99, and 127). Contigs with sizes of < 500 bp and *k*-mer coverage of < 5 were removed from the assemblies. The average coverage for the genome assemblies was calculated using BBmap v36.92 (Bushnell, 2014) and the quality of all assemblies was evaluated using QUAST v5.0.2 (Mikheenko et al., 2018). The assemblies were annotated using the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) server.

A total of 97 *L. monocytogenes* isolates were collected from the factory during 2017–2019 as part of an earlier study (Fagerlund et al., 2022), and of these, 38 were previously subjected to WGS [National Center for Biotechnology Information (NCBI) BioProject accession PRJNA689484 and European Nucleotide Archive (ENA) Project PRJEB56155]. For the 14 isolates in PRJEB56155 (Ivanova et al., 2025), raw sequencing data was subjected to genome assembly as described above. The genomes analyzed in the current study are listed in Supplementary Table 1. Basic Local Alignment Search Tool (BLAST) analyses were performed using blastn v2.12.0+ integrated in CLC Main Workbench 22.0.2 (Qiagen). Alignments were generated using CLC Main Workbench.

2.5 16S rRNA gene amplicon sequencing

16S rRNA gene PCR (V4 region) and paired-end sequencing (2 × 150 bp) using the MiSeq reagent kit v3 on a MiSeq instrument (Illumina) were performed following the protocol by Caporaso et al. (2012) as previously described (Møretrø et al., 2021). The sequences were processed in QIIME 2 (qiime2-2023.5) (Bolyen et al., 2019). Briefly, raw data were demultiplexed using the q2-demux plugin, followed by joining paired ends with vsearch (Rognes et al., 2016). Quality filtering was based on a q-score above 30, and denoising was performed using deblur-16S (Amir et al., 2017). Taxonomy was assigned to sOTUs using the q2-feature-classifier (Bokulich et al., 2018) classify-sklearn naïve Bayes taxonomy classifier against the SILVA 16S database (Silva 138 99% OTUs from the 515F/806R region) (Quast et al., 2013). Singletons were removed, and mitochondrial and chloroplast sequences were filtered out before collapsing the taxonomy tables to genus identity level (L6), converting to relative values, exporting to text files, and further processing in Microsoft Excel. All sOTUs were aligned with mafft (Katoh and Standley, 2013) via q2-alignment and used to construct a phylogeny with fasttree2 (Price et al., 2010) via q2-phylogeny. Alpha and beta diversities were estimated using q2-diversity (core-metrics-phylogenetic) (Bolyen et al., 2019) with a sampling depth of 26,400 sequences. The results for the different metrics were visually inspected, and the results from the Shannon and Bray-Curtis metrics were used for calculation and visualization in Principal Coordinate Analysis (PCoA) plots. Differences in Shannon index were calculated using ANOVA (GLM and one-way) in Minitab v21.4.3, and the bacterial community diversity (Bray-Curtis distance) across different parameters was statistically evaluated using pairwise permutational multivariate analysis of variance (PERMANOVA) tests in QIIME 2 (Anderson, 2001; Anderson and Walsh, 2013).

2.6 Quasimetagenomic shotgun sequencing

For the selected samples from the *L. monocytogenes* culture enrichments, 200 ng of genomic DNA was subjected to paired-end sequencing (2 × 301 bp). Briefly, libraries were prepared as described in the Illumina DNA prep reference guide (Illumina DNA prep kit; Nextera DNA CD indexes; Illumina). Samples were purified, quantified with Qubit HS dsDNA (Invitrogen), normalized, and pooled. The sample pool was purified, quantified, and diluted to 4 nM prior to a denaturation and dilution procedure provided by Illumina, including the use of 8 pM DNA input og 5% PhiX spike.

Taxonomic classification was performed as previously described (Wagner et al., 2021). Illumina reads were filtered on q15 and trimmed of adaptors using fastq-mcf from the ea-utils package (Aronesty, 2011). Taxonomic classification of the filtered Illumina reads was performed using the *k*-mer approach employed in Kraken2 v2.1.1 (Wood et al., 2019) and the available pre-built Kraken2 database PlusPFP (containing indexes for the archaea, bacteria, viral, plasmid, protozoa, fungi, plant, human, and UniVec_Coreplus RefSeq databases from 27 January 2021). A confidence score threshold of 0.05 was selected, and the minimum base quality used in classification was 30.

2.7 MLST and strain-level subtyping

Classical MLST analysis followed the MLST scheme described by Ragon et al. (2008) and the database maintained at the Institute Pasteur's *L. monocytogenes* online MLST repository[3]. The wgMLST analysis was performed using a whole-genome scheme containing 4,797 coding loci from the *L. monocytogenes* pan-genome and the

3 https://bigsdb.pasteur.fr/listeria/

assembly-based BLAST approach, implemented in BioNumerics 8. The minimum spanning tree was constructed using BioNumerics based on the categorical differences in the allelic wgMLST profiles for each isolate. Loci with no allele calls were not considered in the pairwise comparison between two genomes. The number of allelic differences between isolates was read from genetic distance matrices computed from the absolute number of categorical differences between genomes.

Mapping of Illumina reads to the Institute Pasteur's *L. monocytogenes* MLST database (Ragon et al., 2008) was performed as previously described (Wagner et al., 2021). Illumina reads classified to Taxon IDs 1637 (*Listeria* spp.) and 1639 and below (*L. monocytogenes* classified to species and strain level) using Kraken2 were extracted to file using the KrakenTools (Lu et al., 2022) and mapped to the Institute Pasteur's *L. monocytogenes* MLST database using the KMA mapping program (Clausen et al., 2018).

MetaMLST v1.2.1 (Zolfo et al., 2017) was used to detect the dominant ST in metagenomic shotgun sequencing data. A MetaMLST database was built for *L. monocytogenes* using metamlst-index.py, from loci sequence files and MLST profiles downloaded from the Institute Pasteur's *L. monocytogenes* MLST repository on 14.01.2021. Illumina reads were mapped to the MetaMLST database using bowtie2 v2.3.5.1 (Langmead and Salzberg, 2012) and parameters --very-sensitive-local -a --no-unal. MLST loci contained in the sample were detected using metamlst.py and STs were called using metamlst-merge.py.

StrainScan v1.0.14 was used to perform strain-level composition analysis of the metagenomic shotgun sequencing data. Two reference databases were used, one built with one representative genome from each of six CCs; CC9 (MF8690; contains *lmo1118*), CC11-ST451 (MF8691), CC121 (MF8693), CC8 (MF8681), CC199 (MF7408), and CC14-ST91 (MF7327), and the other with all genomes listed in Supplementary Table 1. StrainScan was run using both databases and both with default settings and with the parameter --low_dep 2.

3 Results

3.1 Sampling in the meat processing facility

Six drains from a high risk zone in a meat processing facility, referred to as drains A to F (Figure 1), were selected for sampling. Drains A, B, and F were dry or relatively dry, and located adjacent to each other in the same region of the processing department. They received relatively limited amounts of soiling and effluents from the meat processing operation. Drains C, D, and E were usually humid when sampled, and located in another area of the same department. Drain D, located in a cooling room, had the highest load of soiling and effluent. The sampling was performed in four different weeks in 2022, each time early during the first work shift on each sampling day. The first and second sampling time-points were 6 weeks apart, while the three last sampling occasions occurred in consecutive weeks. These time-points are referred to as weeks 1, 7, 8, and 9. All drains were cleaned with a chlorinated alkaline foam-based cleaning agent, and standard cleaning routines were followed throughout the sampling period.

3.2 *L. monocytogenes* was detected in the majority of drains

The swab cloths were subjected to two-step selective enrichment for *L. monocytogenes,* including a 24 h primary enrichment step in Half Fraser broth followed by 24 h secondary enrichment in Full Fraser broth (International Organization of Standardization [ISO], 2017). The cultures were analyzed for both total bacterial counts (total viable counts; TVC) and counts of *L. monocytogenes* at four time-points: before enrichment, after 4 and 24 h primary enrichment in Half Fraser broth (t4 and t24), and after secondary enrichment; 24 h after subculturing into Full Fraser broth (t48) (Figure 2). Of the 24 analyzed samples, only five were negative for *L. monocytogenes*. Drain A was only positive for *L. monocytogenes* during the first sampling week and drains C and F were negative for *L. monocytogenes* in weeks 8 and 9, respectively. The remaining three drains were positive for *L. monocytogenes* in all four sampling weeks.

All 19 samples that were *L. monocytogenes* positive after secondary enrichment in Full Fraser broth were also positive after 24 h primary enrichment in Half Fraser broth. However, the proportion of *L. monocytogenes* relative to the total bacterial counts in the positive cultures increased in the second step, from median values of 0.3% in Half Fraser (t24) to 55% in Full Fraser broth (t48).

Of the 18 samples collected during weeks 7–9, 13 were positive for *L. monocytogenes*, and for six of these, presumptive *L. monocytogenes* were detected prior to enrichment. The detection limit was 10^3 cfu per sample (International Organization of Standardization [ISO], 2017). As 1–11 colonies were obtained on the selective agar plates, samples from the drains with the highest concentrations appeared to contain around 10^3–10^4 *L. monocytogenes* bacteria. All six samples from week 1, which were not analyzed for *L. monocytogenes* prior to enrichment, tested positive, with two samples showing detection at t4.

A color change from yellow to dark brown in Fraser broth is due to hydrolysis of esculin, and is a presumptive indication of the presence of *L. monocytogenes* and other *Listeria* spp. All the *L. monocytogenes* positive cultures showed this color change after primary enrichment in Half Fraser broth. In addition, one of the five samples that were negative for *L. monocytogenes* – drain C in week 8 – showed the same color change during secondary enrichment in Full Fraser broth, but not in Half Fraser. The other four *L. monocytogenes* negative samples had, on average, 2.9 log lower TVCs (ranging from 2.3 to 3.8 log lower) after 24 h secondary enrichment in Full Fraser (t48) compared to 24 h primary enrichment in Half Fraser broth (t24). In contrast, the sample from drain C in week 8 did not show a lower TVC in Full Fraser compared to in Half Fraser. Thus, for the *L. monocytogenes* negative samples, the presence of bacteria capable of hydrolysing esculin appeared to correlate with the presence of bacteria that could grow well in Full Fraser broth.

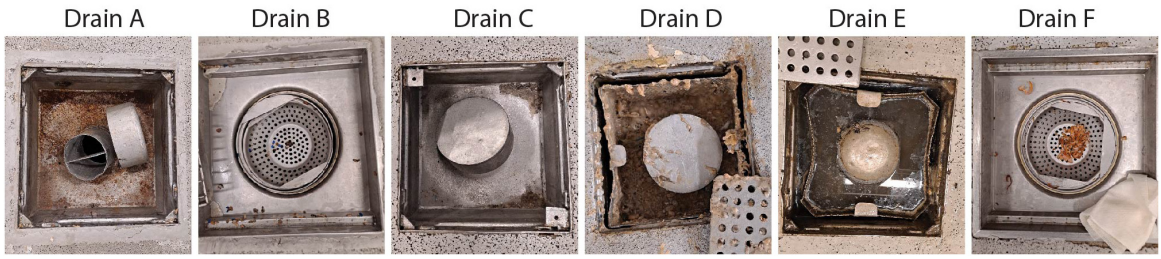

FIGURE 1
Representative pictures of the six sampled drains. The swab cloth used for sampling is shown next to drain F.

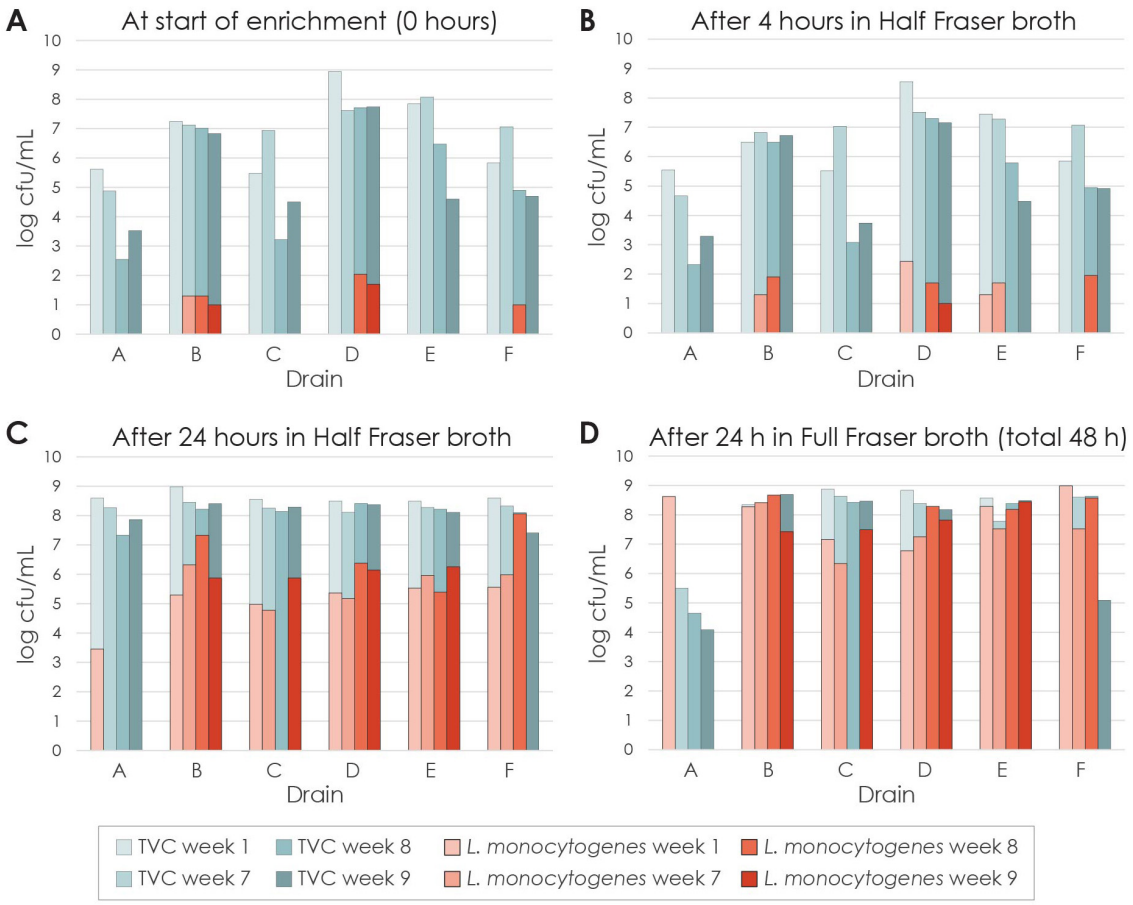

FIGURE 2
Bacterial concentrations measured as total viable counts (TVC) and counts of *L. monocytogenes*. **(A)** Samples from swab sampling cloths after suspension in 100 mL volume, before the start of enrichment. After **(B)** 4 h and **(C)** 24 h of primary enrichment in Half Fraser broth, and **(D)** 24 h secondary enrichment in Full Fraser broth. Samples were not analyzed for *L. monocytogenes* before start of enrichment in week 1. The detection limit was 10 cfu/mL and results below this limit are not indicated. Culture volumes were 100 mL for Half Fraser and 10 mL for Full Fraser enrichments.

3.3 Diversity of total microbiota in the drains

The total counts of bacteria in the drain samples were relatively high overall, with on average 8.1 log TVC (SE ± 0.4) obtained from each swab cloth (prior to enrichment). The variation was relatively large, from 4.5 log to 11.0 log TVC per drain sample (i.e., per total swab cloth). The main effects of both drains and weeks were statistically significant (two-way ANOVA, $p < 0.005$), and the TVCs were significantly lower in drains when sampled in weeks 8 and 9 relative to in weeks 1 and 7 (Tukey's *post-hoc* test, $p < 0.04$). It should be emphasized that no special cleaning measures were implemented during the sampling period, so the explanation behind this reduction in TVC is not known.

The microbiota in the drains and enrichment cultures was studied using Illumina sequencing of 16S rRNA gene amplicons, a technique also known as metabarcoding. Nine samples, including the samples taken prior to enrichment in drains A, B, and C in

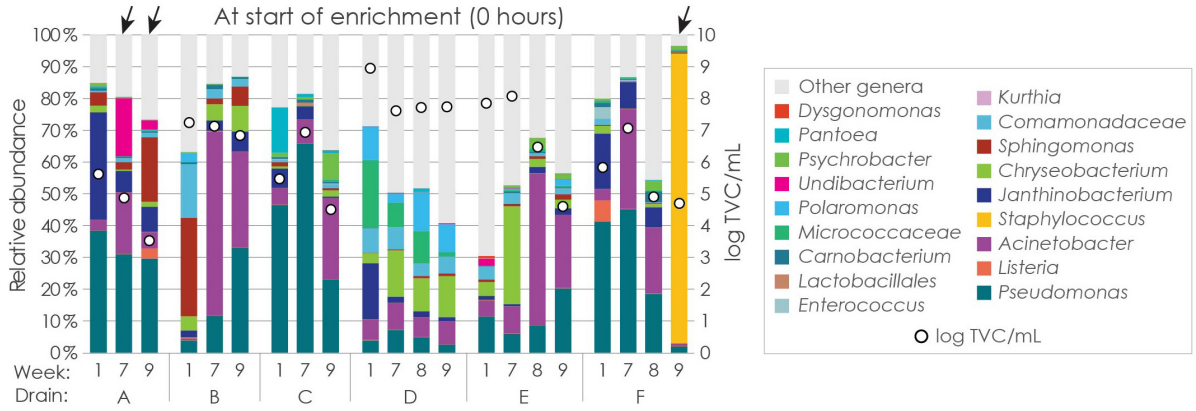

FIGURE 3
Relative abundance (%) of bacterial genera in the drain samples based on 16S rRNA gene amplicon sequencing. Taxa with abundance above 1% across all samples and/or at least 10% abundance in any one sample (considering all time-points) are presented, and remaining taxa are presented in the "other genera" category. Samples negative for *L. monocytogenes* are indicated by arrows. Bacterial concentrations on the secondary axis are for swab sampling cloths after suspension in 100 mL volume.

week 8, failed to generate sufficient DNA for sequencing after PCR due to too low DNA concentration. In total, 87 samples were analyzed, resulting in 6.9 million sequences after denoising and quality filtering. This yielded 1,738 bacterial sequences down to single nucleotide differences (suboperational taxonomic units; sOTUs). Identification to the genus level revealed 527 taxa, of which 18 had an average relative abundance of > 1% across all samples or a maximum average abundance of > 10% in any single sample.

The most commonly identified genus in the drains (prior to enrichment) was *Pseudomonas*, with average and median relative abundances of 22% and 19%, respectively, across the 21 analyzed samples (Figure 3). Other frequently identified genera included *Acinetobacter* (15%), *Janthinobacterium* (6%), and *Chryseobacterium* (5%). *Staphylococcus* dominated with 91% relative abundance in one sample (drain F in week 9), but the median abundance across samples was only 0.05%. *Sphingomonas* was highly prevalent in two samples – drain A in week 9 and drain B in week 1 – with relative abundances of 20% and 31%, respectively. However, the overall median relative abundance of *Sphingomonas* was relatively low at 0.8%.

Listeria constituted only a minority of the total drain microbiota, with average and median relative abundances of 0.6% and 0.1%, respectively. Only two drains had relative abundances of *Listeria* above 0.2%: drain A in week 9 (2.3%) and drain F in week 1 (6.7 %). Notably, drain A in week 9 tested negative for *L. monocytogenes*, however, only one sOTU was identified for the *Listeria* genus, meaning that the analysis cannot distinguish *Listeria* below genus level. Presumably, since *L. monocytogenes* was not detected, the 16S rRNA gene sequencing reads from drain A in week 9 represent *Listeria* spp. other than *L. monocytogenes*.

Due to the inherent limitations of 16S rRNA gene amplicon sequencing, the detection and accurate quantification of low abundance bacteria can be challenging, often leading to increased uncertainty in the results for rare taxa (Bonk et al., 2018). Nevertheless, considering the TVCs and relative abundances of *Listeria* in each sample, the number of *Listeria* cells per drain sample was calculated to range from 10^4 to 10^8 cfu, with a median of 7×10^5 cfu. These estimates for *Listeria* spp. were orders of magnitude higher than those estimated for *L. monocytogenes* from direct plating of samples from weeks 7 to 9 on selective agar plates ($\leq 10^4$ cfu per drain sample).

Sequencing data was obtained prior to enrichment for three of the five drain samples in which *L. monocytogenes* was not detected. Drain A was positive for *L. monocytogenes* in week 1, and negative the other weeks, however, no apparent difference in the microbiota was observed between these weeks (Figure 3). For drain F, however, there was a significant shift in the microbiota in week 9, when no *L. monocytogenes* was detected and the drain was completely dominated by *Staphylococcus*, compared to in weeks 1, 7, and 8, when the drain was positive for *L. monocytogenes* and the microbiota was more similar to that of the other analyzed samples.

The microbiota in the drains prior to enrichment was highly similar to that observed after 4 h of primary enrichment (Figure 4 and Supplementary Figure 1A). No significant difference was seen between samples collected at these two time-points in Shannon diversity (microbial richness, i.e., the number of taxonomic groups, and evenness, i.e., distribution of abundances; alpha diversity, $p = 0.979$) or in Bray-Curtis distances (microbial diversity; beta diversity, $p = 0.999$). This is also evident from the PCoA plot shown in Figure 4, although the samples from drain D (cooling room) diverge somewhat from the samples from the other drains.

For evaluation of the influence of the microbiota on the prevalence of *L. monocytogenes*, samples collected both directly from the drains and after 4 h of enrichment were included ($n = 41$), in order to increase the number of samples for analysis. There was a significant difference in the Shannon diversity between the different drains ($p = 0.017$), where drains B and F (dry or relatively dry) had significantly lower diversity than drains D and E (usually humid) ($p < 0.001$). There was also a significant difference in Bray-Curtis distances between drains ($p = 0.001$, test statistic = 5.19). PERMANOVA analysis also revealed a significant difference in the Bray-Curtis distances between *L. monocytogenes* positive and negative samples ($p = 0.003$, test statistic = 2.46). However, this result was influenced by the low number of *L. monocytogenes* negative drain samples in the dataset and the fact that there was a significant difference between the drains.

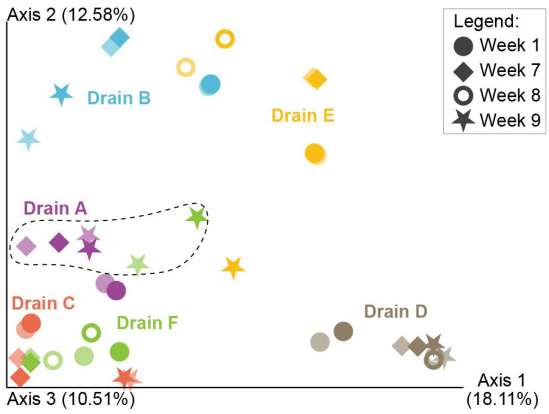

FIGURE 4
Principal Coordinates Analysis (PCoA) based on Bray Curtis dissimilarity depicting taxonomic differences among samples taken before enrichment (represented by weakly colored icons) and after 4 h of primary enrichment (represented by strongly colored icons), analyzed using 16S rRNA gene amplicon sequencing. Distinct colors indicate different drains, while different symbols indicate different weeks. Samples circled by the dashed line were from drains that tested negative for *L. monocytogenes* after selective enrichment.

The diversity in the total microbiota for the remaining samples collected during selective enrichment is shown in Supplementary Figure 1 and further discussed below, along with data on *L. monocytogenes* population dynamics during enrichment.

3.4 qPCR and WGS identified four different CCs

All but one of the presumptive *L. monocytogenes* colonies obtained before enrichment ($n = 22$), all colonies obtained after 4 h of primary enrichment ($n = 59$), and 10 colonies each from positive samples after 24 h enrichment in Half and Full Fraser were isolated for multilocus sequence typing (MLST) identification. In total 460 isolates were collected.

The "Geno*Listeria*" real-time qPCR Taqman subtyping method developed by Félix et al. (2023) was used for MLST subtyping. Initially, qPCR reactions were chosen for the analysis based on which CCs were previously determined to be present in the factory during 2017–2019 (Fagerlund et al., 2022; Ivanova et al., 2025; Supplementary Table 1). In accordance with this, the qPCRs to detect the molecular markers identifying CC9 (PCR-serogroup IIc), CC11-ST451, CC121, CC199, and CC14-ST91-ST160-ST360 (hereafter referred to as CC14-ST91) were sequentially performed for the 460 isolates. According to the published protocol, amplifications should have a cycle threshold (Ct) of less than or equal to 25 cycles ($Ct \leq 25$) to be considered positive (Félix, 2023; Félix et al., 2023).

Using this approach, 186 isolates (40%) were identified as CC9 ($Ct < 17.4$, the remaining 274 had $Ct > 25.5$), 217 isolates (47%) as CC11-ST451 ($Ct < 16.6$, the remaining 57 had $Ct > 27.5$), and 26 isolates (6%) as CC121 ($Ct < 14.6$). In the CC121 qPCR reactions, two samples obtained $Ct \leq 25$ (21.7 and 22.6), but were considered inconclusive in the current study. The remaining 31 isolates were negative also in the CC199 qPCR reaction and inconclusive in the CC14-ST91 qPCR reaction (15 isolates had Ct values in the range 21–25). One of the 31 isolates was then identified as a *Listeria* spp. other than *L. monocytogenes* by qPCR targeting *prs* and *plcA*. In total using this approach, 914 qPCR reactions were run.

The remaining 30 non-identified isolates were typed using WGS. Three isolates belonged to CC8 (0.7%) and 26 isolates (including the two that were inconclusive in the CC121 qPCR) belonged to CC9, bringing the total number of CC9 isolates to 212 (46%). The final isolate was determined to be *Enterococcus faecalis*. The intermediate Ct values observed in the CC14-ST91 qPCR could be attributed to a perfect match in the primer pair and only one mismatch in the TaqMan probe sequence, relative to the genomes of CC8 and CC9.

BLAST analysis showed that the 26 CC9 isolates for which the CC9 qPCR was negative (comprising 12% of all typed CC9 isolates) lacked the genetic marker *lmo1118* used to detect CC9 in the Geno*Listeria* scheme (Félix, 2023; Félix et al., 2023). A total of 22 CC9 isolates collected from the meat processing factory during 2017–2019 were previously typed using WGS (Fagerlund et al., 2022; Ivanova et al., 2025; Supplementary Table 1). Of these isolates, 32% ($n = 7$) lacked *lmo1118*. CC9 isolates lacking *lmo1118* were found in drains B, D, and E. No CC9 isolates from drains A, C, and F were typed using WGS.

The CC9 genome assemblies were fragmented in the region where *lmo1118* is located (when present). Therefore, the region from *lmo1116* to *lmo1122* in the EGD-e genome was aligned with the corresponding regions in the genomes of six completely sequenced CC9 isolates from Norwegian meat processing facilities; MF4626, MF4624, MF4697, MF6172, MF4562, MF4545 (Fagerlund et al., 2018; Supplementary Figure 2). Four of these genomes were identical to EGD-e in this region, while in MF4562, the insertion sequence IS*1542* encoding a transposase (Darini et al., 1999) was inserted on either side of the *lmo1119* and *lmo1118* genes. In MF4545, this cassette was replaced by a third copy of IS*1542*, flanked on either side by an ORF identical to the first half of *lmo2365*.

3.5 Persistence and diversity of *L. monocytogenes*

Whole genome MLST (wgMLST) analysis was performed to examine whether the sequenced isolates collected in 2022 were similar across drains, and/or similar to the 38 previously sequenced isolates collected during 2017 to 2019. In addition to the 29 isolates sequenced as a response to negative or ambiguous qPCR results in the current study (CC8 and CC9), five additional randomly selected isolates (identified using qPCR), were also subjected to WGS (and wgMLST) – two CC9, two ST451, and one CC121 (see Supplementary Table 1).

Previous work (Fagerlund et al., 2022) identified CC9 as persisting in the meat processing facility (factory M8) over a period of 2 years. Several of the CC9 isolates from 2017 to 2019 were from drain D ($n = 4$) or other drains in the same department as drains A to F. All isolates (22 from 2017 to 2019 and 28 from 2022) belonged to the same clone (Figure 5). The 50 CC9 isolates were differentiated by a maximum of 42 wgMLST allelic differences, with a median genetic distance of four alleles, showing

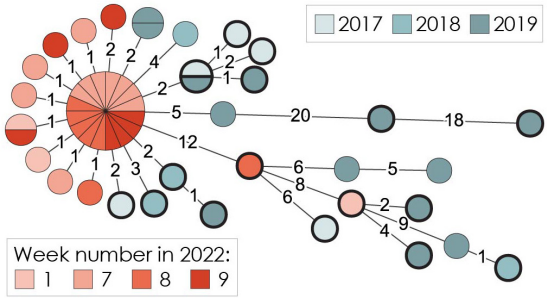

FIGURE 5
Minimum spanning tree for 50 *L. monocytogenes* CC9 genomes from the meat processing factory, based on wgMLST analysis using a scheme of 4,797 loci. The area of each circle is proportional to the number of isolates represented, and the number of allelic differences between isolates is indicated on edges connecting the nodes. Nodes representing isolates containing the *lmo1118* marker gene are highlighted by a thick black ring.

that the same strain had persisted in the facility for at least 5 years (Figure 5). Isolates containing the *lmo1118* qPCR target gene were not genetically distinct from those lacking *lmo1118*.

The two sequenced CC11-ST451 isolates from 2022, from drain A in week 1 and drain F in week 8, differed by only 1 wgMLST allele. An isolate from a product sample collected in 2019 was identical by wgMLST to the currently sequenced isolate from drain A. Thus, it seemed that CC11-ST451 had persisted in the factory for at least 3 years. The sequenced CC121 isolate from drain C in week 7, 2022, showed 2 to 23 wgMLST allelic differences toward the ten CC121 sequenced isolates collected during 2017–2019, including 2 and 3 wgMLST allelic differences toward two isolates collected from drain D in 2019 and 2018, respectively. This means that CC121 had persisted in the facility for 5 years. The three CC8 isolates identified in 2022, from drain D in week 7 and drain E in week 8, showed 3 to 9 wgMLST allelic differences, indicating that the same strain was present in both drains.

In summary, the same clones of CC9, CC11-ST451, and CC121 appeared to have persisted in the facility for 3–5 years. CC8 was only identified in 2022, while CC199 and CC14-ST91 were only detected during 2017–2019. At least one of the four previously analyzed CC199 isolates were from the same department in the factory as drains A to F sampled in the current study.

The CC9 and CC121 persistent clones both carried premature stop codon mutations in the *inlA* gene, which encodes the virulence factor internalin A [mutation types 12 and 6, respectively (Van Stelten et al., 2010)]. These mutations indicate that these clones were hypovirulent (Food and Agriculture Organization [FAO] and World Health Organization [WHO], 2022). The CC11-ST451 persistent clone carried a full-length *inlA*. None of the isolates carried the *Listeria* pathogenicity islands LIPI-3 or LIPI-4. All three persistent clones carried a *repA*-family theta-replicating plasmid, with sizes of 26 kb, 61 kb and 66 kb in the CC9, CC121, and CC11-ST451 clones, respectively. The plasmids in the CC9 and CC121 clones contained a cadmium resistance operon (*cadA1C1*) and the *clpL* heat resistance determinant. Additionally, both CC9 and CC121 harbored the Tn*6188* transposon encoding QacH, conferring resistance to quaternary ammonium compound (QAC) sanitizers (Müller et al., 2014). The CC9 clone also carried stress survival islet 1 (SSI-1), promoting growth under low pH and high salt stress conditions, while the CC121 clone contained SSI-2, which provides protection against alkaline or oxidative stress (Ryan et al., 2010, Harter et al., 2017). Furthermore, the CC9 clone harbored the Tn*554*-like transposon carrying the arsenic resistance cassette *arsCBADR*. These features suggest that both the CC9 and CC121 clones are classical food-processing-associated clones, well-adapted to such environments. In contrast, the CC11-ST451 clone lacked all queried stress and resistance genes (Fagerlund et al., 2022).

3.6 Population dynamics during selective enrichment

Different CCs dominated different drains (Figure 6). The three driest drains (A, B, and F) located in one end of the production room were almost completely dominated by CC11-ST451, while the predominant type in the three more humid drains located in another area of the room (C, D, and E) was CC9. CC121 was detected sporadically in two drains (D and E) and 2 weeks in a row in one (drain C), and no isolates belonging to CC121 or CC8 were detected during week 1.

As presented above, the microbiota in the drains prior to enrichment was highly similar to that observed after 4 h of primary enrichment (Figures 3, 4 and Supplementary Figure 1A). In the five drains where *L. monocytogenes* colonies were obtained at these two time-points, the same CC was exclusively obtained from each drain, except in the case of drain D in week 9, where one of ten colonies obtained after 4 h enrichment belonged to a different CC (Figure 6). This indicates a stable microbial community composition, consistent with the absence of enrichment bias in the *L. monocytogenes* population. However, it should be noted that the number of *L. monocytogenes* colonies was low at the early sampling times.

After 24 h of primary enrichment, the dominance of *Pseudomonas* had increased to 67% for both average and median relative abundances, and a substantial increase in the phylum *Firmicutes* was observed (Supplementary Figure 1B). After secondary enrichment in Full Fraser broth, the microbiota changed dramatically and became much less diverse compared to in Half Fraser broth (Supplementary Figure 1C). All secondary enrichments for drains that were positive for *L. monocytogenes* were dominated by *Listeria*, except drain B in week 1 which contained 78% *Enterococcus*. These changes coincided with a noticeable trend of higher proportions of CC121 after 24 h primary enrichment in Half Fraser broth, which seemed to be outcompeted by CC9 after secondary enrichment in Full Fraser broth. The opposite tendency was found for CC8, which was only isolated after secondary enrichment. Given the limited data, it would be highly speculative to conclude whether the microbiota in the enrichment cultures contributed to the observed trends in CCs, or if the observed trends were due to enrichment bias favoring certain CCs in Half Fraser and Full Fraser. No clear associations between the microbiota during enrichment and the presence of specific CCs were apparent from the data.

Regarding the microbiota in the secondary enrichment cultures for drains that did not contain *L. monocytogenes*, four of the five

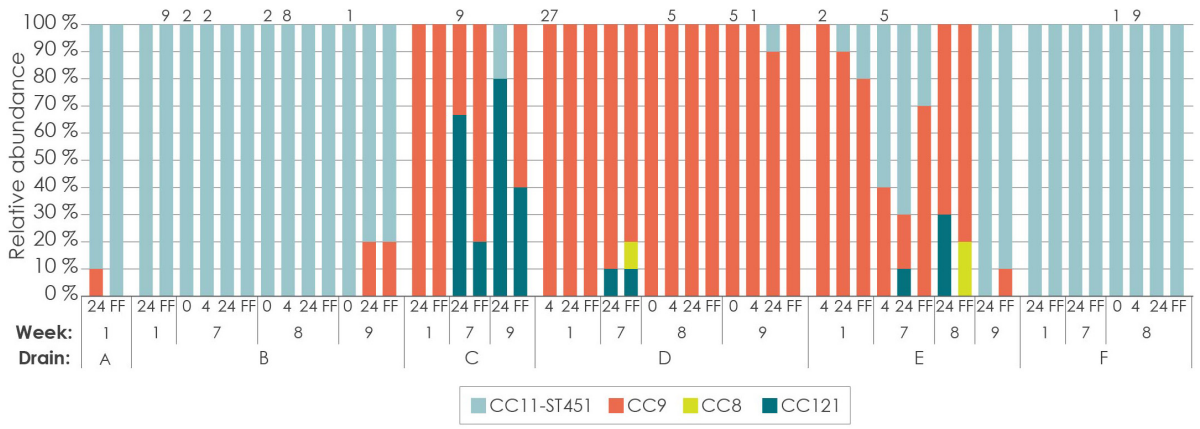

FIGURE 6
Distribution of *L. monocytogenes* CCs determined by subtyping of single isolates. Each column shows results from identification of 10 isolates, except where otherwise indicated by a number above the column. Labels 0, 4, and 24 refers to time-points before enrichment, 4 and 24 h primary enrichment in Half Fraser broth, respectively. FF indicates isolates sampled after secondary enrichment in Full Fraser broth.

samples were dominated by either *Pseudomonas* or *Staphylococcus* (Supplementary Figure 1C). The fifth *L. monocytogenes* negative sample, from drain C in week 8, showed a color change to brown in Full Fraser broth, indicating the presence of bacteria capable of hydrolysing esculin. This sample had a relative abundance of 99% *Listeria* in the secondary enrichment culture, presumably representing reads from *Listeria* spp. other than *L. monocytogenes*.

3.7 Diversity at species-level analyzed by shotgun metagenomics

To examine species distribution below genus level, samples from one of the drains which contained more than one *L. monocytogenes* ST – drain E in week 1 – was selected for further characterization. The samples collected prior to enrichment, during primary enrichment (t4 and t24), and after secondary enrichment were analyzed using shotgun metagenomics with Illumina sequencing. This analysis is usually termed "quasimetagenomics" when applied to selective enrichment cultures (Ottesen et al., 2020). The sequencing yielded between 3.6 and 7.2 million paired-end reads per sample. The selected drain sample was determined to be positive for *L. monocytogenes* after 4 h of primary enrichment, and ST9 and CC11-ST451 were detected after both primary and secondary enrichment.

The genus level results from the shotgun sequencing analysis shown in Figure 7 corresponded well with the data from 16S rRNA gene amplicon sequencing (Figure 3 and Supplementary Figure 1). For *Pseudomonas*, amplicon sequencing yielded relative abundances of 11%, 10%, 57% in samples collected prior to enrichment and at t4 and t24 during primary enrichment. The corresponding values from the shotgun metagenomic data were 13%, 12%, and 70%. The most dominant *Pseudomonas* species were *Pseudomonas extremaustralis* and *Pseudomonas lurida*, reaching 34% and 19%, respectively, of the reads classified to the *Pseudomonas* genus after 24 h of primary enrichment.

For *Listeria*, however, 16S rRNA gene amplicon sequencing yielded relative abundances of 0.08%, 0.10%, and 2.0% in these three samples, while shotgun metagenomics yielded notably lower values of 0.0019%, 0.0024%, and 0.01%. Of the 26 reads mapping to *Listeria* from shotgun sequencing of the sample collected prior to enrichment, 22 mapped specifically to the species *L. monocytogenes*.

Considering the total bacterial count in the sample prior to enrichment (7×10^9 TVC in 100 mL analyzed sample), the number of *Listeria* cells in the drain sample was estimated to 5.6×10^6 cfu per drain sample based on the relative abundance determined by the 16S rRNA gene amplicon sequencing, and 1.3×10^5 cfu per drain sample based on the shotgun sequencing data (1.6 log difference). For *Pseudomonas*, the corresponding values were slightly below 10^9 cfu per drain sample using both datasets (0.1 log difference). Assuming that the concentration of *L. monocytogenes* in the primary enrichment culture did not decrease during the initial 4 h of enrichment, and that the *Listeria* population was composed primarily of *L. monocytogenes*, these numbers can be compared with that obtained by plating on selective agar after 4 h of enrichment. Here, two colonies were obtained, and the detection limit was 10^3 cfu per sample, giving an estimated number of *L. monocytogenes* in the drain prior to enrichment of $\leq 2 \times 10^3$ cfu per sample. Both estimates based on sequencing data were substantially higher than this (3.5 log and 1.8 log higher), suggesting that they overestimated the relative abundance of *Listeria*.

For the secondary enrichment sample (t48), 16S rRNA gene amplicon sequencing indicated the presence of 74% *Listeria* and 24% *Enterococcus*, while the corresponding values obtained with the quasimetagenomics approach were 50% and 46%. *Enterococcus* is a major source of false positive test results with rapid swab tests for detection of *Listeria* spp. (Schirmer et al., 2012). The majority of the *Enterococcus* shotgun sequencing reads mapped either to *Enterococcus* sp. CR-Ec1 (10%), *Enterococcus gallinarum* (9%), *Enterococcus* sp. FDAARGOS_375 (7%), or *Enterococcus casseliflavus* (9%). Of the reads classified as *Listeria* spp., 2.0 million reads were classified as *L. monocytogenes* (93%), 13 056 as *Listeria innocua* (0.6%), 10,496 as *Listeria welshimeri* (0.5%), and 396 as other named *Listeria* spp. (*Listeria grayi*, *Listeria seeligeri*, *Listeria ivanovii*, and *Listeria weihenstephanensis*).

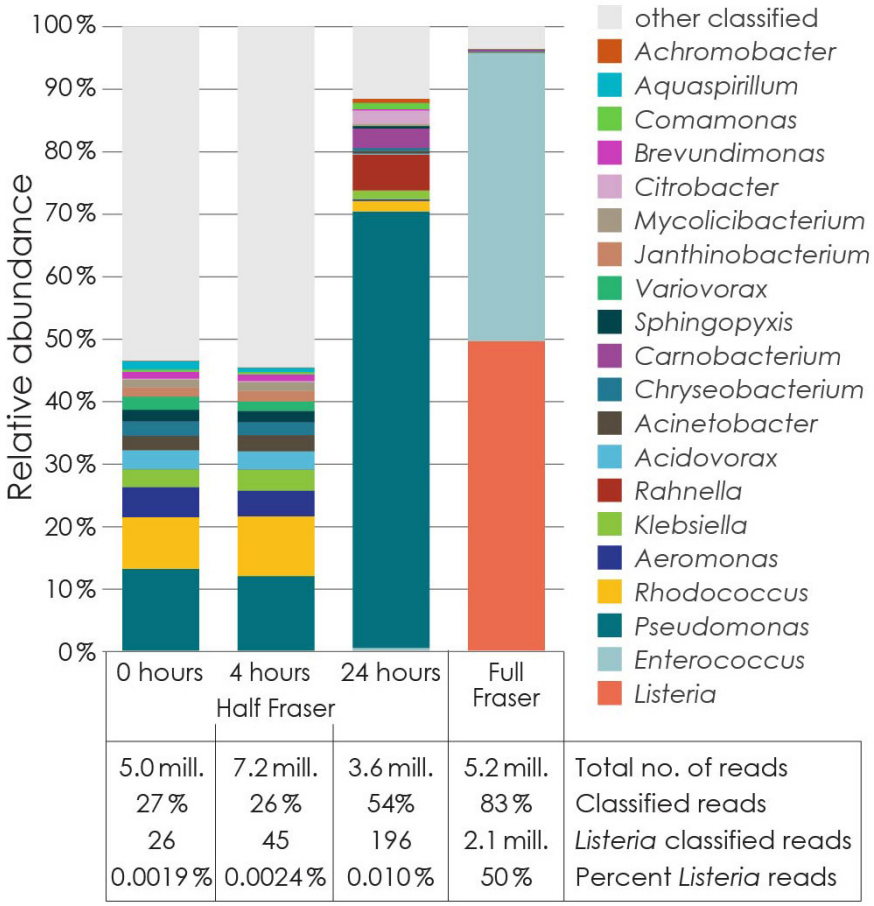

FIGURE 7
Genus level taxonomic assignment of reads from Illumina MiSeq metagenomic and quasimetagenomic shotgun sequencing of samples from drain E in week 1. Classified reads from genera with < 0.3% abundance (of total reads) in at least one of the four samples were combined and represented as "other classified." The table at the bottom shows, for each sample, the total number of reads, the percentage of classified reads, the number of classified reads classified as *Listeria* spp., and the proportion of classified reads classified as *Listeria* spp.

3.8 MLST and strain-level typing of shotgun sequencing data

The two *L. monocytogenes* colonies obtained for drain E in week 1 by plating on selective agar after 4 h of primary enrichment were identified as CC9. The 10 colonies picked after 24 h primary enrichment in Half Fraser and after the subsequent 24 h secondary enrichment in Full Fraser were identified as CC9 ($n = 9$ and $n = 8$, respectively) and CC11-ST451 ($n = 1$ and $n = 2$, respectively) (Figure 6). According to these results, the drain E week 1 enrichment culture was dominated by CC9 (~85%) with a minor proportion of CC11-ST451 (~15%).

To examine whether similar results could be obtained directly from the metagenomic or quasimetagenomic sequencing data, the data was analyzed using MetaMLST, which is designed to identify the dominant MLST profile in a sample by read mapping to a database of MLST alleles (Zolfo et al., 2017). MetaMLST detected ST9, belonging to CC9, with 100% confidence in the sample from the secondary enrichment in Full Fraser broth. Allele read depths were reported to range from 277x to 424x. As expected, due to few *Listeria* reads in the samples from the drain or from the primary enrichment, no ST was detected by MetaMLST in these samples.

As an alternative MLST subtyping approach, the *Listeria* spp. and *L. monocytogenes* reads from of each Illumina shotgun sequencing sample were mapped to the Institute Pasteur's *L. monocytogenes* MLST database using the KMA (*k*-mer alignment) method (Clausen et al., 2018). As for MetaMLST, no hits in the MLST profile database were obtained for the samples from the drain or from the primary enrichment. For the sample from the secondary enrichment, 15 MLST alleles were identified as perfect matches (100% identity and 100% coverage) by KMA (Supplementary Table 2). Seven alleles had read depths in the range 290x–430x, while the remaining alleles had read depths of 43x or lower. The seven alleles with read depths > 290x all had perfect matches in the KMA analysis, and corresponded to MLST profile 6-5-6-4-1-4-1, sequence type ST9. Furthermore, *bglA* allele 176, also a perfect match (read depth 4x), had exclusively been identified in ST1164, also belonging to CC9. This part of the analysis aligns with the output from MetaMLST.

Five of the eight remaining alleles with perfect matches identified in the KMA analysis had exclusively been identified in *L. innocua* and *L. welshmeri* strains (*abcZ* alleles 25 and 462, *bglA* allele 82, *dat* allele 20 and *ldh* allele 20). The last two perfect matches were *abcZ* allele 7 (read depth 11x) and *cat* allele 10

(read depth 43x). When examining all the possible MLST profile combinations containing these two alleles plus *bglA*, *dapE*, *dat*, *ldh* and *lhkA* alleles – including alleles that were not perfect matches – three possible MLST profiles were identified. All three belonged to CC11, and were ST451 with MLST profile 7-5-10-21-1-4-1, ST1558 with MLST profile 7-5-10-21-127-4-1 and ST2088 with MLST profile 7-332-10-21-1-4-1. The *dapE* allele 21 (present in all of these three STs) had a read depth of 6x, the *bglA* allele 332 had read depth 4x, while the *dat* allele 127 had read depth 1.4x. The percentage match (for both identity and coverage) for these three alleles were 96%, 99%–100%, and 76%, respectively.

Finally, the data was analyzed using the strain-typing tool StrainScan (Liao et al., 2023). This tool clusters a set of selected reference genomes based on *k*-mer Jaccard similarity and uses a hierarchical *k*-mer based indexing structure to compare sequencing reads to the reference genomes within each cluster. A database was built using one representative genome from each of the six CCs identified in the meat processing factory (CC9, CC11-ST451, CC121, CC8, CC199, and CC14-ST91), which resulted in six cluster search trees, each containing one strain. When running StrainScan with default settings, the relative abundances of *L. monocytogenes* strains in the secondary enrichment culture was predicted to be 85% of the representative CC9 genome and 15% of the representative CC11-ST451 genome. Predicted read depths were 165x and 29x, respectively. Notably, slightly different results were obtained when the database was built using the genomes of all 72 sequenced isolates from the processing factory (listed in Supplementary Table 1). In this case, the secondary enrichment culture was predicted to contain 94% CC9 and 6% CC11-ST451. The bias in representation of the two CC groups in this database (50 CC9 genomes and only three CC11-ST451 genomes) potentially influenced the outcome. No clusters were detected in the drain or primary enrichment samples when running StrainScan with default settings.

StrainScan was also run using settings that adjust the filtering cutoffs of the *k*-mer tree to better accommodate low sequencing depths, with a trade-off of lower confidence predictions. In this mode, the program detected 75% CC9, 13% CC11-ST451, 9% CC8, and 2% CC199 in the secondary enrichment culture, with associated read depths of 165x, 29x, 21x, and 5x, respectively. Additionally, for the 24 h primary enrichment sample, the program detected 17% CC9 and 83% CC8, with read depths of 1x and 5x, respectively. These results did not align with the MLST subtyping of single isolates (Figure 6), however, they indicated the possible presence of additional CCs at very low prevalence.

Overall, the data showed that corresponding results were obtained by picking and subtyping single colonies after primary or secondary enrichment and performing metagenomic sequencing of the secondary enrichment culture. All applied approaches showed that the secondary enrichment culture was dominated by CC9. Analysis using KMA and StrainScan was also able to predict a minor proportion of CC11 strains of (or related to) ST451.

4 Discussion

4.1 *L. monocytogenes* genetic diversity and persistence

Four different *L. monocytogenes* CCs were identified in the 24 samples from six floor drains examined in the current study (CC9, CC11-ST451, CC121, and CC8). Different CCs dominated in the different drains, and this remained consistent during the examined period of 9 weeks. The dominating CC appeared to correlate with the proximity of drains and whether the drains were wet or dry: Drains A, B, and F were in close proximity, all were usually dry and received limited levels of soiling and effluent, and all were dominated by CC11-ST451 isolates, with only a minority of CC9 detected. In contrast, drains C, D, and E, which were located in another part of the same processing area, were usually humid, dominated by CC9 isolates, but with higher diversity than the other drains. Three or four CCs were detected in each of these drains during the course of the monitoring period. Interestingly, the overall microbial richness (Shannon diversity) was significantly higher in the humid than the dry drains, mirroring the diversity of the CCs.

It is not necessarily evident whether a *L. monocytogenes* strain persists in a drain or whether the drain acts as a collector site for strains that persist in other niches in a facility. Potentially, drains in close proximity are more likely to be exposed to the same contamination source, e.g., during cleaning. An unknown factor in the current study was also whether or not any of the examined drains were directly connected by drainpipes beneath the floor. However, the observed stability of the microbial composition (both on genus and CC level) probably indicates that the drains represent a stable niche in which *L. monocytogenes* can persist. In either case, floor drains, along with other floor-associated sampling points, are important hygiene indicator sites for monitoring of *L. monocytogenes* in food processing facilities (Dzieciol et al., 2016; Simmons and Wiedmann, 2018).

4.2 Detection and diversity during selective enrichment

In three of the six examined drains, up to three co-occurring CCs were detected in the same sample. In some cases, different minority CCs were detected after primary and secondary enrichment. This underscores the importance of selecting more than one isolate for subtyping during investigation of contamination routes or persistence, as subtyping of only one single isolate from each sample may result in erroneous conclusions, e.g., during source tracking (Döpfer et al., 2008; Chen et al., 2020; Stessl et al., 2020; Wagner et al., 2021; Acciari et al., 2022).

L. monocytogenes was detected by plating on selective agar without prior selective enrichment in one-third of the examined samples. Culture-independent metagenomic shotgun sequencing also detected *L. monocytogenes* reads prior to enrichment. However, few colonies and very few shotgun sequencing reads were retrieved without enrichment. This approach was therefore not suited to capturing the diversity of CCs in the samples. Equivalent

results were obtained for samples collected after 4 h of primary enrichment, in line with the limited growth and shift in the microbiota observed during the first 4 h of primary enrichment. Given the low number of shotgun sequencing reads classified as *L. monocytogenes* prior to enrichment, the higher values of relative abundance of *Listeria* in the drains estimated from sequencing data compared to from culturing methods, and known technical issues related to quantifying low abundance species in metagenomics data (further discussed below), it is challenging to ascertain whether *L. monocytogenes* was truly detected by direct metagenomic sequencing of drain microbiomes from naturally contaminated drains prior to selective enrichment.

The metagenomic approach applied to the secondary enrichment culture, which had a high relative abundance of *L. monocytogenes*, successfully detected both CCs identified by subtyping of 22 single isolates from the same culture. Additionally, this approach matched the relative abundances of CC9 and CC11-ST451 found by isolate subtyping. To capture diversity and the correct relative abundances of CCs, it is therefore advisable to pick and subtype multiple single colonies after 24 h of primary enrichment, or perform metagenomic sequencing on secondary enrichment cultures.

Analysis of metagenomic sequence data is still challenging, and requires selecting appropriate methods depending on the research question and dataset (Anyansi et al., 2020). An assembly-based approach involving metagenome-assembled genomes (MAGs) has been used for phylogenetic analysis of *L. monocytogenes* in food processing environments, but this method is unsuitable for multi-strain samples and requires high sequencing depth (Kocurek et al., 2023). In this study, the aim was to identify multiple MLST subtypes, including low-abundance subtypes, in multi-strain samples. For this purpose, alignment-based methods were selected, in which sequencing reads are aligned to reference databases. Two MLST database mapping approaches were used. While only the most dominant subtype was identified using MetaMLST (Zolfo et al., 2017), two different subtypes could be inferred from the KMA analysis output (Clausen et al., 2018) based on differential read depth coverage for each MLST allele. An algorithm integrating allele combinations (STs), CC prevalence data, and read depth would have simplified the analysis of the KMA output. Nevertheless, both MLST-based methods require significant sequencing depth, as they utilize only a small portion of the *L. monocytogenes* metagenome data.

Full genome alignment-based methods aim to differentiate metagenomic sequencing data at the strain level by aligning reads to a reference database of complete genomes, and assigning strains to the closest reference using probabilistic models (Anyansi et al., 2020). StrainScan, used in this study, organizes reference genomes into a hierarchical cluster tree and assigns metagenomic data to the nearest cluster, and within the cluster, to the nearest reference, using a *k*-mer based search strategy (Liao et al., 2023). The definition of a strain is, however, not clear-cut (Lindner et al., 2024), and assigning to a reference genome does not inherently solve assignment to a MLST subtype. The analysis requires that the CC type in the metagenome matches a CC in the reference database, allowing inference of the subtype. However, with adequate database representation, clustering methods like StrainScan are expected to work well for the species *L. monocytogenes*, as CCs largely correspond to clear and well-separated sublineages defined by phylogenetic analyses (Chen et al., 2016; Moura et al., 2016). Accordingly, StrainScan's reference genome clusters showed a one-to-one correspondence with the six CCs included in the reference databases in the current study. Further analysis is, however, needed to confirm this for all *L. monocytogenes* CCs. The authors of StrainScan reported that the program showed high specificity and sensitivity in assigning strains down to 1x read depth (Liao et al., 2023). However, our findings indicate limitations in estimating the relative abundances of low-abundance strains and potential biases in these abundance estimates introduced by the composition of the reference database.

Estimates of absolute abundances, based on total bacterial counts, suggested that the 16S rRNA gene amplicon and shotgun metagenomics sequencing data overestimated the relative abundance of *Listeria* in the drains compared to values obtained from plating on *L. monocytogenes* selective agar plates. Such overestimation could be due to "sample bleeding," i.e., incorrect assignment of an index (barcode) to a sequence from an adjacent cluster on the flow cell (Mitra et al., 2015). These issues are particularly noticeable in sequencing runs that include libraries with low sequence diversity, which was the case for most of the secondary enrichment samples in this study, which almost exclusively contained *Listeria*. Furthermore, the 16S rRNA gene amplicon sequencing approach, which used single barcodes, was expected to be more affected compared to the shotgun sequencing approach, which used dual barcodes. Quantification of taxa-specific abundances in microbial communities using sequencing based methods are known to be subject to various errors limiting direct comparison with culture-based quantifications, including DNA extraction efficiency, PCR-associated bias, variation in 16S rRNA operon copy numbers per genome, and inability to easily distinguish between viable and non-viable cells (Bonk et al., 2018). These errors can lead to significant deviations from true values, especially for low-abundance taxa ($< 10\%$) (Tettamanti Boshier et al., 2020).

4.3 Molecular subtyping to CC level using qPCR

The Geno*Listeria* Multiplex qPCR scheme (Félix, 2023; Félix et al., 2023) has been referred to as a frontline screening tool for *L. monocytogenes* CCs, suitable for initial identification prior to selection of isolates for in-depth characterization using WGS, in the event of e.g., outbreaks (Jashari et al., 2024). The method was also a well-suited and cost-effective approach for the current study, where 460 isolates were subtyped. Sequential identification of CCs and selection of relevant qPCR reactions, based on previously identified CCs in the sampled environment, enabled the identification of 93.5% of the isolates in the dataset using approximately twice as many qPCR reactions as there were isolates to be subtyped. In our hands, the total costs (time cost plus direct costs) of strain typing would have been around six-fold more expensive had WGS been run for all DNA samples, compared with the combined qPCR / WGS approach. The use of the method, however, requires careful consideration of the chosen amplification cycle threshold (Ct) that differentiates positive and negative analysis results. The original method indicates that reactions with Ct $\leq$ 25 should be considered

positive (Félix, 2023; Félix et al., 2023), while another study set the Ct cutoff at ≤ 30 (Jashari et al., 2024). In the present work, amplifications with Ct < 18 were considered positive, and in several cases, calling detections with Ct values in the range 20–25 as positive would have given erroneous results.

The genetic marker *lmo1118* used to detect CC9 in the Geno*Listeria* scheme (Félix, 2023; Félix et al., 2023) is the same as the marker used to identify PCR-serogroup IIc (Doumith et al., 2004; Vitullo et al., 2013). Félix et al., 2023 reported that 3% of CC9 genomes in a panel comprising 142 CC9 isolates lacked *lmo1118*, while no false-negative results were reported for CC9 (n = 38) in another report (Jashari et al., 2024). In the current study, 12% of all typed CC9 isolates (n = 212), and 32% (n = 22) of CC9 isolates previously identified by WGS from the same meat processing factory (Fagerlund et al., 2022; Ivanova et al., 2025), lacked the *lmo1118* marker. Potentially, it is possible to identify a more suitable molecular target for CC9. In comparison, an *in silico* analysis of 1241 Norwegian *L. monocytogenes* isolates using the GENE-UP Typer from bioMérieux was able to correctly identify 99.7% of the 313 CC9 isolates included in the analysis (Fagerlund et al., 2024). This method uses a kit with eight duplex qPCR reactions comprising 16 target marker genes, followed by analysis using probabilistic algorithms to predict the subtype based on an underlying WGS-based phylogeny.

Although WGS is the gold standard subtyping method (Lakicevic et al., 2023), new simpler methods such as Geno*Listeria* Multiplex and GENE-UP Typer continue to emerge, suggesting that there is still a need for alternative genotyping methods for pathogens such as *L. monocytogenes*. This likely reflects current challenges related to the cost and analysis time of WGS. Ideally, an alternative subtyping method should be fast, practical, and able to distinguish known genetic groups identified from WGS-based studies (i.e., CCs for *L. monocytogenes*) with high sensitivity and specificity. Compared to methods like PFGE and MLVA, PCR-based assays have the advantage of being concordant with *in silico* predictions of subtyping results from WGS data, also when WGS is performed using short read sequencing technology (Kwong et al., 2016). This is particularly relevant when a combination of methods is used, such as using simple genotyping on many (or most) isolates and WGS on a selection or for untypeable isolates, or when there is a need to compare results with externally available WGS data. In this context, PCR-based genotyping methods represent a sustainable option until technological developments or increased usage reduce the cost and analysis time for WGS. Additionally, it would be interesting to explore whether PCR-based genotyping is suitable for analyzing DNA obtained directly from enrichment culture samples and if it can identify more than one CC present in a sample.

4.4 Microbial composition and association with *L. monocytogenes*

Floor drains are colonized by different microbes that can persist over time despite cleaning and disinfection (Dzieciol et al., 2016; Belk et al., 2022). This resident microbiota consists mainly of non-pathogenic bacteria but may also be a reservoir for pathogens like *L. monocytogenes* (Fagerlund et al., 2021), and several studies have identified drains as common sites for persistent *L. monocytogenes* (Burnett et al., 2020; Belias et al., 2022; EFSA Panel on Biological Hazards (BIOHAZ) et al., 2024). There is ongoing debate regarding the association between the microbial composition and the presence or absence of *L. monocytogenes* in samples or biofilms from the food industry. For example, it has been shown that *L. monocytogenes* can be established in *Pseudomonas* biofilms (Fagerlund et al., 2021), while other studies have shown lower abundance of specific *Pseudomonas* species in *Listeria*-positive compared to *Listeria*-negative samples (Pracser et al., 2024). A wide range of biofilm producers have been associated with the presence of *Listeria* spp. in food processing plants, including the most abundant genera observed in drains in the current study; i.e., *Pseudomonas*, *Acinetobacter* and *Janthinobacterium* (Zwirzitz et al., 2021; Belk et al., 2022; Pracser et al., 2024). The presence of *Janthinobacterium* has, however, also been associated with lower prevalence of *L. monocytogenes* positive drains (Fox et al., 2014).

Pseudomonas was the most abundant and consistently found genus in the majority of drains in the current study. It is frequently found in food processing plants (Møretrø and Langsrud, 2017). It was also recently reported to be the most abundant and stable component of the microbiota on floor surfaces and drain in a RTE meat factory (Diaz et al., 2025), and in drains in a small meat production facility (Belk et al., 2022). Both studies found that the drain microbiota composition remained relatively stable over time.

Since *Pseudomonas* is commonly found on both raw materials and food contact surfaces (Møretrø and Langsrud, 2004), its presence in drains is likely not significant for the food quality of the final product. In this study, all drains were positive for *L. monocytogenes* in at least one sampling week, while Diaz et al. (2025) found few *L. monocytogenes* positive samples overall. Although it has been suggested that *Pseudomonas* may affect the growth and survival of *L. monocytogenes* in biofilms (Fagerlund et al., 2021), our study does not support this claim, as no clear association between resident drain microbiota and the presence or absence of *L. monocytogenes* was found for either study.

Drains are among the most common harbourage locations for *Listeria* spp. in food processing facilities. Current monitoring approaches may underestimate the persistence of the same clone over time. Therefore, implementing methodology capable of detecting multiple clones within shorter timeframes should be encouraged. Corrective actions beyond regular cleaning and disinfection should be initiated in case of repeated detection of *L. monocytogenes* in drains. According to guidelines for food processers, extended exposure time (overnight) of cleaning agents or disinfectants combined with brushing may resolve issues with persistent *L. monocytogenes* (Fagerlund et al., 2020, Tuytschaever et al., 2023).

5 Conclusion

The current study highlights the use of alternative techniques such as qPCR and quasimetagenomics, which may be more cost-effective and rapid, for monitoring and mitigating the risk of contamination in processing environments. Despite being the method with highest sensitivity and specificity, WGS may not always be practical, e.g., when multiple subtypes of

L. monocytogenes are present in the same sample. In that regard, the study demonstrated that shotgun metagenomic sequencing of a secondary enrichment culture provided information about the relative abundance of *L. monocytogenes* clonal complexes (CCs) in a sample, comparable to the information obtained from identifying multiple single isolates after primary or secondary enrichment. It should be noted, however, that results from both metagenomic sequencing analyses and the Geno*Listeria* Multiplex qPCR may be challenging to interpret, taking into account e.g., potential biases and errors in metagenomic sequencing and data processing, and challenges with lack of genetic markers and selection of Ct cutoffs for qPCR reactions.

In conclusion, this study provides valuable insights into the microbiota and population dynamics of *L. monocytogenes* in drains in a meat processing environment. The persistence and genetic diversity of *L. monocytogenes* underscore the importance of implementing new tools for surveillance of this pathogen in food processing facilities. Future research should focus on further elucidating the interactions between *L. monocytogenes* and the resident microbiota, as well as developing and validating more effective monitoring techniques to mitigate the risk of *L. monocytogenes* contamination in the food supply chain.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

AF: Writing – original draft, Conceptualization, Data curation, Formal Analysis, Visualization, Writing – review and editing. TM: Writing – review and editing, Conceptualization, Supervision. MJ: Writing – review and editing, Data curation, Formal Analysis, Investigation. SL: Writing – review and editing, Conceptualization, Project administration. BM: Writing – original draft, Conceptualization, Data curation, Formal Analysis, Visualization, Writing – review and editing.

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Conflict of interest

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

Generative AI statement

The authors declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

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Exploration of the biodiversity and mining novel target genes of *Listeria monocytogenes* strains isolated from beef through comparative genomics analysis

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L. monocytogenes is a significant foodborne pathogen. This study aims to explore the biodiversity and evolutionary characteristics of *L. monocytogenes* isolated from beef through pan-genome analysis, and to provide important reference value for its specific molecular detection. This study conducted an in-depth analysis of the virulence genes, antimicrobial resistance genes, and environmental resistance genes of 344 *L. monocytogenes* strains isolated from beef. Pan-genomic analysis revealed that *L. monocytogenes* from beef have open genomes, providing a solid genetic basis for adaptation to different environments. MLST analysis revealed that the most prevalent types of *L. monocytogenes* isolated from beef were ST9 and CC9. A total of 50 virulence genes were detected in these strains, with 26 virulence genes such as *inlA*, *inlB*, *plcA*, *plcB*, and *prfA*, present in all *L. monocytogenes* strains. The four most prevalent antibiotic resistance genes in *L. monocytogenes* were *norB*, *lin*, *mprF*, and *FosX*, indicating high resistance to fluoroquinolones, lincosamides, peptides, and phosphonic acid antibiotics. A total of 416 potential target genes were identified through pan-genomic screening, which were then further filtered using a hub gene selection method to mining novel target genes. Ultimately, 10 highly connected hub genes were selected: *bglF_2*, *tilS*, *group_2105*, *group_2431*, *oleD*, *ndk*, *flgG*, *purB*, *pbpB*, and *fni*. These genes play a crucial role in the pathogenesis of *L. monocytogenes*. The PCR results demonstrated the excellent specificity of the *bglF_2* gene for *L. monocytogenes*. Moreover, in the artificial contamination experiment, the *bglF_2* gene was able to effectively detect *L. monocytogenes* in beef samples. Therefore, the *bglF_2* gene holds potential as a specific molecular target for the detection of *L. monocytogenes* strains in beef samples.

KEYWORDS

***Listeria monocytogenes*, comparative genomics, pan-genomics, genetic evaluations, target genes**

Introduction

L. monocytogenes is a foodborne pathogen belonging to Gram-positive bacteria. This highly adaptable bacterium can thrive under adverse conditions, including low temperatures, high salinity, and extreme pH levels. It is commonly associated with food contamination, particularly in meat products (Zhang et al., 2021; Montero et al., 2015). It can cause listeriosis in humans, particularly in populations with compromised immune function, such as neonates, the elderly, pregnant women, and individuals with weakened immune systems. The onset of the disease may be accompanied by various symptoms, including mild diarrhea, meningitis, and septicemia (Matle et al., 2020; Colomba et al., 2020). Listeriosis is typically caused by the consumption of contaminated food, making food safety particularly important for the prevention and control of the disease.

The detection of *L. monocytogenes* in food typically involves biochemical and molecular methods (Gupta and Adhikari, 2022; Law et al., 2015). Biochemical methods are conventional and accurate, but require a 7-day bacterial incubation period followed by morphological, biochemical, and serological confirmation. This approach is labor-intensive and time-consuming, making it unsuitable for rapid detection (Sloan et al., 2017). Molecular methods, on the other hand, can shorten the detection time to a few hours, allowing for precise and rapid identification of the bacteria (Buszewski et al., 2017; Feng et al., 2020). Molecular techniques developed for *L. monocytogenes* detection include PCR, quantitative PCR (qPCR), and multiplex PCR (Liu, 2013). The *hly* gene is an important virulence factor in the infection process of *L. monocytogenes*. It encodes a toxin called listeriolysin O (LLO), which facilitates the escape and infection of the host by *L. monocytogenes*. As a result, the *hly* gene has been widely used in PCR-based detection for identifying *L. monocytogenes* (Le et al., 2011). However, it is important to note that not all *L. monocytogenes* strains carry the *hly* gene (Dapgh and Salem, 2022; Ata et al., 2017; Li et al., 2021), indicating the need to mining more specific genes for the detection of *L. monocytogenes*.

In recent years, with the widespread application of next-generation sequencing and third-generation sequencing technologies, a large number of *L. monocytogenes* genomes have been sequenced and shared (Lakicevic et al., 2023; Fagerlund et al., 2022; Fox et al., 2016). This study aims to explore the biodiversity and evolutionary characteristics of *L. monocytogenes* isolated from beef through pan-genome analysis, and to provide important reference value for its specific molecular detection. Therefore, we conducted a comparative genomics study on all *L. monocytogenes* strains isolated from beef in the NCBI database. A pan-genomic analysis and Multilocus Sequence Type (MLST) phenotypic analysis were performed for each *L. monocytogenes* strain. Pan-genomic analysis enables the identification of potential target genes in the strains. Functional analysis of the potential target genes in *L. monocytogenes* was conducted using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations. Furthermore, a protein–protein interaction (PPI) network was constructed for potential target genes of *L. monocytogenes*, and eight different hub gene analysis methods were utilized to screen novel target genes from the potential target genes. Finally, the virulence genes, antimicrobial resistance genes, CRISPR-Cas system, plasmids, and environmental resistance genes of each *L. monocytogenes* strain were investigated to explore their biodiversity and genetic determinants.

Materials and methods

Data availability and processing

A total of 356 genomic sequences were retrieved and downloaded from the NCBI Genomic Database (last accessed on July 13, 2024), including 344 *L. monocytogenes* strains isolated from beef, as well as genomic sequences from 5 other *Listeria* species and 7 *non-Listeria* bacterial species. The comparative genomic analysis of *L. monocytogenes* with 5 other *Listeria* species effectively distinguishes *L. monocytogenes* from other *Listeria* species. Similarly, the comparative genomic analysis with 7 *non-Listeria* bacterial species allows for effective differentiation of *L. monocytogenes* from *non-Listeria* bacteria. The concrete information of *L. monocytogenes* in research is summarized in Supplementary Tables S1, S2, including GenBank accession numbers, strain names, genome size, GC content, number of contigs and N50.

Pan-genomics analysis of *Listeria monocytogenes* strains isolated from beef and non-targeted strains

The analysis of pan-genomic comparison of *L. monocytogenes* and non-target strains can be used to screen potential target genes. The potential target genes refer to those genes that are unique to *L. monocytogenes* strains and are absent in non-target strains (Li et al., 2021; Zhang et al., 2024). Our study found that the commonly used molecular detection target gene, *hly*, is not present in all strains of *L. monocytogenes*. Therefore, it is essential to identify potential target genes that are present in all strains of *L. monocytogenes* as new detection targets (Dapgh and Salem, 2022; Ata et al., 2017; Li et al., 2021). Perform pan-genomic analysis on 344 target strains of *L. monocytogenes* and 12 non-target reference strains. In brief, all analyzed genome sequences were annotated using Prokka v1.14.6 (Seemann, 2014), and the output results of Prokka were used for pan-genomic analysis with Roary v3.11.2 (Page et al., 2015). A core genome was determined for each isolate using a 99% cutoff, with a BLASTP identity cutoff of 85% (Pang et al., 2019). Genes that matched with all *L. monocytogenes* strains genome sequences were considered highly conserved and used for subsequent comparisons with other *Listeria* species and *non-Listeria* bacterial genomes.

Pan-genome clusters were defined as core-genes: present in all isolates; soft-core genes: present in at least 95% of isolates; shell-genes (accessory genes): present between 15 and 95% of isolates; and cloud-genes (unique genes): present in less than 15% of isolates (Mafuna et al., 2022).

The potential target genes were screened according to the following criteria: 100% presence in *L. monocytogenes* strains and no presence in non-target bacterial strains. Then, these potential target genes were used screened against the nucleotide collection (nr/nt) databases using the online BLAST program to ensure specificity (Zhang et al., 2024).

MLST and phylogenetic analysis

Perform MLST analysis on the genome of *L. monocytogenes* to predict sequence types (STs), clonal complexes (CCs), and lineages.

This analysis involved retrieving seven housekeeping genes from each *L. monocytogenes* genome using the MLST database: *abcZ* (ABC transporter), *bglA* (beta-glucosidase), *cat* (catalase), *dapE* (succinyldiaminopimelate desuccinylase), *dat* (D-alanine aminotransferase), *ldh* (lactate dehydrogenase), and *lhkA* (histidine kinase). These genes were employed to determine the STs, CCs, and lineages of *L. monocytogenes* (Wu et al., 2016; Wang et al., 2021).

To investigate the phylogenetic relationships among 344 strains of *L. monocytogenes* isolated from beef, MEGA software was utilized to construct a phylogenetic tree based on all core single-copy genes of *L. monocytogenes* (Li et al., 2021). Additionally, the ST and CC typing of each strain were annotated on the tree.

Functional characteristics of potential target genes

In order to investigate the functional characteristics of genes present exclusively in *L. monocytogenes* strains and absent in non-target bacterial strains (potential target genes), annotation analysis was performed using GO enrichment analysis and KEGG enrichment analysis (Adnan et al., 2022), and the results were integrated.

PPI network analysis and screening of novel target genes

In this study, the STRING database was used to establish PPI networks, and these networks were visualized using Cytoscape v3.10.2 (Lu et al., 2022). Cytoscape is a general-purpose modeling environment for integrating biomolecular interaction networks and states. The CytoHubba function in Cytoscape was used to identify hub genes (novel target genes) from the PPI network. CytoHubba ranks genes in the PPI network using eight different algorithms, including Degree, Betweenness, BottleNeck, Closeness, Edge Percolated Component (EPC), Maximum Neighborhood Component (MNC), Maximum Clique Centrality (MCC), and Stress. The top 10 genes with the highest scores in each algorithm were selected as hub genes (Zhang et al., 2024).

Prediction of virulence factors and antimicrobial resistance genes of *Listeria monocytogenes*

Identifying the virulence and antimicrobial resistance genes of *L. monocytogenes* is crucial for understanding its genetic determinants. Therefore, we used the Virulence Factors of Pathogenic Bacteria (VFDB) database and the Comprehensive Antibiotic Resistance Database (CARD) to predict the virulence and resistance genes of *L. monocytogenes* strains (Zhu et al., 2023), and integrated the results for presentation using a heatmap.

Prediction of CRISPR-Cas system types and plasmids of *Listeria monocytogenes*

The presence of the CRISPR-Cas system and plasmids can facilitate the evolution of *L. monocytogenes*, contributing to our understanding of its genetics. Therefore, we used CRISPRCasFinder and the PLSDB plasmid database to predict the CRISPR-Cas system and plasmids in *L. monocytogenes* strains (Di et al., 2014; Zhang et al., 2016), and integrated the results for presentation using a heatmap.

Prediction of *Listeria* genomic Islands and stress survival Islands of *Listeria monocytogenes*

The presence of *Listeria* genomic Islands (LGIs) and survival Islands (SSIs) can enhance the resistance of *L. monocytogenes* to harsh environments, contributing to our understanding of its adaptability. Therefore, we used the BIGSdb database to predict the LGIs and SSIs in *L. monocytogenes* strains (Mafuna et al., 2021), and integrated the results for presentation using a heatmap.

Design and validation of specific primers for *Listeria monocytogenes*

Primers for the *bglF_2* gene sequence were designed using Primer Premier 5 software (Table 1) (He et al., 2022). The primers were synthesized by Sangon Biotech Co., Ltd., Shanghai, China. PCR experiments on some of the analyzed strains to detect primer specificity. Total reaction volume was 25 μL, including 12.5 μL of 2 × Es Taq MasterMix (CWBIO, Beijing, China), 1 μL each of for ward and reverse primers (10 μM), 8.5 μL of sterile water, and 2 μL of the purified bacterial genomic DNA as a template. An equal volume of sterile distilled water was used instead of the template as a negative control. PCR thermal cycling involved an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min, with a final elongation at 72°C for 10 min. PCR products were evaluated by 2% agarose electrophoresis.

Artificial contamination experiments

To ensure the specificity and efficacy of the *bglF_2* gene, the primers designed for this gene were validated through artificial contamination

TABLE 1 Specific target gene and primers used for the detection of *L. monocytogenes* isolated from beef.

Gene	Sequence length/bp	Primer	Sequence (5′/3′)	Encoded protein	Product size/bp
bglF_2	1,853	bglF_2F	TCGGAAATGACGTGCCTAAAGTGTT	PTS system beta-glucoside-specific EIIBCA component	464
		bglF_2R	ATCGGAATAACAGAGTAAGC		

experiments to assess their effectiveness in detecting *L. monocytogenes* in beef samples. In brief, the *L. monocytogenes* EGD-e strain was cultured overnight, and bacterial concentration was determined using the plate count method. Beef samples (10 g) obtained from a slaughterhouse in Changchun, China were minced and exposed to ultraviolet light for 30 min to ensure sterility. The samples were then added to 88 mL of Milli-Q water and incubated for 30 min. The mixture was centrifuged at 8,000 rpm for 20 min, and the supernatant was filtered through a 0.22 μm filter to obtain the simulated detection solution. Finally, 2 mL of the cultured EGD-e was added to the simulated detection solution, and the mixture was thoroughly mixed to complete the artificial contamination. After that, the mixtures were incubated at 37°C for 4 h,6 h, 8 h,12 h, and 24 h. Genomic DNA was extracted at the indicated time points from sample, and then analyzed by PCR (Li et al., 2021).

Results

Genome statistics and general features

In the NCBI database, we downloaded the whole-genome sequences of all *L. monocytogenes* strains isolated from beef, totaling 344 strains, and compiled the corresponding information for these strains (Supplementary Tables S1, S2). They possess an average genome size of 3.061 (2.9–3.6) Mbp, an average GC content of 37.986 (37.5–38)%, the number of contigs ≤ 312, and an average N50 of 387.121 Kbp.

Pan-genomic analysis results

According to the pan-genome analysis, the distribution of pan-genes in 344 *L. monocytogenes* strains is as follows: 2001 core genes (15.58%), 187 soft core genes (1.46%), 1,374 shell genes (10.70%), and 9,278 cloud genes (72.26%) (Figure 1A). As the number of strains increases, the number of pan-genes gradually increases, while the number of core genes tends to stabilize, indicating that *L. monocytogenes* possesses an open genome, which is closely related to their adaptability and virulence expression in beef environments under different conditions. To identify potential target genes, we also performed pan-genome analysis on 344 *L. monocytogenes* strains and 12 non-target strains. The results of the pan-genome analysis are as follows: the number of core genes is 0, soft core genes are 2,098, shell genes are 1,439, cloud genes are 41,690, and the total number of pan-genes is 45,227. Due to the presence of non-target strains, no common core genes were identified. However, potential target genes can be screened by comparing genes present in the non-target strains. Based on the aforementioned screening method, a total of 416 potential target genes were identified in *L. monocytogenes* strains (Supplementary Table S3). These potential target genes were present in 100% of the *L. monocytogenes* strains isolated from beef, but absent in non-target strains. These potential target genes may serve as novel target genes for *L. monocytogenes* strains isolated from beef, but further screening is required to validate these potential target genes.

MLST and phylogenetic analysis

By uploading the whole-genome sequences of 344 *L. monocytogenes* strains to the database for comparison, these strains were classified into 52 distinct ST types and 34 different CC types. However, 18 strains did not successfully match any corresponding types in the database. The most common ST types were ST9 (28.20%) and ST5 (8.43%), while the most common CC type was CC9 (30.23%). The strains were divided into two evolutionary lineages: lineage I (30.23%) and lineage II (64.24%) (Figure 1B; Supplementary Table S4).

To elucidate the evolutionary patterns of *L. monocytogenes* strains isolated from beef, we performed a phylogenetic analysis of *L. monocytogenes* using the conserved amino acid sequences of all single-copy genes (Figure 1B). Phylogenetic tree analysis revealed that the *L. monocytogenes* strains isolated from beef share a common ancestor and belong to the main root of the *Listeria* genus. Most *L. monocytogenes* strains isolated from the same region clustered into the same branch, suggesting a distinct regional pattern in the evolution of *L. monocytogenes*. However, some strains, although isolated from different regions, were grouped into the same branch, indicating a certain level of evolutionary similarity and strong adaptability of *L. monocytogenes* to different environments.

Enrichment analysis of the functional characteristics of potential target genes using the GO and KEGG databases

To investigate the functional characteristics of the 416 potential target genes from *L. monocytogenes* strains isolated from beef, we performed functional annotation and classification of these genes using the GO and KEGG databases. The detailed information of the potential target genes is presented in Supplementary Table S3. The pathway database of KEGG is the most widely used public database for metabolic pathways, which classifies biological metabolic pathways into six categories: Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, Organismal Systems, and Human Diseases. The potential target genes were annotated according to six pathway categories in the KEGG database. Among these, the most enriched pathways in the Metabolism category were metabolism of cofactors and vitamins ($n = 49$, 11.78%), carbohydrate metabolism ($n = 43$, 15.87%), and amino acid metabolism ($n = 24$, 5.78%). In the Genetic Information Processing category, replication and repair ($n = 9$, 2.16%) was the most enriched pathways. In the Environmental Information Processing category, membrane transport ($n = 18$, 4.33%) and signal transduction ($n = 5$, 1.20%) were the most enriched pathways. In the Cellular Processes category, cellular community—eukaryotes ($n = 2$, 0.48%) and cell motility ($n = 2$, 0.48%) were the most enriched pathways. In the Organismal Systems category, endocrine system ($n = 2$, 0.48%) was the most enriched pathways. In the Human Diseases category, drug resistance: antimicrobial ($n = 5$, 1.2%) was the most enriched pathways (Figure 2A). We integrated and ranked the KEGG enrichment analysis results of all potential target genes, and generated bubble maps to show the top 20 functional features in KEGG enrichment analysis based on the significance of gene number and *p*-value (Figure 2C).

The GO database categorizes gene functions into three main categories, namely Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF). In the BP category, the most enriched biological processes were primary metabolic process ($n = 84$, 20.19%), cellular metabolic process

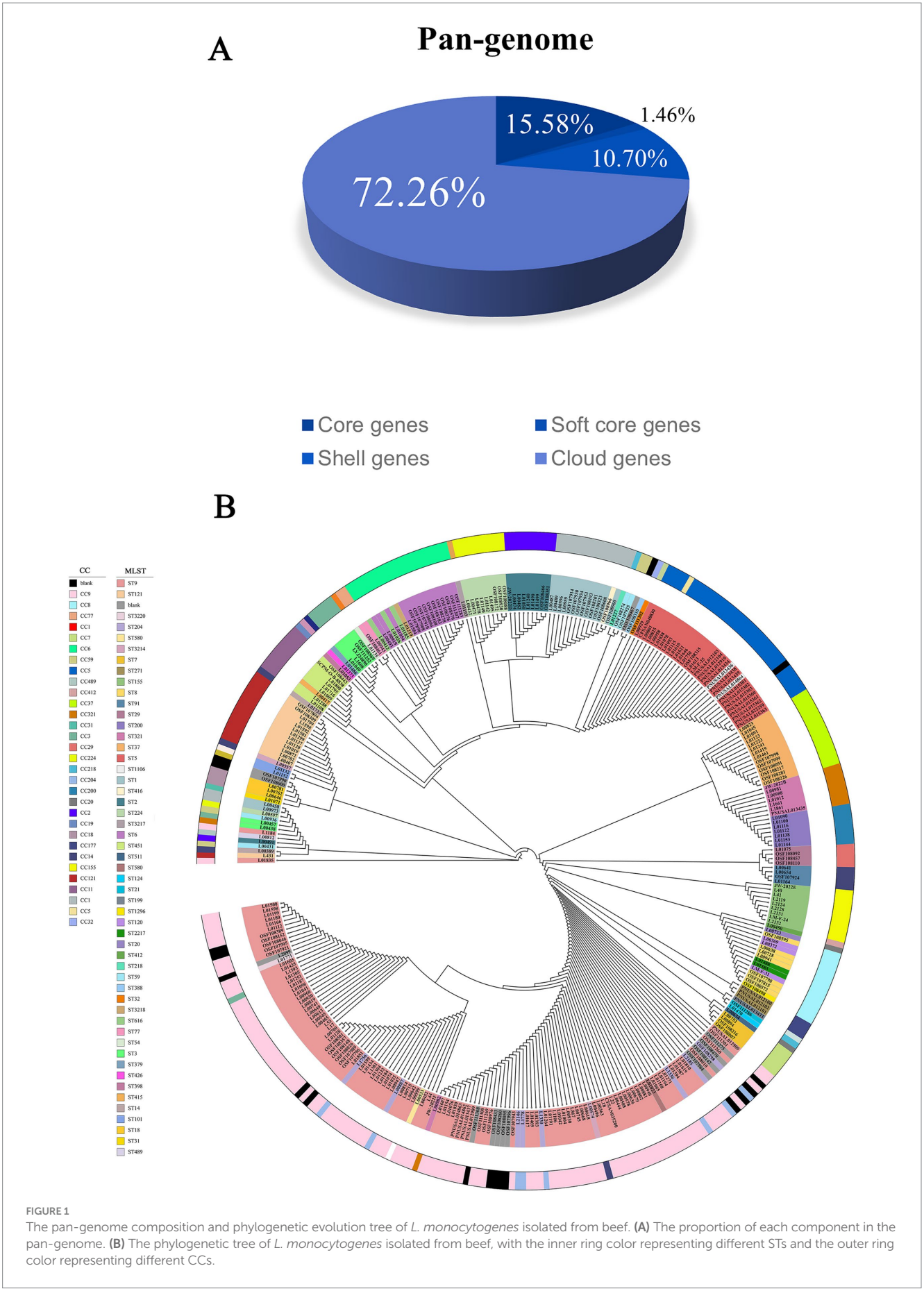

FIGURE 1
The pan-genome composition and phylogenetic evolution tree of *L. monocytogenes* isolated from beef. **(A)** The proportion of each component in the pan-genome. **(B)** The phylogenetic tree of *L. monocytogenes* isolated from beef, with the inner ring color representing different STs and the outer ring color representing different CCs.

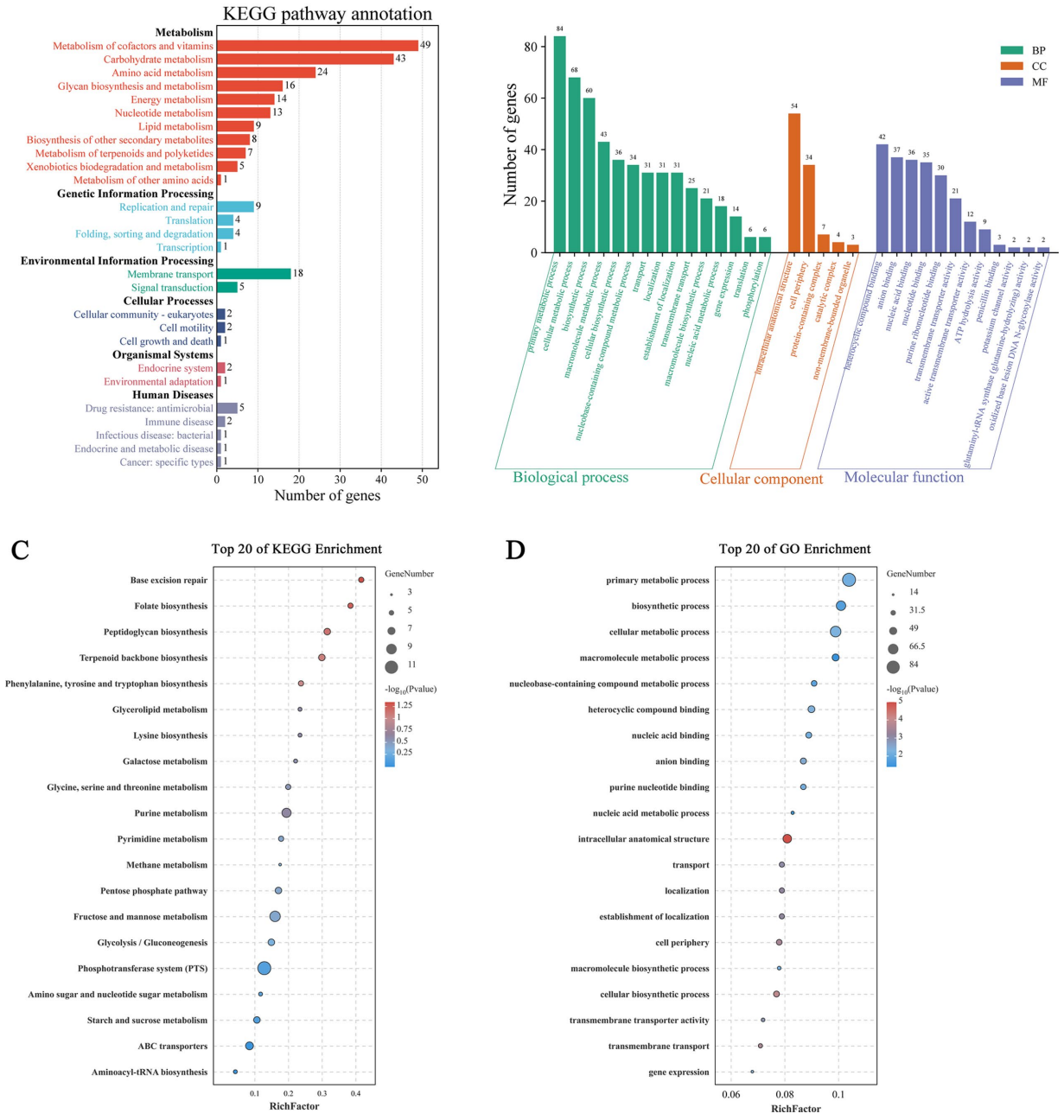

FIGURE 2
Enrichment analysis of potential target genes of *L. monocytogenes* isolated from beef based on GO and KEGG databases. **(A)** Enrichment analysis based on the KEGG database. **(B)** Enrichment analysis based on the GO database. **(C)** Enrichment analysis based on KEGG database with the top 20 enriched terms listed. **(D)** Enrichment analysis based on GO database with the top 20 enriched terms listed.

(n = 68, 16.35%), biosynthetic process (n = 60, 14.42%), and macromolecule metabolic process (n = 43, 10.34%). Within the CC category, the most abundant cellular components were intracellular anatomical structure (n = 54, 12.98%), cell periphery (n = 34, 8.17%). In the MF category, the most enriched molecular functions were heterocyclic compound binding (n = 42, 10.09%), and anion binding (n = 37, 8.89%) (Figure 2B). We integrated and ranked all the GO enrichment analysis results of potential target genes, and generated a bubble chart to display the top 20 functional features in GO enrichment analysis based on the number of genes and the significance of p-values (Figure 2D).

Enrichment analysis of the functional characteristics of the 416 potential target genes using the GO and KEGG databases revealed that these genes are primarily associated with metabolic processes, compound binding, protein localization, and transmembrane transport in *L. monocytogenes*. Specifically, these genes are involved in cellular metabolic processes, carbohydrate metabolism, and biosynthesis of organic matter. For example, carbohydrate metabolism

is critical for energy acquisition in *L. monocytogenes* because it involves converting sugars into a form of energy that cells can use directly. These potential target genes are closely related to the basic biological processes of *L. monocytogenes* and the pathogenesis of infection. They not only support the basic metabolic activities of the bacteria, but also involve the invasion and pathogenic effects of the bacteria on the host, including how the bacteria survive in the host cell, replicate and evade the innate immune response of the host. However, there were still some genes with unclear functional information, which warrants further investigation in future studies. In conclusion, the functional characteristics of these genes provide deeper insights into the biological properties of *L. monocytogenes* and offer potential molecular targets for the development of new control strategies.

PPI network analysis of potential target genes and identification of novel target genes

To further explore the relationship between potential target genes of *L. monocytogenes* PPI analysis was carried out using the STRING database. The PPI network of potential target genes comprised 416 genes, and visualization of the PPI network was performed using Cytoscape v3.10.1 software. Cytoscape is a general-purpose modeling environment for integrating biomolecular interaction networks and states. Potential target genes in the PPI network were screened using eight different algorithms in the CytoHubba plugin of Cytoscape software. The top 10 genes with the highest scores were selected as hub genes, and their rankings are shown in Table 2. Finally, the top 10 hub genes with the highest scores obtained from the Degree algorithm were identified as novel target genes. A PPI network was constructed based on their scores. These 10 hub genes include *bglF_2*, *tilS*, *group_2105*, *group_2431*, *oleD*, *ndk*, *flgG*, *purB*, *pbpB*, and *fni* (Figure 3). Detailed information on these 10 hub genes, including their functional roles, gene lengths, and other characteristics, is presented in Table 3. These 10 hub genes play a crucial role in maintaining the basic life activities and infection processes of *L. monocytogenes* strains and may serve as novel target genes for *L. monocytogenes*, particularly the top-scoring gene, *bglF_2*.

Prediction results of virulence and antimicrobial resistance genes

To investigate the virulence relationships and pathogenic mechanisms among *L. monocytogenes* strains isolated from beef, we predicted the virulence factor encoding genes in the whole genome of *L. monocytogenes* strains. Based on VFDB prediction and annotation, virulence factors of *L. monocytogenes* were classified into 12 categories including Adherence, Bile resistance, Enzyme, Immune modulator, Intracellular survival, Invasion, Iron uptake, Nucleation-promoting factor, Peptidoglycan modification, Regulation, Surface protein anchoring, and Toxin. The VFDB prediction revealed that 26 virulence genes were present in all *L. monocytogenes* strains isolated from beef, including *dltA*, *inlJ*, *lap*, *bsh*, *plcB*, *plcA*, *stp*, *inlC*, *inlK*, *lntA*, *oppA*, *prsA2*, *inlA*, *inlB*, *lpeA*, *hbp2*, *pdgA*, *agrA*, *agrC*, *cheY*, *lisK*, *lisR*, *prfA*, *virR*, *virS*, and *lspA* (Figure 4). During the process of invading and infecting the host, the genes *inlA*, *inlB*, *plcA*, *plcB*, *prfA*, *hly*, and *actA* play critical roles. In this study, the *inlA*, *inlB*, *plcA*, *plcB*, and *prfA* genes were present in 100% of the *L. monocytogenes* strains, while the *hly* and *actA* genes were not present in all strains, but their presence probability exceeded 97%. The prediction of virulence genes indicates that *L. monocytogenes* strains isolated from beef contain a large number of virulence genes, suggesting that these strains possess high virulence and pathogenicity.

With the widespread use of antibiotics, bacterial resistance has become a major concern. The changes in antimicrobial resistance genes during bacterial evolution help us gain a deeper understanding of the trends in bacterial antimicrobial resistance. Using the CARD database, a total of 9 antimicrobial resistance genes were predicted in 344 *L. monocytogenes* strains isolated from beef, belonging to 9 drug categories and exhibiting 5 resistance mechanisms. The identified antimicrobial resistance genes include *norB*, *lin*, *mprF*, *FosX*, *dfrG*, *APH(3′)-IIIa*, *ANT(6)-Ia*, *lsaE*, and *catA8* (Figure 5). The most common 4 antimicrobial resistance genes in *L. monocytogenes* strains isolated from beef are *norB*, *lin*, *mprF*, and *FosX*, indicating high

TABLE 2 Top 10 hub genes ranked by scoring in eight different algorithms.

Catelogy	Rank methods in cytoHubba							
	Betweenness	BottleNeck	Closeness	Degree	EPC	MCC	MNC	Stress
Gene symbol top 10	group_2105	group_2105	tilS	bglF_2	bglF_2	gatY_3	bglF_2	tilS
	tilS	ndk	group_2105	tilS	tilS	gatY_2	tilS	group_2105
	bglF_2	flgG	bglF_2	group_2105	oleD	frwD	group_2431	bglF_2
	flgG	bglF_2	oleD	group_2431	group_2431	mngA_3	gatY_3	ndk
	ndk	group_5668	ndk	oleD	purB	bglF_2	gatY_2	flgG
	oleD	tilS	group_2431	ndk	ndk	yhaP	cpoA	oleD
	group_2431	oleD	prkC	flgG	group_2105	mshD_2	purB	group_2431
	group_5795	fmt	pyrE	purB	pyrE	group_10780	group_11281	cysG
	prkC	hemH	fmt	pbpB	gatY_3	pgcA_1	group_5806	pphA
	cysG	cysG	purB	fni	pgcA_1	pgcA_2	group_45051	group_5795

bglF_2 gene highlighted in bold are the most promising candidate to serve as novel target genes.

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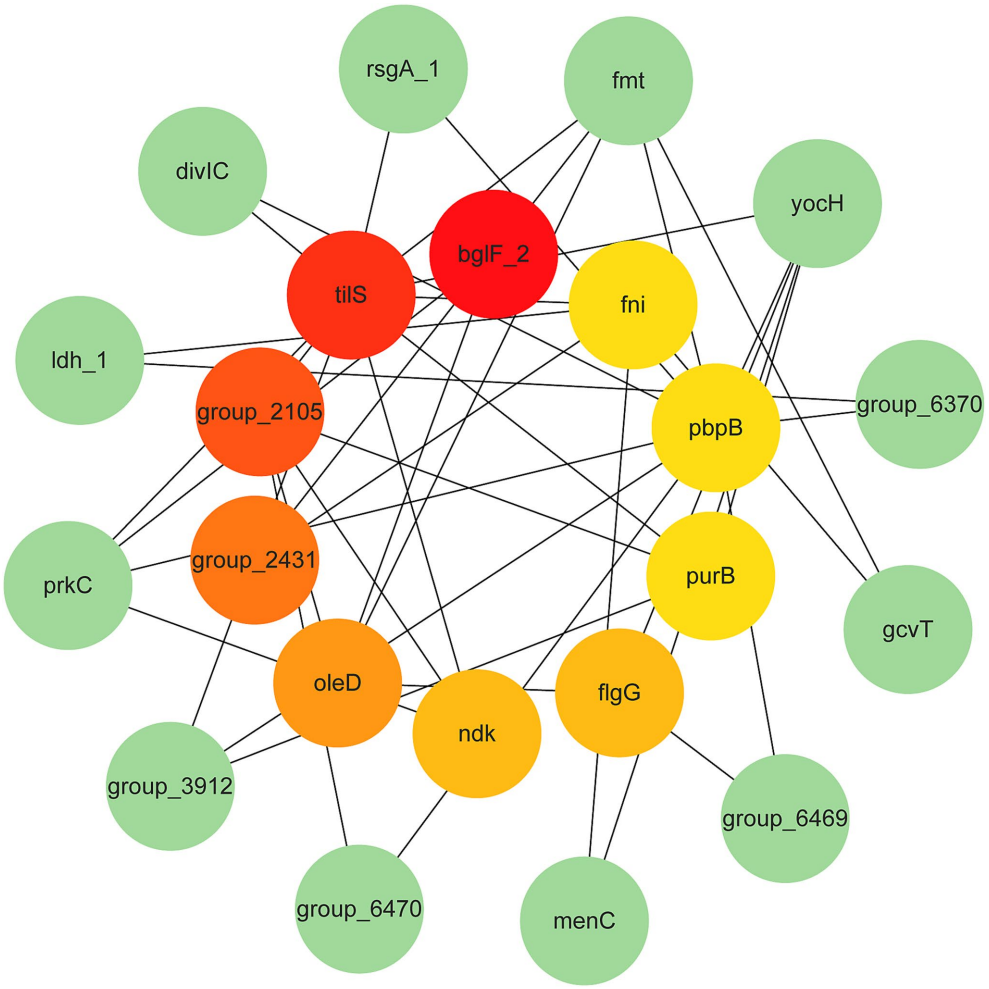

FIGURE 3
PPI network analysis of novel target genes of *L. monocytogenes* isolates from beef. The darker the red, the higher the gene score.

resistance to fluoroquinolones, lincosamides, peptides and phosphonic acid antibiotics.

Prediction results of CRISPR-Cas system types and plasmids

The CRISPR-Cas system is an adaptive immune system in bacteria that defends against the invasion of foreign genetic material, such as phages and plasmids. It serves as a defense mechanism found in most bacteria to eliminate foreign plasmid or phage DNA. An analysis of the CRISPR-Cas system in 344 *L. monocytogenes* strains isolated from beef identified 4 types of CRISPR-Cas systems, each exhibiting distinct Cas genes. The CRISPR-Cas system types detected in *L. monocytogenes* included CAS-TypeIA (2/344), CAS-TypeIB (105/344), CAS-TypeIIA (30/344), and CAS-TypeIIIA (23/344). In total, 39.24% (135/344) of the *L. monocytogenes* strains contained at least one CRISPR-Cas system, with CAS-TypeIB (30.52%) and CAS-TypeIIA (8.72%) being the most prevalent (Figure 5). CAS-TypeIA was detected only in ST451 and ST2.

Mobile Genetic Elements (MGEs) refer to a class of genetic elements capable of spreading or transferring within a genome, such as plasmids, which can facilitate the evolution of microorganisms. Therefore, the prediction of plasmids can provide further insights into the evolution of *L. monocytogenes*. We employed PLSDB databases for the detection of plasmids, and the results only documented plasmids with an identity score of 1. A total of 23 plasmids were predicted in *L. monocytogenes* strains, with the most prevalent plasmids being pMF4545 (10/344) and pCFSAN100570 (10/344), followed by pLM33 (8/344) and pLIS18 (8/344). The plasmids pMF4545 and pCFSAN100570 were exclusively found in ST9 (Figure 5).

Prediction results of LGIs and SSIs

We predicted the presence of genomic LGIs in *L. monocytogenes* strains and found that LGI-2 was present in all strains. LGI-1 was less common, detected in only 3 *L. monocytogenes* strains, while LGI-3 was found in 155 strains (Figure 5).

The SSIs associated with resistance genes in *Listeria* consist of 2 islands: SSI-1 and SSI-2. SSI-1 consists of the genes *lmo0444*, *lmo0445*, *pva*, *gadD*, and *gadT*, while SSI-2 is composed of the genes *lin0464* and *lin0465*. SSI-1 is associated with the tolerance of *Listeria* to

TABLE 3 Detailed information of top 10 hub genes ranked by scoring in Degree algorithm.

Gene	Name of target ganes	Sequence length/bp	Presence profile		Encoded protein	Source
			In target	In non-target		
bglF_2	lmo2772	1,853	344 (100%)	0	PTS system beta-glucoside-specific EIIBCA component	This study
tilS	lmo0219	1,945	344 (100%)	0	tRNA(Ile)-lysidine synthase	This study
group_2105	lmo1266	955	344 (100%)	0	O-succinylbenzoic acid--CoA ligase	This study
group_2431	lmo0078	1,350	344 (100%)	0	Endonuclease MutS2	This study
oleD	lmo1477	1,027	344 (100%)	0	Putative N-acetyl-LL-diaminopimelate aminotransferase	This study
ndk	ndk	443	344 (100%)	0	5-dehydro-2-deoxygluconokinase	This study
flgG	lmo0682	779	344 (100%)	0	Glutathione biosynthesis bifunctional protein GshAB	This study
purB	purB	1,292	344 (100%)	0	Siroheme synthase	This study
pbpB	pbpB	2,216	344 (100%)	0	Tryptophan synthase alpha chain	This study
fni	lmo1383	1,073	344 (100%)	0	Histidinol-phosphate aminotransferase	This study

environmental factors such as high NaCl concentrations, high bile salt concentrations, and low pH. In contrast, SSI-2 contributes to the survival of strains under alkaline and oxidative stress, enabling them to persist in food processing environments. Initially, the two genes of SSI-2 were thought to be unique to *L. innocua*, however, studies have found that these genes are also present in *L. monocytogenes* of the ST121. Prediction of SSIs revealed that 330 strains (95.93%) of *L. monocytogenes* harbor SSI-1, while 14 strains (4.07%) of *L. monocytogenes* harbor SSI-2 (Figure 5). It is noteworthy that all 14 *L. monocytogenes* strains harboring SSI-2 were identified as ST121. Among these 14 *L. monocytogenes* strains, 12 were isolated from the Netherlands, 1 from Spain, and 1 from Australia.

Detection of *Listeria monocytogenes* using specific primers by PCR

To validate the specificity of the *bglF_2* gene for *L. monocytogenes*, primers targeting the *bglF_2* gene were designed, and its specificity was evaluated through PCR. PCR results showed that in the primer system designed for the *bglF_2* gene, a distinct band was observed at 464 bp only in *L. monocytogenes*, while no bands were detected in other *non-L. monocytogenes* strains (Figure 6A). The results indicate that the *bglF_2* gene exhibits good specificity for *L. monocytogenes*, and primers designed for the *bglF_2* gene can effectively detect *L. monocytogenes*.

The results of the artificial contamination experiment

To ensure the primers designed for the *bglF_2* gene can effectively detect *L. monocytogenes* in beef samples, their specificity and efficacy were validated through artificial contamination experiments. *L. monocytogenes* at a concentration of 5.2×10^4 CFU/mL was added to the simulated detection solution and subjected to 24 h of enrichment culture. DNA was extracted from the samples at 4 h, 6 h, 8 h, 12 h, and 24 h, and PCR validation was performed using primers designed for the *bglF_2* gene. The results showed distinct bands at 4 h, 6 h, 8 h, 12 h, and 24 h, indicating that the primers designed for the *bglF_2* gene can effectively detect *L. monocytogenes* in beef samples (Figure 6B). The *bglF_2* gene demonstrated excellent specificity and holds promise as a specific molecular target for detecting *L. monocytogenes* strains in beef samples.

Discussion

Food safety is a critical issue in the field of public health, and *L. monocytogenes*, as a significant foodborne pathogen, often poses serious food safety risks (Chowdhury and Anand, 2023). Therefore, we conducted a comparative genomic analysis of *L. monocytogenes* strains isolated from beef to explore their phenotypic and genetic evaluations, revealing their biodiversity and evolutionary traits. We also identified potential target genes and mining novel targets. The aim was to gain a deeper understanding of *L. monocytogenes* strains and provide important reference value for strain-specific molecular detection, thereby reducing food safety issues in public health.

In this study, we conducted a pan-genome comparative analysis of 344 *L. monocytogenes* strains isolated from beef to investigate strain phenotypes and genetic evaluations, revealing their biodiversity and evolutionary features. To assess the genomic diversity of *L. monocytogenes* strains isolated from beef, we performed core/pan-genome analysis. The pan-genome represents all the genes present in the strains, while the core genome represents the essential portion necessary for the presence and shared phenotypic features of specific strains, which are critical for maintaining basic survival and infection capability (Lee et al., 2019). Therefore, we conducted an in-depth study of the core genome to identify potential target genes. A total of 2,001 core genes, accounting for 15.58%, were identified among all *L. monocytogenes* strains isolated from beef in the NCBI database. These genes constitute the fundamental components essential for the survival and development of *L. monocytogenes* strains. This suggests that despite evolving in different regions and

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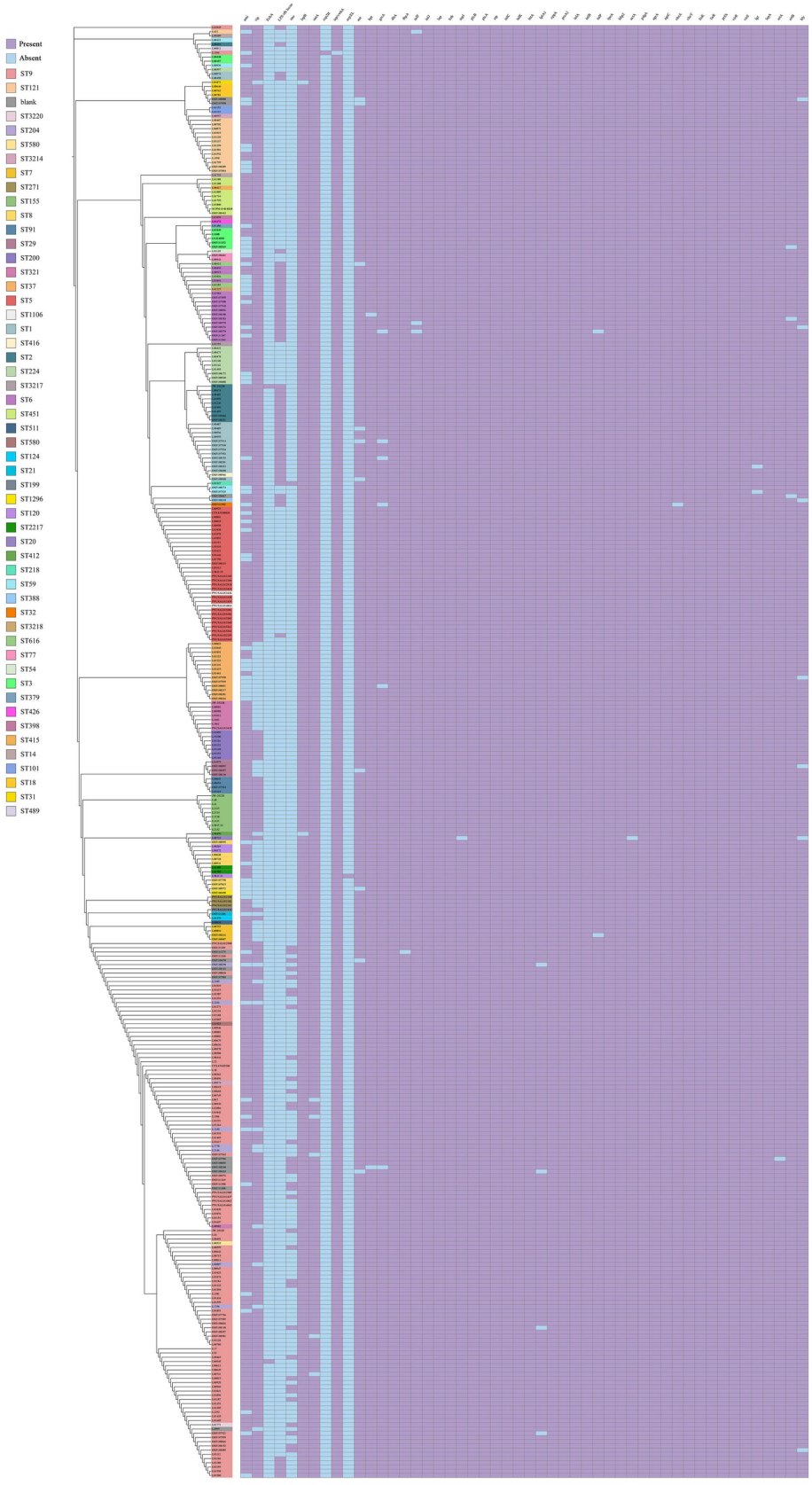

FIGURE 4
The distribution of virulence genes in *L. monocytogenes* isolated from beef.

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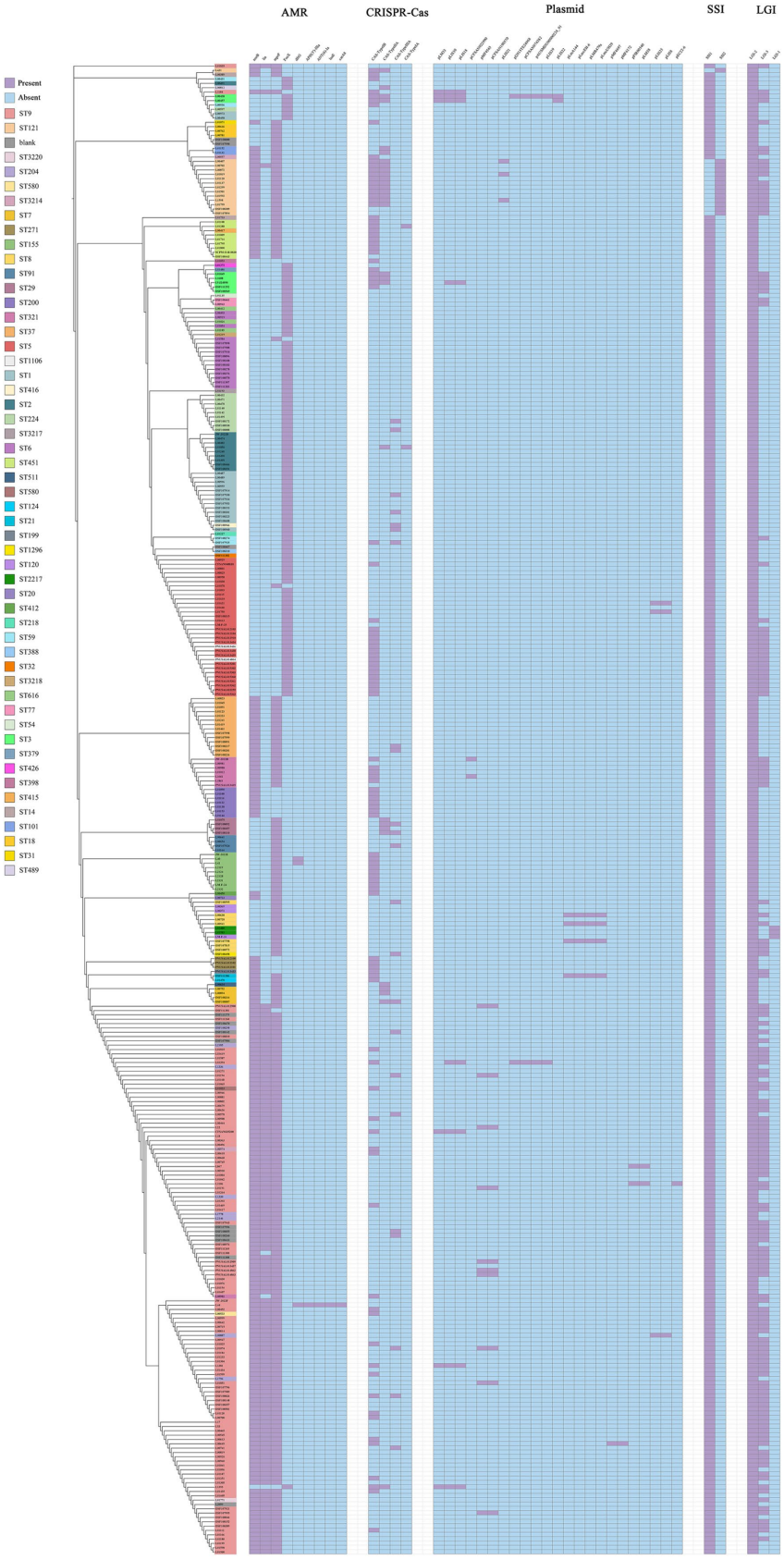

FIGURE 5
The distribution of antimicrobial resistance genes, CRISPR-Cas system, plasmids, SSIs and LGIs in *L. monocytogenes* isolated from beef.

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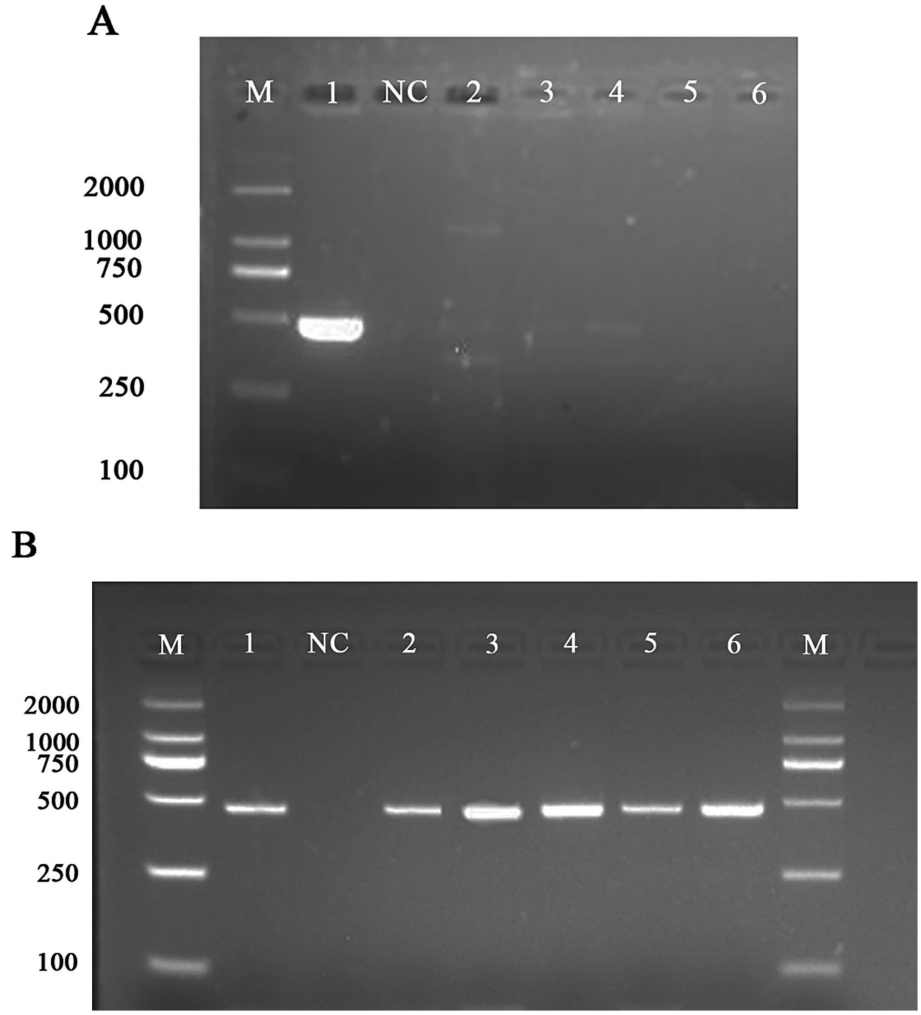

FIGURE 6
Verification of primer specificity for the *bglF_2* gene of *L. monocytogenes* and artificial contamination experiments. **(A)** Lane M: DL DNA 2000 marker, lane NC: negative control, and lanes 1–6: represent 6 different strains, including lane 1: *Listeria monocytogenes*, lane 2: *Listeria innocua*, lane 3: *Listeria ivanovii*, lane 4: *Listeria welshimeri*, lane 5: *Escherichia coli*, lane 6: *Salmonella enterica*. **(B)** Lane M: DL DNA 2000 marker, lane 1: positive control, lane NC: negative control, lane 2: DNA extracted from samples at 4 h, lane 3: DNA extracted from samples at 6 h, lane 4: DNA extracted from samples at 8 h, lane 5: DNA extracted from samples at 12 h, lane 6: DNA extracted from samples at 24 h.

environments, *L. monocytogenes* strains still share a significant number of common phenotypic features. In addition to the core genes, the pan-genome also contains many accessory genes, which provide the strains with unique traits (Nwaiwu, 2022). As *L. monocytogenes* strains are found in various environments, they have evolved specific accessory genes to adapt to these distinct environments and counteract environmental pressures. Through pangenomic analysis, we identified the core genes of *L. monocytogenes* strains isolated from beef, and subsequently used these core genes to determine potential target genes. The selection criterion for potential target genes was that they were present exclusively in *L. monocytogenes* strains isolated from beef, and absent in non-target strains. This suggests that the potential target genes are essential and unique to *L. monocytogenes*, playing a crucial role in its basic survival and pathogenic invasion. Therefore, studying the potential target genes of *L. monocytogenes* can enhance our understanding of its phenotypic and genetic evaluations, and these potential target genes could also serve as specific molecular detection targets for *L. monocytogenes*.

To investigate the biodiversity and evolutionary characteristics of *L. monocytogenes* strains isolated from beef, we performed MLST and phylogenetic analyses. MLST analysis is a crucial tool in the classification and epidemiological study of *L. monocytogenes*, providing important insights into the genetic diversity and transmission routes of *L. monocytogenes* strains (Stessl et al., 2014). Our MLST analysis of *L. monocytogenes* strains isolated from beef revealed that ST9 and CC9 were the most prevalent, followed by ST5 and CC5. Similar findings were reported by Henri et al. (2016) who conducted MLST analysis on *L. monocytogenes* isolated from food, with ST9 and ST121 being the most common. Manqele et al. (2024) performed MLST analysis of *L. monocytogenes* in beef and beef products and identified ST9, ST204, ST1, and ST5. Our results are consistent with the findings reported in the above-mentioned literature. This indicates that *L. monocytogenes* strains in beef predominantly belong to ST9 and CC9 types. By constructing a phylogenetic tree, we found that although the *L. monocytogenes* strains studied were isolated from different regions and environments, some

of the strains were still grouped into the same branch. This suggests that while *L. monocytogenes* strains have evolved unique traits to adapt to the specific environmental pressures they encounter, they still exhibit a certain degree of genetic similarity in their evolutionary characteristics.

We obtained the core genes of *L. monocytogenes* strains isolated from beef through pan-genomic analysis, and then identified 416 potential target genes by screening these core genes. To explore the functional roles of these potential target genes, we conducted GO and KEGG enrichment analyses (Zhang et al., 2024). The functional annotation results indicated that the potential target genes are mainly associated with the metabolic processes, compound binding, protein localization, and transmembrane transport of *L. monocytogenes*, which are closely related to the bacterium's basic life activities and its ability to invade and infect hosts. The potential target genes are promising candidates for specific targeting of *L. monocytogenes* (Zhang et al., 2024). However, due to the large number of potential target genes, further screening was performed. A PPI network was constructed, and the hub genes were identified using the Cytohubba function in Cytoscape software. Hub genes are the most critical genes in the PPI network and are used to discover novel target genes (Zhang et al., 2024). Ultimately, 10 hub genes were selected from the potential target genes (*bglF_2*, *tilS*, *group_2105*, *group_2431*, *oleD*, *ndk*, *flgG*, *purB*, *pbpB*, and *fni*). These hub genes play a crucial role in the basic life activities and infection invasiveness of *L. monocytogenes*. Among them, the *bglF_2* gene scored the highest and exhibited stronger connections with other proteins, indicating its potential as a specific molecular target for *L. monocytogenes* detection.

L. monocytogenes is a recognized pathogenic strain of *Listeria* (Disson et al., 2021). To further understand the genetic determinants behind the virulence of *L. monocytogenes*, we predicted the virulence genes of *L. monocytogenes* strains isolated from beef. During invasion of the host by *L. monocytogenes*, the bacterium first utilizes the *inlA* and *inlB* genes to bind with the E-Cadherin and Met receptors of the host's eukaryotic cell membrane, respectively, thereby inducing bacterial uptake through receptor-mediated endocytosis. After internalization, the bacterium is encapsulated within a vacuole, and releases the *hly*, *plcA*, and *plcB* genes to mediate vacuole escape. Subsequently, the *actA* gene is utilized to induce actin polymerization and generate sufficient force for the bacterium to spread from one cell to another (Osek and Wieczorek, 2022). Our predictive results revealed that 50 virulence genes are present in *L. monocytogenes* strains isolated from beef, with 26 of these virulence genes being consistently present in all strains. These include key virulence genes involved in the infection and invasion process of *L. monocytogenes*, such as *inlA*, *inlB*, *plcA*, *plcB*, and *prfA* genes. Interestingly, *hly* and *actA* genes were not present in all strains, although their occurrence exceeded 97%. This discrepancy could be attributed to prediction errors in the database or genetic variations occurring in individual strains under specific environmental conditions. The presence of a large number of virulence genes contributes to the strong pathogenicity of *L. monocytogenes*, making it a virulent strain. The genetic determinants behind *L. monocytogenes* virulence are governed by its virulence genes. These results suggest that although some virulence genes may undergo genetic variation during the evolutionary process, critical virulence genes essential for the infection and invasion of the host remain conserved, playing a crucial role in *L. monocytogenes* pathogenicity.

To further investigate the genetic determinants behind the antibiotic and environmental resistance of *L. monocytogenes*, we predicted the presence of antimicrobial resistance genes, CRISPR-Cas systems, plasmids, SSIs, and LGIs in *L. monocytogenes* strains isolated from beef. Antibiotic resistance has long been a concerning issue (Asghar et al., 2024). Our predictions revealed that the most common antibiotic resistance genes in *L. monocytogenes* are *norB*, *lin*, *mprF*, and *FosX*, indicating high resistance to fluoroquinolones, lincosamides, peptides, and phosphonic acid antibiotics. The recommended antibiotics for treating *L. monocytogenes* infections are ampicillin and gentamicin. The CRISPR-Cas system is an important adaptive immune mechanism in bacteria, used to defend against foreign genetic elements such as phages and plasmids (Liu et al., 2024). Our predictions revealed the presence of a significant number of CAS-TypeIB and CAS-TypeIIA in *L. monocytogenes* strains, suggesting that *L. monocytogenes* evolves an environment-specific CRISPR-Cas system during its evolutionary process. Predicting the presence of plasmids in *L. monocytogenes* helps analyze the variations in MGEs and provides further insights into the evolution of the strains. Our prediction of plasmids revealed that, despite *L. monocytogenes* being exposed to different external environments, there remains a certain degree of similarity in bacterial genome evolution. The prediction of SSI and LGI in *L. monocytogenes* provides valuable insights into the changes in the environmental resistance of the bacterium (Hein et al., 2011). We found that LGI-2 was present in all strains, whereas LGI-3 was only present in 155 strains, suggesting that the presence of LGI-2 plays a crucial role in the resistance to environmental stress during the evolution of *L. monocytogenes*. SSI prediction revealed that SSI-1 was present in the majority of *L. monocytogenes* strains, whereas SSI-2 was found only in the ST121 strains, indicating that the presence of SSI-1 helps *L. monocytogenes* survive in harsh environments. Therefore, LGI-2 and SSI-1 are a genetic determinant for environmental resistance in the evolution of *L. monocytogenes*.

To validate the specificity of the *bglF_2* gene, primers were designed for the *bglF_2* gene, and its specificity was confirmed through PCR. The results indicate that the *bglF_2* gene exhibits excellent specificity for *L. monocytogenes*. The rationale for selecting genes different from those used in previous studies for detecting *L. monocytogenes* is that the *bglF_2* gene yielded the best results in the analysis of *L. monocytogenes* strains isolated from beef (Zhang et al., 2024). Meanwhile, the *bglF_2* gene, as a component of the PTS system, is typically strain-specific, which helps to avoid cross-reactivity with other *Listeria* species and *non-Listeria* bacteria. Therefore, the *bglF_2* gene exhibits better specificity in these strains, making it a more suitable choice for detecting *L. monocytogenes* in beef. Subsequently, an artificial contamination experiment was conducted to verify whether the *bglF_2* gene could successfully detect *L. monocytogenes* in beef samples. The results showed that the *bglF_2* gene effectively detected *L. monocytogenes* at five different time points, indicating that *bglF_2* not only efficiently detects *L. monocytogenes* in beef samples but also performs excellently. Therefore, the *bglF_2* gene is expected to be a specific molecular target for detecting *L. monocytogenes* in beef samples.

Conclusion

In conclusion, we conducted a comparative genomics study to explore the phenotypic and genetic evaluations of *L. monocytogenes* strains isolated from beef, revealing the biodiversity and evolutionary traits of these strains. A large number of virulence genes were identified in the *L. monocytogenes* strains, which form the basis for the high pathogenicity of *L. monocytogenes*. Although the strains analyzed are from different environments and have evolved unique genes to cope with various environmental pressures, they all isolated from beef, and thus share many common features in their evolutionary process. These common features allowed us to identify potential target genes, which were further explored to discover novel targets. Specificity tests and artificial contamination experiments confirmed that the *bglF_2* gene holds promise as a specific molecular target for detecting *L. monocytogenes* strains in beef samples. This study further enhances our understanding of the pathogenicity and adaptability of *L. monocytogenes*, while providing significant reference value for the development of specific molecular detection targets for this pathogen.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

BZ: Data curation, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. WS: Data curation, Writing – original draft. XW: Supervision, Writing – review & editing. HR: Supervision, Writing – review & editing. YaW: Validation, Writing – original draft. SH: Validation, Writing – original draft. CL: Validation, Writing – original draft. YuW: Validation, Writing – original draft. JH: Validation, Writing – original draft. XH: Validation, Writing – original draft. RS: Supervision, Writing – review & editing. YL: Supervision, Writing – review & editing. SL: Supervision, Writing – review & editing. QL: Supervision, Writing – review & editing. ZL: Supervision, Writing – review & editing. PH: Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1560974/full#supplementary-material

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The cultivation conditions of leafy vegetables influence the structures of phyllosphere bacterial communities and ultimately impact the *L. monocytogenes* growth post-harvest

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Cultivation conditions, including plant species, variety, cultivation method, and seasonality, are all at least co-factors of epiphytic *Listeria monocytogenes* growth. Meanwhile, phyllosphere-associated bacteria were found to influence the colonization of invading pathogens. Thus, the main objective of this study was to determine whether cultivation conditions are factors in the development of the bacterial phyllosphere community on leafy vegetables, which consequently correlates positively or negatively with *L. monocytogenes* growth. Indeed, this study revealed that vegetable cultivation conditions are a more significant determinant of phyllosphere development than plant species. Of the identified phyllosphere-associated bacteria, the presence of Pseudomonadaceae had a positive correlation with *L. monocytogenes* populations on all tested produce. Hitherto, *Pseudomonadaceae* content appeared to be more critical for *L. monocytogenes* growth on spinach F1 Trumpet. For days 7–9 of storage, *Pseudomonadaceae* increased abundance on open field spinach F1 Trumpet were associated with *L. monocytogenes'* most significant increase (0.94 $\log_{10}$ colony-forming unit (cfu) g^{-1}). In contrast, *Pseudomonadaceae* content decreased for polytunnel spinach F1 Trumpet, and the corresponding *L. monocytogenes* populations remained unchanged. *Carnobacteriaceae* were present on spinach F1 Trumpet from the polytunnel but not on other spinach products, with higher associated *L. monocytogenes* growth. *Pectobacteriaceae* (genus *Dickeya*) increased for spinach F1 Trumpet polytunnel but decreased for other spinach produce with lower associated *L. monocytogenes* growth. Similarly, polytunnel rocket Esmee had an increasing relative abundance of *Pectobacteriaceae*, whereas it remained constant for polytunnel rocket Buzz. Compared to summer spinach F1 Trumpet produce, winter produce had significantly greater *Streptococcaceae* content and was correlated with a decrease in *L. monocytogenes* growth. Finally, higher phyllosphere alpha diversity putatively limited *L. monocytogenes* growth. Ultimately, this study revealed that cultivation conditions determine the bacterial phyllosphere community structure, which consequently influences *L. monocytogenes* growth.

KEYWORDS

variety, season, lactic acid bacteria, *Pseudomonadaceae*, *Spinacia oleracea*, *Eruca sativa*, *Listeria monocytogenes*

1 Introduction

Leafy vegetables such as rocket and spinach are commonly consumed due to their vitamin, mineral, antioxidant, and phytochemical content (Colonna et al., 2016; Van der Avoort et al., 2018; Venu et al., 2019). To meet the demand for such leafy vegetables, global production of spinach has increased by 218% from 2001 to 2021 (FAO, 2021). Polytunnels enable all year-round production of such high-quality leafy vegetables in winter months or in countries where production may not be possible due to challenging weather conditions (Sagar, 2020). However, cultivation in polytunnels is also altering environmental conditions not only for plant growth but also for the growth of the plant microbiome.

While the increasing demand for vegetables has resulted in the adoption of cost-effective and fast production methods, less concern is given to the safety of their produce, that is, microbial contamination with foodborne pathogens such as *Listeria monocytogenes* (Balali et al., 2020). Potential sources of contamination include irrigation water and manures (re-harvest), as well as the handling of the produce (post-harvest) (Balali et al., 2020). In terms of *L. monocytogenes* growth on spinach and rocket produce, there have been conflicting results from studies with differing experimental and pre-harvest cultivation conditions (Sant'Ana et al., 2012; Lokerse et al., 2016; Söderqvist et al., 2017b; Ziegler et al., 2019; Culliney and Schmalenberger, 2020). However, Culliney and Schmalenberger (2022) revealed that cultivation conditions, that is, plant species and variety, cultivation method (polytunnel vs. open field), and seasonality of harvest, are at least partly responsible for differing levels of *L. monocytogenes* growth (Culliney and Schmalenberger, 2022).

Foodborne pathogens, such as *L. monocytogenes* do not grow in isolation but within a microbial community within the phyllosphere. The phyllosphere refers to the aerial parts of the plant, primarily the surface of the leaves, which harbor diverse and rich communities of bacteria, fungi, viruses, nematodes, and protozoans (Bashir et al., 2022). Plant species and genotype, as well as abiotic factors, such as geographical location, solar radiation, pollution, and nutrients, and biotic factors, including leaf age and presence of other microorganisms, are all drivers of the development of the phyllosphere (Xu et al., 2022). Although the phyllosphere harbors a highly diverse community, at the phylum level, the phyllospheres of different plant species, even from various geographical locations, exhibit high levels of similarity. They primarily consist of *Pseudomonadota* (*Proteobacteria*), *Actinomycetota* (*Actinobacteria*), *Bacteroidota* (*Bacteroidetes*), and *Bacillota* (*Firmicutes*) (Liu et al., 2020).

Phyllosphere-inhabiting microorganisms and their metabolites interact with their environment and may play protective roles against invading opportunistic foodborne pathogens (Saleem, 2021). A previous study revealed that bacterial isolates from ready-to-eat (RTE) lettuce influence the colonization of *Listeria innocua* in co-cultures (Francis and O'Beirne, 2002). However, a paucity of studies has investigated the *in situ* influence of the food microbiome or vegetable phyllosphere on the *L. monocytogenes* growth. A cultivation-based study did not identify any differences in resident bacteria present between cut leaves of broad-leaved endive associated with high and low levels of *L. monocytogenes* growth (Carlin et al., 1995). To date, there have been no attempts to correlate the phyllosphere bacteriome of rocket or kale with *L. monocytogenes* growth.

Lactic acid bacteria (LAB) are often naturally present as indigenous, spoilage bacteria and negatively impact *L. monocytogenes* due to their competitive growth capabilities (Østergaard et al., 2014). Additionally, LAB produce organic acids which reduce pH by lowering intracellular dissociation and intracellular leakage through porins or permeases to values beneath the pH at which *L. monocytogenes* performs optimally, that is, pH 7 (Webb et al., 2022). Moreover, LAB produce other metabolites or bio-preservative agents such as reuterin, bacteriocins, diacetyl, reutericyclin, organic acids, acetoin, and hydrogen peroxide (Ibrahim et al., 2021). *Lactiplantibacillus plantarum* is a LAB previously isolated from rocket produce, which harbors genes that encode for the production of Coagulin A and the active peptide Pediocin ACH. These can act as anti-listerial agents, thus displaying particular inhibition capacities of *L. monocytogenes* (Le Marrec et al., 2000; Espitia et al., 2016; Barbosa et al., 2021). Conversely, several members of the *Pseudomonadaceae* family cause hydrolysis of proteins, which could provide free amino acids likely to stimulate the *L. monocytogenes* growth (Marshall et al., 1992; Zilelidou and Skandamis, 2018). *Pseudomonadaceae* spp. can also increase nutrient availability, for example, carbon and nitrogen for pathogen colonization by altering ion transport across the plant cell plasma membranes (Hutchison, 1995). Additionally, *P. putida* has the ability to produce and release plant growth regulators, for example, indole-3-acetic acid, which promotes nutrient leakage and microbial fitness (Brandl and Lindow, 1998; Leveau and Lindow, 2005). Further research is needed to determine whether a higher diversity of the phyllosphere indigenous bacterial community is related to the reduction of the competitiveness of transient opportunistic pathogenic microorganisms (Darlison et al., 2019).

The objective of the present study was to utilize Illumina-based 16S amplicon sequencing to describe the bacterial composition of leafy vegetable phyllospheres. Different plant species (spinach, rocket, and kale), cultivars (F1 Trumpet vs. F1 Cello; and Buzz vs. Esmee), cultivation methods (polytunnel vs. open field), and seasonality (summer vs. winter spinach) were tested to identify the presence of certain bacteria of importance to *L. monocytogenes* growth. Changes in their relative abundance were correlated with shifts in the abundance of *L. monocytogenes* populations. This study hypothesized that differences in the relative abundance of certain phyllosphere-associated bacterial taxa attributed to differing cultivation conditions are essential co-factors responsible for divergent levels of *L. monocytogenes* growth. Consequently, the present study aimed to analyze the bacterial community structures of leafy vegetables cultivated differently, including spinach, rocket, and kale.

2 Materials and methods

2.1 Spinach, rocket, and kale produce

All spinach, rocket, and kale produce (Caryophyllales for spinach, Brassicales for rocket, and kale, referred here as species) used in this study were cultivated as described by Culliney and Schmalenberger (2022). A total of 160 samples from *L. monocytogenes* growth potential experiments were selected: open field and polytunnel spinach (F1 Trumpet; summer harvest), open field and polytunnel rocket (Buzz), polytunnel spinach (F1 Cello), polytunnel rocket (Esmee), open field spinach (F1 Trumpet; winter harvest). Samples were stored for days 0,

2, and 5 at 7°C and for days 7–9 at 12°C for days, where *L. monocytogenes* and total bacteria counts (TBCs) were enumerated on cultivation media (Culliney and Schmalenberger, 2022).

2.2 *L. monocytogenes* content of spinach, rocket, and kale produce

Growth experiments were executed as described in accordance with the European Union (EU) guidance document's guidelines for conducting growth potential studies (European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm); EURL Lm, 2019). The rationale behind selecting these guidelines is to provide a robust representation of real-life scenarios involving low-level contaminations with the potential to grow under realistic storage conditions. Each sample consisted of 25 g of produce inoculated with 100 cfu g^{-1} of a three-strain mix of *L. monocytogenes*, that is, 959 (vegetable isolate), 1,382 (EURL *Lm* reference strain), and 6,179 (food processing plant isolate). The contents of each were transferred into separate stomacher bags and homogenized in 25 mL of phosphate-buffered saline (PBS) using a stomacher (Seward 400, AGB Scientific, Dublin, Ireland) for 120 s at a high speed (260 rpm). These homogenates were used for all types of microbial analysis.

Growth potentials (log_{10} cfu g^{-1}) calculated from median values were open field spinach (F1 Trumpet; summer harvest) = 2.59, polytunnel spinach (F1 Trumpet) = 1.40, open field rocket (Buzz) = 1.28, polytunnel rocket (Buzz) = 1.45, polytunnel rocket (Esmee) = 1.23, polytunnel spinach (F1 Cello) = 1.84, polytunnel kale (Nero di Toscana) = 2.56, and open field spinach (F1 Trumpet; winter harvest) = 1.65 as described recently (Culliney and Schmalenberger, 2022).

The associated average *L. monocytogenes* counts (log_{10} cfu g^{-1}) across the five time points (± the relative increase or decrease from the previous time point) are displayed in Table 1.

2.3 DNA extraction

The remaining homogenate suspensions obtained after microbial analysis were transferred into 50 mL conical tubes and centrifuged at 4,500*g* (15 min at 4°C). Supernatants were discarded, and the derived pellets were stored at −20°C. For DNA extraction, pellets were resuspended in 400 µL of PBS, and 100 µL was used for DNA extraction with the PowerFood DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and quality of the extracted DNA were determined with the Take3 plate in an Eon plate reader/incubator (BioTek, Winooski, VT, USA) (Culliney and Schmalenberger, 2024).

2.4 Next generation sequencing (NGS) analysis

All 160 samples were sent to the University of Minnesota Genomics Center (UMGC) for indexing and Illumina MiSeq (San Diego, CA) sequencing. Raw sequencing files were deposited in the Sequence Read Archive (National Center for Biotechnology Information [NCBI]) under the BioProject identification [ID] number: PRJNA117723.4. Bioinformatics analysis was performed using QIIME2 2021.11 (https://qiime2.org/) (Bolyen et al., 2019) as described recently by Culliney and Schmalenberger (2024). The paired-end sequences with quality of each group of 20 samples were demultiplexed and imported with metadata separately via a ManifestPhred33V2 file. This was followed by trimming and truncating (quality filtering at Q20) using the q2-dada2 plugin. Following this, the "qiime feature-table merge" and "qiime feature-table merge-seqs" plug-ins to merge feature tables and the representative amplicon sequence variants (ASV) were conducted, so the following group comparisons could be performed:

Comparison 1 (open field vs. polytunnel vs. plant species) consisted of open field spinach (F1 Trumpet), polytunnel spinach (F1 Trumpet), open field rocket (Buzz), and polytunnel rocket (Buzz).

Comparison 2 (variety vs. species) consisted of polytunnel spinach (F1 Trumpet), polytunnel rocket (Buzz), polytunnel rocket (Esmee), polytunnel spinach (F1 Cello), and polytunnel kale (Nero di Toscana).

Comparison 3 (seasonality) included open field spinach (F1 Trumpet; summer harvest) and open field spinach (F1 Trumpet; winter harvest).

Assigning taxonomic information to the ASV sequences was conducted using a pre-trained Naïve Bayes taxonomic classifier, which was trained on the Silva version 138.99% reference dataset where sequences were trimmed to represent only the region between the

TABLE 1 Average *Listeria monocytogenes* counts (log_{10} cfu g^{-1} ± the relative increase or decrease from the previous time point) over time.

Product	Day 0	Day 2	Day 5	Day 7	Day 9
Open field spinach (F1 Trumpet; summer harvest)	1.99	2.31 (+0.32)	2.90 (+0.59)	3.48 (+0.58)	4.58 (+1.10)
Polytunnel spinach (F1 Trumpet)	1.94	3.04 (+1.10)	3.34 (+0.30)	3.33 (− 0.01)	3.36 (+0.03)
Open field rocket (Buzz)	1.89	2.45 (+0.56)	2.69 (+0.24)	3.14 (+0.45)	3.23 (+0.09)
Polytunnel rocket (Buzz)	1.91	2.48 (+0.57)	2.94 (+0.46)	3.45 (+0.51)	3.52 (+0.07)
Polytunnel rocket (Esmee)	1.94	2.32 (+0.38)	2.78 (+0.46)	2.89 (+0.11)	3.29 (+0.40)
Polytunnel spinach (F1 Cello)	1.91	2.77 (+0.86)	3.30 (+0.53)	3.38 (+0.08)	3.88 (+0.50)
Polytunnel kale (Nero di Toscana)	2.02	2.78 (+0.76)	3.55 (+0.77)	4.03 (+0.48)	4.48 (+0.45)
Open field spinach (F1 Trumpet; winter harvest)	2.12	2.69 (+0.57)	3.24 (+0.55)	3.33 (+0.09)	3.76 (+0.43)

Adapted from Culliney and Schmalenberger (2022).

515F/806R primers (V3–V4 region) as described previously (Culliney and Schmalenberger, 2025). Sequences not assigned to a phylum level, chloroplast, and mitochondrial sequences were removed using the filter-table method in the q2-taxa plugin. All subsequent analyses were conducted with both rarefied and unrarefied data. Even sampling depths for use in diversity metrics were for comparison 1: 11,519 → Retained 921,520 (29.48%) features in 80 (100.00%) samples at the specified sampling depth; for comparison 2: 3,117 → Retained 240,009 (9.99%) features in 77 (79.38%) samples at the specified sampling depth; and for comparison 3: 15,015 → Retained 600,600 (40.99%) features in 40 (100.00%) samples at the specified sampling depth. Alpha diversity metrics (observed ASVs, Shannon index, Pielou's evenness, and Faith's Phylogenetic Diversity) and beta diversity metrics (weighted unique fraction metric or UniFrac (Lozupone et al., 2007) and Bray–Curtis dissimilarity) using q2-diversity were estimated and viewed on Principal Coordinates Analysis (PCoA) Emperor plots. Analysis of Composition of Microbiomes (ANCOM) test in the q2-composition plugin was used to identify differentially abundant features. ANCOM identified individual taxa whose relative abundances are significantly different across groups. Relative abundance was calculated after conversion of the biome tables from QIIME2 to tsv files (phylum and family levels). Pearson's correlation coefficient was determined to measure the strength and direction of the linear association between two variables (i.e., between *L. monocytogenes* populations and the corresponding relative abundance of each of the 20 most abundant families) for all groups over time (Sedgwick, 2012). Pearson's correlation coefficient from <0.10 is a negligible correlation, 0.10–0.39 indicates weak correlations, 0.40–0.69 represents moderate correlations, while 0.70–0.89 indicates strong correlations, with >0.90 being very strong (Schober et al., 2018). Absolute abundances of bacterial taxa at the family and genus level were estimated by using the total heterotrophic counts published elsewhere (Culliney and Schmalenberger, 2022). Input, filtered, denoised, merged, non-chimeric reads, as well as chloroplast to total DNA content, are reported in Supplementary Tables S1–S3.

2.5 Statistical analysis

RStudio software (Posit, Boston, MA; version 4.1.1) was used for statistical analysis. In situations of normality (Shapiro–Wilk test) and homoscedasticity (Levene's), a one-way analysis of variance (ANOVA) was conducted to compare input, filtered, denoised, merged, and non-chimeric reads between groups. The remainder of the statistical analysis for alpha and beta diversity metrics was conducted in QIIME2. For alpha diversity (observed ASVs, Shannon index, Pielou's evenness, and Faith's Phylogenetic Diversity (Faith, 1992)), comparisons among groups and pairwise comparisons were conducted through Kruskal–Wallis tests. Beta diversity was analyzed through the non-parametric permutation test, permutational multivariate analysis of variance (PERMANOVA) (999 permutations) (Anderson, 2017). Statistical significance was tested at $p \leq 0.05$. In situations of normality (Shapiro–Wilk test) and homoscedasticity (Levene's), a one-way ANOVA Tukey honestly significant difference (HSD) *post hoc* test applying Benjamini–Hochberg correction for multiple testing was conducted to compare relative abundances for all alpha diversity metrics and relative abundances across subgroups. In situations of non-normality, the Kruskal–Wallis rank sum test, using the kruskal.test function, and Dunn test *post hoc* analysis for multiple pairwise comparisons between groups were conducted, applying the Benjamini–Hochberg correction for multiple testing (false discovery rate was set at 10%). In situations of unequal variance, the oneway.test function was employed with var = F, and Games–Howell *post hoc* analysis.

3 Results

3.1 Comparison 1 (open field vs. polytunnel vs. plant species)

This section describes how alpha and beta diversities were shaped by the selection of different leafy vegetable plants (spinach and rocket) as well as other cultivation methods (open field and polytunnel) and how diversities evolved with storage. The primary assumption was that both plant species and environment affect alpha and beta diversities. In turn, differently developing phyllosphere communities were expected to affect *L. monocytogenes* growth over time, as well as the succession of the phyllosphere community over time.

3.1.1 Influence of cultivation method (polytunnel and open field) and plant species (spinach and rocket) on alpha diversity of a *L. monocytogenes* inoculated phyllosphere

On average, the richness and diversity for rocket (observed features, Shannon index and Faith's Phylogenetic Diversity) were significantly greater for open field rocket (*L. monocytogenes* growth potential = 1.28 $\log_{10}$ cfu g^{-1}) compared to polytunnel rocket produce (*L. monocytogenes* growth potential = 1.45 $\log_{10}$ cfu g^{-1}). Pielou's evenness was not significantly different between the open field rocket and polytunnel ($p > 0.05$). For spinach, Pielou's evenness and diversity (Shannon index) were significantly higher for polytunnel spinach (*L. monocytogenes* growth potential = 1.40 $\log_{10}$ cfu g^{-1}) compared to open field spinach (*L. monocytogenes* growth potential = 2.59 $\log_{10}$ cfu g^{-1}). However, average observed features and Faith's Phylogenetic Diversity values did not differ between spinach produce ($p > 0.05$). Except for Pielou's evenness and Shannon index of polytunnel rocket, no other significant differences over time were observed for all alpha diversity metrics. Significant changes over time were only identified for polytunnel rocket (Shannon index and Pielou's evenness) and open field rocket (Pielou's evenness) (Supplementary Table S4), Rarefaction of sequencing reads did not influence alpha diversity metrics (Supplementary Table S5).

3.1.2 Influence of cultivation method (polytunnel and open field) and plant species (spinach and rocket) on beta diversity of a *L. monocytogenes* inoculated phyllosphere

All four groups, that is, open field rocket, polytunnel rocket, open field spinach, and polytunnel spinach produce, were all significantly different from each other ($p = 0.001$). When grouped by produce type, spinach and rocket produce were also significantly different ($p = 0.001$). Furthermore, when grouped by cultivation

method, all polytunnel produce vs. all open field bacterial communities were significantly different ($p = 0.001$). While all four produce groups were significantly different, this was not always the case when compared at individual time points. The bacterial communities of all 5 time points of open field spinach were significantly different compared to polytunnel spinach produce ($p = 0.026–0.038$). The same was observed for polytunnel rocket compared to polytunnel spinach produce ($p = 0.029–0.035$). However, for open field vs. polytunnel rocket, significant differences between their bacterial communities were limited to days 0, 2, 5, and 9 ($p = 0.019–0.037$) and not day 7 ($p = 0.057$). Bacterial communities of open field rocket and spinach showed significant differences on day 0, 7, and 9 ($p = 0.024–0.030$) but not on day 5 or 7 ($p = 0.069–0.084$). Adjusting the p-value significance threshold with Benjamini–Hochberg correction did not change the statistical outcome of the tests. A visual representation of the bacterial beta diversity on a PCoA plot showed that communities of polytunnel rocket and spinach, as well as open field spinach and rocket, partially overlapped, while polytunnel rocket and open field rocket, as well as polytunnel spinach and open field spinach, were separated (Figure 1). Overall, beta diversity analyses highlighted the differences between the bacterial phyllosphere communities at the plant species and environment levels.

The phyllosphere of open field rocket produce changed significantly over time, that is, from days 0–9 and 2–9 ($p = 0.028$ and 0.030). For polytunnel rocket produce changes in phyllosphere structure occurred from days 0–9, 2–7, and 2–9 ($p = 0.021–0.048$). Open field spinach produce demonstrated significant changes in its phyllosphere from days 0–9, 2–9, and 5–9 ($p = 0.014–0.030$). Finally, polytunnel spinach exhibited the most significant changes in its phyllosphere community over time, that is, days 0–5, 0–7, 0–9, 2–7, 2–9, 5–7, and 5–9 ($p = 0.019–0.041$). Correcting the p-value significance threshold with Benjamini–Hochberg correction changed the outcome of only two statistical tests to non-significant, that is, polytunnel spinach produce from days 0–7 and polytunnel rocket produce from days 7–9. Overall, these findings demonstrate that bacterial phyllosphere communities change substantially during the storage period, even if they do not significantly change at each sampling time.

3.1.3 Influence of cultivation method (polytunnel and open field) and plant species (spinach and rocket) on phyla and family relative abundances of a *L. monocytogenes* inoculated phyllosphere

For all four groups, the three most abundant phyla were *Pseudomonadota*, *Actinomycetota*, and *Bacteroidota*, which comprised 89.64–94.82% of the phyllosphere bacterial communities (Figure 2). Over time, the total abundance of these three most abundant phyla ranged for (i) open field rocket from 88.96 to 94.44%, (ii) open field spinach from 92.28 to 96.15%, (iii) polytunnel rocket from 87.71 to 95.02%, and (iv) for polytunnel spinach from 88.21 to 91.26% of total phyla. At the phylum level, cultivation methods appeared to be a more influential determinant of relative bacterial community structure compared to plant species (Figure 2).

A total of 35 families common to all four groups were detected, albeit with some significant differences across groups and low relative abundances (Supplementary Table S6). Out of the 20 most abundant families of each group, 12 were shared by all four groups with substantially higher relative abundances. Open field rocket and polytunnel rocket produce shared 14 of their 20 most abundant families, four of which were significantly different in relative abundance. Open field rocket and open field spinach produce shared 16 families of their 20 most abundant, eight of which had significantly different relative abundances between the two groups. Open field spinach and polytunnel spinach produce had 16 families of their most abundant 20 in common, nine of which were significantly different. Polytunnel rocket and polytunnel spinach shared 16 out of 20 most abundant families, seven of which were significantly different (Table 2). Of the 15 families that showed differences in relative abundance, eight appeared to group by cultivation type (polytunnel and open field), while only four grouped by plant species. However, when total heterotrophic counts were used to estimate total abundances, the higher total abundance of bacteria in the spinach

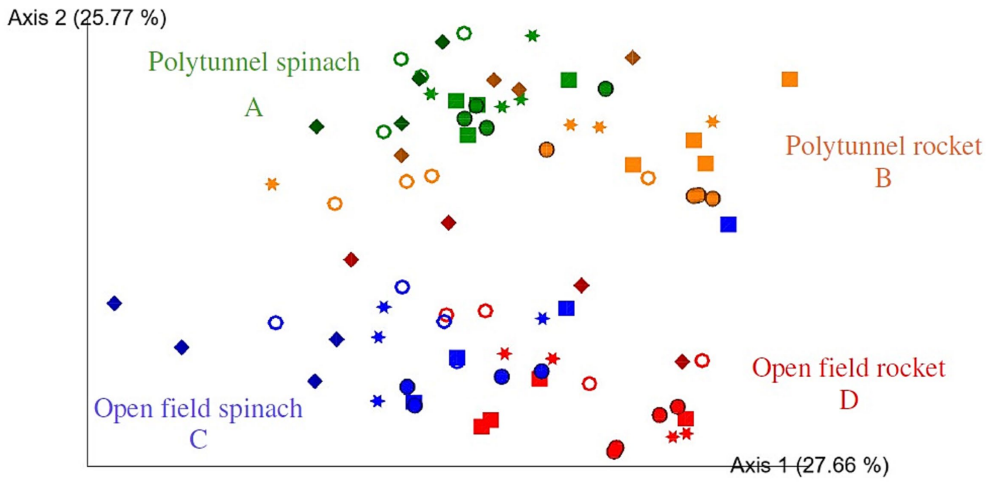

FIGURE 1
Two-dimensional Emperor (PCoA) plots showing beta diversity distances, that is, weighted UniFrac, among the different samples across open field rocket Buzz (red), open field spinach F1 Trumpet (blue), polytunnel rocket Buzz (orange), and polytunnel spinach F1 Trumpet (green) groups with rarefaction applied. Shapes revealed separations over time are day 0 = circle, day 2 = square, day 5 = star, day 7 = ring, and day 9 = diamond. Letters A-D indicate significant differences.

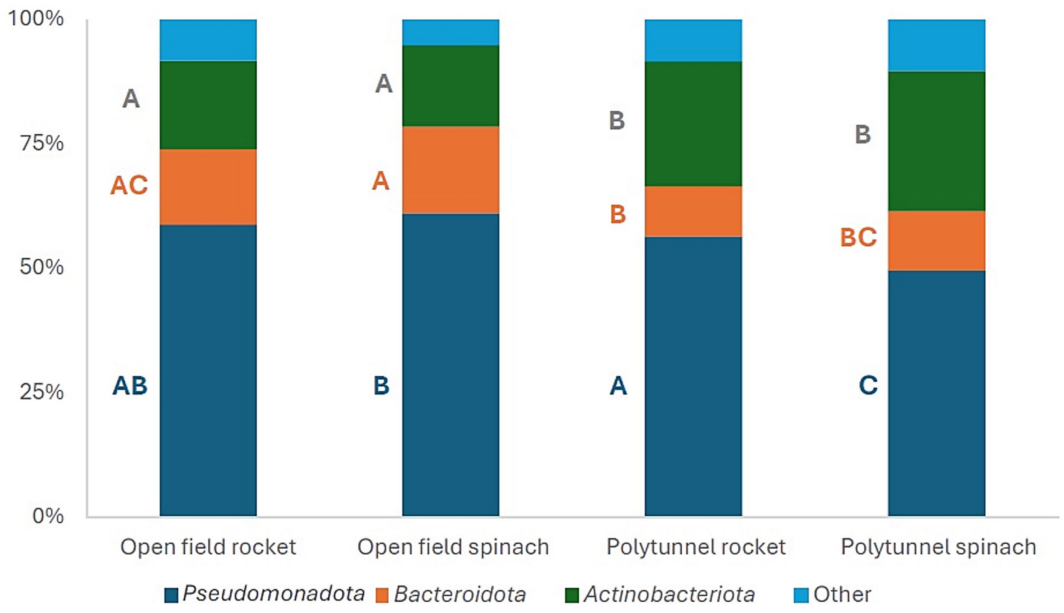

FIGURE 2
Mean relative abundances (%) of the three most abundant phyla of the 16S gene of the open field rocket Buzz, open field spinach F1 Trumpet, polytunnel rocket Buzz, and polytunnel spinach F1 Trumpet groups, with rarefaction applied. All remaining lower abundant phyla are combined in "Other." A–C indicate significant differences between groups.

TABLE 2 Average relative abundance ± the standard error of families present different relative abundances in the phyllosphere of the open field vs. polytunnel and rocket vs. spinach with rarefaction applied. Letters a-c indicate significant differences.

Family	Rocket	Spinach	Rocket	Spinach
	Open fields	Open fields	Polytunnel	Polytunnel
Hymenobacteraceae	6.08 ± 0.67^{a}	5.05 ± 0.76^{a}	1.93 ± 0.32^{b}	0.48 ± 0.09^{c}
Rhizobiaceae	4.53 ± 0.39^{a}	2.96 ± 0.24^{b}	4.07 ± 0.39^{ab}	3.76 ± 0.32^{ab}
Sphingobacteriaceae	3.78 ± 0.37^{ab}	6.26 ± 0.93^{b}	2.39 ± 0.47^{a}	5.82 ± 0.60^{b}
Microbacteriaceae	5.48 ± 0.33^{a}	8.01 ± 0.51^{b}	5.37 ± 0.41^{a}	9.83 ± 0.60^{c}
Pectobacteriaceae	3.28 ± 0.29^{a}	7.79 ± 1.16^{b}	2.20 ± 0.21^{a}	7.46 ± 1.05^{b}
Caulobacteraceae	0.54 ± 0.05^{a}	0.81 ± 0.10^{a}	2.79 ± 0.25^{b}	4.51 ± 0.30^{c}
Xanthomonadaceae	1.04 ± 0.14^{a}	2.65 ± 0.28^{b}	1.61 ± 0.37^{a}	1.39 ± 0.13^{a}
Nocardiaceae	5.85 ± 0.46^{a}	5.76 ± 1.00^{a}	8.26 ± 0.74^{b}	11.11 ± 0.53^{c}
Sphingomonadaceae	14.32 ± 0.65^{a}	16.63 ± 0.98^{a}	8.77 ± 1.15^{b}	6.63 ± 0.48^{b}
Comamonadaceae	2.75 ± 0.33^{a}	1.47 ± 0.13^{c}	0.96 ± 0.19^{b}	0.83 ± 0.13^{b}
Beijerinckiaceae	12.89 ± 0.66^{a}	7.67 ± 1.05^{b}	11.84 ± 0.91^{a}	3.87 ± 0.45^{c}
Micrococcaceae	0.78 ± 0.12^{a}	0.45 ± 0.05^{a}	3.95 ± 0.56^{b}	1.47 ± 0.12^{c}
Nocardioidaceae	1.85 ± 0.18^{a}	0.72 ± 0.10^{b}	5.71 ± 0.62^{c}	2.90 ± 0.14^{c}
Oxalobacteraceae	1.90 ± 0.22^{a}	2.94 ± 0.31^{b}	1.01 ± 0.15^{c}	1.33 ± 0.17^{ac}
Exiguobacteraceae	0.52 ± 0.11^{a}	0.94 ± 0.28^{a}	3.26 ± 0.58^{b}	4.68 ± 0.46^{b}

Families listed are present with greater than 2% relative abundance in at least one of the four groups.

phyllospheres (open field and polytunnel) resulted in all but one family grouping according to plant species (Table 3 and Supplementary Table S7).

Overall, *L. monocytogenes* populations for all four groups showed common negative correlations with families *Sphingomonadaceae* and *Beijerinckiaceae* (Table 4 and Supplementary Tables S8–S11). Similarly, only two common positive correlations were identified, namely, *Pseudomonadaceae* and *Xanthomonadaceae*, between all four groups. *L. monocytogenes* populations of open field rocket had a strong positive correlation with three families, whereas a strong to very strong negative correlation was identified with seven families. *L. monocytogenes* populations of polytunnel rocket had a strong positive correlation with five families, and a strong to very strong negative correlation

TABLE 3 Average absolute abundance (log 10, sequence data linked to total cfu counts) of the 20 most abundant families with significantly different abundances in the phyllosphere of the open field vs. polytunnel and rocket vs. spinach with rarefaction applied. Letters a-c indicate significant differences.

Family	Rocket		Spinach	
	Open fields	Polytunnel	Open fields	Polytunnel
Pseudomonadaceae	6.42^{a}	5.97^{a}	7.08^{b}	7.12^{b}
Sphingomonadaceae	6.10^{ab}	5.44^{a}	7.12^{c}	6.80^{bc}
Nocardiaceae	5.83^{ab}	5.70^{a}	6.70^{bc}	7.04^{c}
Pectobacteriaceae	5.60^{a}	5.10^{a}	6.76^{b}	7.01^{b}
Microbacteriaceae	5.77^{a}	5.28^{a}	6.80^{b}	6.97^{b}
Sphingobacteriaceae	5.54^{a}	5.09^{a}	6.81^{b}	6.78^{b}
Beijerinckiaceae	6.05ab	5.68^{a}	6.77^{c}	6.51^{bc}
Weeksellaceae	5.37^{a}	5.10^{a}	6.60^{b}	6.49^{b}
Rhizobiaceae	5.78^{a}	5.43^{a}	6.43^{b}	6.54^{b}
Paenibacillaceae	4.40^{a}	4.17^{a}	6.39^{b}	6.55^{b}
Caulobacteraceae	4.55^{a}	4.97^{ab}	5.86^{bc}	6.64^{c}
Exiguobacteraceae	4.53^{a}	5.07^{ab}	5.85^{bc}	6.61^{c}
Xanthomonadaceae	5.00^{a}	4.88^{a}	6.38^{b}	6.20^{b}
Nocardioidaceae	5.28^{a}	5.33^{ab}	5.81^{bc}	6.48^{c}
Hymenobacteraceae	5.59^{ab}	4.58^{a}	6.51^{c}	5.57^{bc}
Oxalobacteraceae	5.15^{ab}	4.41^{a}	6.41^{c}	5.98^{bc}
Flavobacteriaceae	5.21^{a}	4.92^{a}	6.30^{b}	5.99^{b}
Micrococcaceae	5.13^{a}	5.48^{a}	5.58^{ab}	6.18^{b}
Comamonadaceae	5.26^{ab}	4.78^{a}	6.11^{c}	5.85^{bc}
Moraxellaceae	4.28^{a}	5.72^{ab}	5.21^{ab}	6.02^{b}

was revealed with seven families. For open field spinach *L. monocytogenes* populations showed a strong to very strong correlation with six families, while strong to very strong negative correlations were identified with only three. Finally, *L. monocytogenes* populations in polytunnel spinach showed a strong positive correlation with four families, and a strong to very strong negative correlation was identified among six families (Table 4).

Pseudomonadaceae content was not significantly different between all four groups ($p = 0.277–0.849$). On average, open field spinach displayed the highest average *Pseudomonadaceae* content, that is, 13.42%, followed by open field rocket 12.79%, polytunnel rocket 10.44%, and, finally, polytunnel spinach 9.60% (Supplementary Tables S8–S11). Therefore, open field spinach, which displayed the highest growth potential of 2.59 $\log_{10}$ cfu g^{-1} was associated with the highest average *Pseudomonadaceae* content, compared to spinach grown in a polytunnel setting, which displayed only 1.40 $\log_{10}$ cfu g^{-1}. Relative abundance of *Pseudomonadaceae* content was compared for all four groups across the five different time points: At day 0, open fields spinach and open field rocket were significantly different ($p < 0.001$) and open field spinach and polytunnel spinach were significantly different ($p < 0.001$), remaining comparisons were not significantly different ($p = 0.154–0.995$). However, at days 2, 5, 7, and 9, no groups were significantly different from one another ($p = 0.448–0.896$, 0.161–0.984, 0.999, and 0.252–0.748). From days 7–9, *Pseudomonadaceae* content increased for open field rocket from 13.35 to 29.11% and polytunnel rocket from 14.12 to 20.91% (Supplementary Tables S8–S11). Open field spinach showed a moderate correlation (+0.66) between *L. monocytogenes and Pseudomonadaceae* compared to the strong positive correlation in polytunnel spinach. Similarly, open field and polytunnel rocket were strongly positively correlated between the two taxa (Table 4 and Supplementary Tables S8–S11).

Relative *Pectobacteriaceae* content (of which genus *Dickeya* was the sole genus) of polytunnel spinach produce displayed an increasing trend in relative abundance (3.9–13.3%) and strong positive correlation with *L. monocytogenes* populations from days 0–9 compared to open field spinach produce which displayed a decreasing trend (12.6–5.6%) and a moderate negative correlation with *L. monocytogenes* for the same period. The *Pectobacteriaceae* content remained consistently lower for rocket than for spinach. Moreover, the content of *Pectobacteriaceae* was positively correlated with *L. monocytogenes* in polytunnel rocket, which had a higher *L. monocytogenes* growth potential than open field rocket. Indeed, *Pectobacteriaceae* content of open field rocket correlated moderately negatively with decreasing *L. monocytogenes* populations (4.4–4.1%, Table 4 and Supplementary Tables S7–S10).

Polytunnel spinach retained the largest relative content of *Lactobacillales* (order level) (0.31%), followed by open field spinach (0.20%), open field rocket (0.16%), and, finally, polytunnel rocket (0.04%). Only the *Lactobacillales* content of open fields rocket vs. polytunnel rocket and polytunnel spinach vs. polytunnel rocket were significantly different ($p = 0.019$ and 0.027). Moreover, over time, significant differences were observed only at day 2, where the following comparisons were significantly different ($p = 0.007$, 0.019,

TABLE 4 Average relative abundance (% ± standard error) of families (16S ribosomal DNA [rDNA]) of open field, polytunnel, spinach and rocket across days 0, 2, 5, 7, and 9 rarefied with strong or very strong Pearson's correlation coefficient (i.e., the strength and direction of the relationship between that specific family's relative abundances and the corresponding *Listeria monocytogenes* populations over time). Letters a-c indicate significant differences between the groups.

	Day 0	Day 2	Day 5	Day 7	Day 9	Pearson's correlation
Open field rocket						
Sphingomonadaceae	16.94 ± 0.93^{a}	14.43 ± 1.26^{ab}	16.01 ± 0.71^{a}	13.06 ± 0.87^{ab}	11.14 ± 1.59^{b}	−0.87, strong
Beijerinckiaceae	14.41 ± 0.28^{a}	13.25 ± 1.60^{a}	15.03 ± 0.95^{a}	12.19 ± 1.08^{a}	9.56 ± 1.76^{a}	−0.73, strong
Pseudomonadaceae	5.04 ± 0.57^{a}	6.01 ± 1.84^{a}	10.45 ± 2.58^{a}	13.35 ± 2.99^{a}	29.11 ± 6.81^{a}	+0.80, strong
Nocardiaceae	4.24 ± 0.59^{a}	5.36 ± 1.23^{a}	5.57 ± 0.54^{a}	8.16 ± 0.35^{a}	5.93 ± 1.27^{a}	+0.79, strong
Rhizobiaceae	3.94 ± 0.45^{a}	3.47 ± 0.84^{a}	3.23 ± 0.70^{a}	6.04 ± 0.91^{a}	5.95 ± 0.37^{a}	+0.73, strong
Hymenobacteraceae	9.06 ± 0.80^{a}	6.59 ± 1.39^{ab}	6.77 ± 1.37^{ab}	5.16 ± 0.66^{ab}	2.84 ± 1.47^{b}	−0.93, very strong
Comamonadaceae	3.55 ± 0.79^{a}	4.16 ± 0.89^{a}	2.42 ± 0.46^{a}	2.25 ± 0.37^{a}	1.35 ± 0.25^{a}	−0.81, strong
Oxalobacteraceae	2.64 ± 0.14^{a}	1.78 ± 0.27^{a}	2.05 ± 0.70^{a}	1.99 ± 0.57^{a}	1.05 ± 0.37^{a}	−0.78, strong
Chthoniobacteraceae	2.17 ± 0.40^{a}	1.06 ± 0.26^{a}	1.68 ± 0.64^{a}	1.35 ± 0.64^{a}	0.86 ± 0.47^{a}	−0.75, strong
Xanthobacteraceae	1.19 ± 0.25^{a}	0.76 ± 0.12^{a}	0.93 ± 0.36^{a}	0.61 ± 0.27^{a}	0.43 ± 0.16^{a}	−0.92, very strong
Polytunnel rocket						
Sphingomonadaceae	16.03 ± 2.85^{a}	10.47 ± 1.66^{ab}	6.75 ± 0.98^{b}	5.63 ± 1.33^{b}	4.96 ± 0.13^{b}	−0.97, very strong
Beijerinckiaceae	14.83 ± 1.09^{a}	14.69 ± 0.68^{a}	10.73 ± 2.37^{a}	10.44 ± 1.87^{a}	8.49 ± 2.22^{a}	−0.93, very strong
Pseudomonadaceae	4.46 ± 2.49^{a}	4.12 ± 1.73^{a}	10.57 ± 1.76^{ab}	14.12 ± 2.67^{bc}	20.91 ± 3.54^{c}	+0.78, strong
Microbacteriaceae	7.82 ± 0.91^{a}	6.24 ± 0.36^{ab}	4.97 ± 0.67^{ab}	4.22 ± 0.44^{b}	3.62 ± 0.17^{b}	−0.99, very strong
Rhizobiaceae	3.09 ± 0.15^{a}	2.87 ± 0.45^{a}	3.68 ± 0.65^{a}	4.85 ± 0.80^{a}	5.84 ± 1.22^{a}	+0.88, strong
Sphingobacteriaceae	1.32 ± 0.14^{a}	0.77 ± 0.24^{a}	2.99 ± 1.30^{a}	4.66 ± 1.03^{a}	2.22 ± 1.03^{a}	+0.70, strong
Pectobacteriaceae	1.91 ± 0.61^{a}	1.81 ± 0.42^{a}	1.95 ± 0.32^{a}	2.60 ± 0.57^{a}	2.75 ± 0.41^{a}	+0.85, strong
Nocardioidaceae	6.24 ± 0.78^{a}	8.49 ± 1.77^{a}	5.58 ± 1.06^{a}	4.86 ± 0.87^{a}	3.40 ± 1.35^{a}	−0.71, strong
Hymenobacteraceae	2.61 ± 0.57^{ab}	3.37 ± 0.54^{a}	1.91 ± 0.48^{ab}	1.47 ± 0.81^{ab}	0.31 ± 0.12^{b}	−0.81, strong
Moraxellaceae	4.39 ± 1.49^{a}	2.42 ± 0.64^{a}	4.79 ± 2.49^{a}	7.27 ± 1.48^{a}	11.73 ± 2.25^{a}	+0.75, strong
Caulobacteraceae	3.93 ± 0.36^{a}	2.86 ± 0.77^{ab}	2.73 ± 0.35^{ab}	2.83 ± 0.41^{ab}	1.57 ± 0.20^{b}	−0.84, strong
Rhodobacteraceae	2.91 ± 0.19^{ab}	4.21 ± 0.61^{a}	2.80 ± 0.47^{ab}	1.47 ± 0.23^{b}	1.41 ± 0.16^{b}	−0.74, strong
Open field spinach						
Sphingomonadaceae	19.87 ± 1.27^{a}	17.07 ± 2.08^{a}	16.97 ± 2.06^{a}	16.57 ± 2.71^{a}	12.67 ± 2.08^{a}	−0.93, very strong
Beijerinckiaceae	7.64 ± 1.03^{a}	12.12 ± 3.48^{a}	8.97 ± 2.37^{a}	6.47 ± 1.18^{a}	3.14 ± 0.54^{a}	−0.81, strong
Sphingobacteriaceae	2.96 ± 1.21^{a}	3.73 ± 1.57^{a}	5.78 ± 1.72^{a}	7.04 ± 1.66^{ab}	11.80 ± 1.31^{b}	+0.99, very strong
Weeksellaceae	2.88 ± 1.11^{a}	3.99 ± 1.66^{a}	3.85 ± 0.91^{a}	3.72 ± 0.81^{a}	6.11 ± 0.63^{a}	+0.88, strong
Xanthomonadaceae	2.04 ± 0.54^{a}	2.60 ± 0.98^{a}	2.20 ± 0.58^{a}	3.04 ± 0.46^{a}	3.37 ± 0.57^{a}	+0.88, strong
Unknown family (*Enterobacterales* order)	1.10 ± 0.36^{a}	1.74 ± 0.57^{a}	2.86 ± 0.55^{a}	5.39 ± 2.73^{a}	5.68 ± 2.05^{a}	+0.94, very strong
Hymenobacteraceae	10.15 ± 1.88^{a}	5.61 ± 0.38^{a}	4.22 ± 0.66^{a}	3.13 ± 0.66^{a}	2.11 ± 0.84^{a}	−0.86, strong
Flavobacteriaceae	0.08 ± 0.03^{a}	0.22 ± 0.07^{ab}	1.34 ± 0.26^{ab}	1.74 ± 1.53^{ab}	4.52 ± 2.03^{b}	+0.97, very strong
Oxalobacteraceae	2.69 ± 0.51^{a}	2.18 ± 0.66^{a}	2.89 ± 0.60^{a}	2.61 ± 0.70^{a}	4.34 ± 0.82^{a}	+0.84, strong
Polytunnel spinach						
Sphingomonadaceae	7.74 ± 0.61^{ab}	7.20 ± 0.79^{ab}	8.59 ± 0.91^{a}	5.65 ± 0.71^{bc}	3.98 ± 0.77^{c}	−0.63, moderate
Beijerinckiaceae	6.52 ± 1.43^{a}	4.21 ± 0.15^{ab}	3.33 ± 0.63^{b}	2.76 ± 0.66^{b}	2.50 ± 0.24^{b}	−0.98, very strong
Pseudomonadaceae	4.19 ± 0.56^{a}	5.09 ± 0.71^{a}	10.31 ± 1.88^{b}	15.72 ± 1.93^{c}	12.67 ± 1.23^{bc}	+0.83, strong
Microbacteriaceae	11.41 ± 0.66^{a}	10.50 ± 2.03^{a}	10.68 ± 1.33^{a}	7.93 ± 0.68^{a}	8.64 ± 1.29^{a}	−0.77, strong
Pectobacteriaceae	3.88 ± 1.25^{ab}	2.97 ± 1.03^{a}	8.43 ± 1.44^{ab}	8.73 ± 1.12^{ab}	13.29 ± 2.39^{b}	+0.87, strong

(Continued)

TABLE 4 (Continued)

	Day 0	Day 2	Day 5	Day 7	Day 9	Pearson's correlation
Weeksellaceae	5.01 ± 0.72[a]	4.05 ± 0.72[a]	3.14 ± 0.50[a]	3.07 ± 0.91[a]	2.31 ± 0.47[a]	−1.00, very strong
Unknown family (*Enterobacterales* order)	0.52 ± 0.24[a]	0.57 ± 0.15[a]	2.44 ± 0.77[a]	6.76 ± 2.40[a]	8.08 ± 2.89[a]	+0.83, strong
Oxalobacteraceae	2.37 ± 0.20[a]	1.80 ± 0.20[a]	1.16 ± 0.08[b]	0.69 ± 0.15[b]	0.69 ± 0.15[b]	−0.96, very strong
Exiguobacteraceae	6.90 ± 0.86[a]	4.91 ± 1.11[ab]	5.43 ± 0.35[a]	4.06 ± 0.72[ab]	2.11 ± 0.15[b]	−0.90, very strong
Rhodobacteraceae	3.46 ± 0.66[a]	3.06 ± 0.23[a]	2.76 ± 0.24[ab]	1.63 ± 0.15[b]	1.56 ± 0.22[ab]	−0.88, strong
Sanguibacteraceae	0.97 ± 0.09[a]	0.94 ± 0.11[a]	1.62 ± 0.19[ab]	1.49 ± 0.22[ab]	2.01 ± 0.31[b]	+0.89, strong

and 0.019): open field rocket and polytunnel rocket; open field rocket and polytunnel spinach; and polytunnel rocket and open field spinach are significantly different. The relative abundance of *Carnobacteriaceae* (family of *Lactobacillales*) was significantly different between open field rocket and polytunnel spinach, as well as open field spinach and polytunnel spinach ($p = 0.037$ and 0.001), while all remaining group comparisons were not significantly different ($p = 0.101–0.582$). The *Carnobacteriaceae* content was on average 0.26% for polytunnel spinach, but was not at all present in open field spinach. On polytunnel and open field rocket, the relative abundance of *Carnobacteriaceae* was on average 0.01 and 0.02%, respectively.

Although detected and enumerated on *Listeria* selective agar, the *Listeria* genus, belonging to the *Lactobacillales* order, was not detected using NGS on open field spinach or open field rocket produce and was detected on only two of 20 samples belonging to polytunnel rocket produce, and in only one of 20 samples belonging to polytunnel spinach produce.

Overall, the majority of bacterial phyla and families were detected on spinach and rocket in both open fields and polytunnels. However, specific taxa and their change in abundance over time could be correlated with *L. monocytogenes* growth.

3.2 Comparison 2 (variety vs. species)

This section describes how their plant hosts shaped alpha and beta diversities at different taxonomic levels, that is, order (Caryophyllales for spinach, Brassicales for rocket and kale, referred here as species) vs. variety (Trumpet and Cello for spinach, Buzz and Esmee for rocket) and how diversity evolved during storage. The primary assumption was that both plant species and variety affect alpha and beta diversities. In turn, differently developing phyllosphere communities were expected to affect *L. monocytogenes* growth over time, as well as the succession of the phyllosphere community over time.

3.2.1 Influence of spinach and rocket cultivars as well as kale on alpha diversity of a *L. monocytogenes* inoculated phyllosphere

Rocket Buzz demonstrated the highest richness and diversity, followed by spinach F1 Trumpet, rocket Esmee, spinach F1 Cello, and, finally, kale Nero di Toscana (Supplementary Table S12). On average, observed features were all significantly different, with kale being the lowest, while rocket Buzz was the highest at day 5. For Faith's Phylogenetic Diversity, kale Nero di Toscana and spinach F1 Cello were statistically similar; and spinach F1 Trumpet and rocket Esmee were statistically identical ($p > 0.05$). Only spinach F1 Trumpet and rocket Buzz had a significantly higher Shannon index than the remaining leafy vegetables. Spinach F1 Trumpet and rocket Buzz displayed the highest evenness (Pielou's) that was substantially higher than for rocket Esmee, spinach F1 Cello, and kale Nero di Toscana.

Indeed, kale Nero di Toscana, with the lowest diversity, was associated with increased *L. monocytogenes* (growth potential = 2.56 $\log_{10}$ cfu g^{-1}), and spinach F1 Cello, with the second-lowest diversity measurements, was associated with the second-highest growth potential, that is, 1.84 $\log_{10}$ cfu g^{-1}. In contrast, the higher diversity groups spinach F1 Trumpet, rocket Esmee, and rocket Buzz were associated with lower growth potentials of *L. monocytogenes* (i.e., 1.23–1.45 $\log_{10}$ cfu g^{-1}). Few significant differences were observed over time for alpha diversity metrics, which were limited to rocket Buzz for Shannon diversity (significantly highest on days 5 and 7) and Pielou's evenness (significantly highest at day 5) (Supplementary Table S12).

The primary observation in this section is that differences in bacterial alpha diversity are related to plant taxonomic relatedness. These, in turn, may limit the growth and potential of *L. monocytogenes* when diversity is high.

3.2.2 Influence of spinach and rocket cultivars and kale on beta diversity of a *L. monocytogenes* inoculated phyllosphere

Based on the PCoA bi-plot, rocket Esmee was more separated on axis two from all other groups (Figure 3). Moreover, spinach F1 Cello and spinach F1 Trumpet partially overlapped, while spinach Trumpet also partially overlapped with rocket Buzz. However, significant differences were identified among all bacterial communities ($p = 0.002–0.036$), indicating that bacterial community structures are determined down to the plant variety level. When rocket and spinach varieties were grouped together, respectively, kale and rocket as well as kale and spinach were no longer significantly different ($p = 0.140$ and 0.059, respectively). However, spinach and rocket remained significantly different ($p = 0.003$). Adjusting the p-value significance threshold with Benjamini–Hochberg correction did not influence the outcome of the PERMANOVA tests. Comparisons within a vegetable variety over time were compromised for kale Nero di Toscana and rocket Esmee due to low sequence reads on days one to seven and day one, respectively.

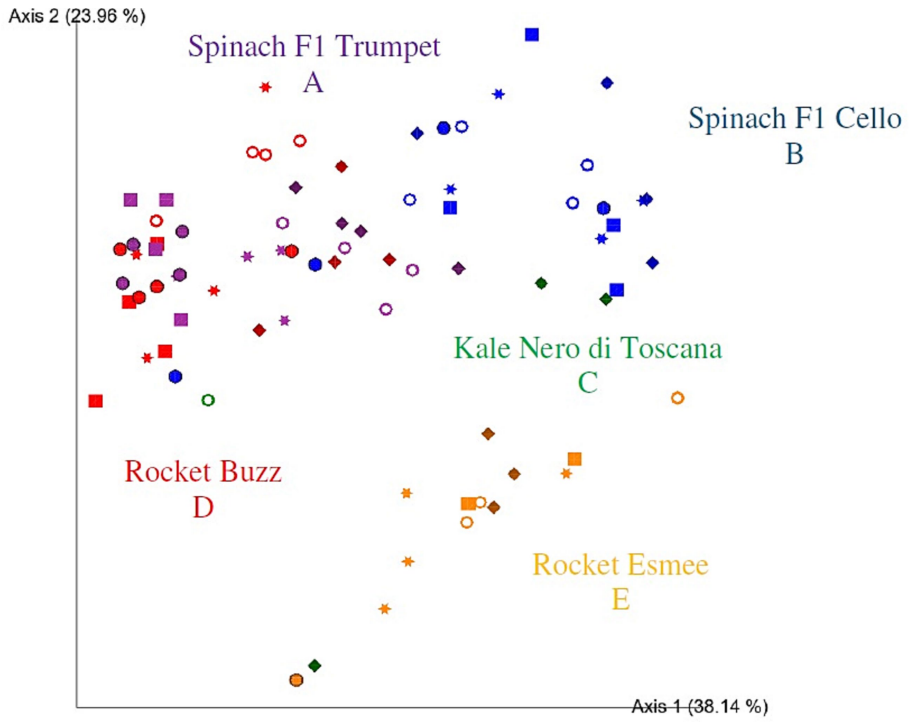

FIGURE 3
Two-dimensional Emperor (PCoA) plots showing beta diversity distances, that is, weighted UniFrac, among the different samples across polytunnel produce: rocket Esmee (orange), spinach F1 Cello (blue), kale Nero di Toscana (green), rocket Buzz (red) and spinach F1 Trumpet (purple) with rarefaction applied. Shapes revealed separations over time where day 0 = circle, day 2 = square, day 5 = star, day 7 = ring, and day 9 = diamond. 16 and 7 samples with low bacterial reads were removed for kale Nero di Toscana and rocket Esmee, respectively. Letters (A-E) indicate significant differences.

3.2.3 Influence of spinach and rocket cultivars and kale on phyla and family relative abundances of a *L. monocytogenes* inoculated phyllosphere

For all five groups, the four most abundant phyla were *Pseudomonadota*, *Actinobacteriota*, *Bacteroidota*, and *Bacillota*, which comprised 95.61–99.58% of the phyllosphere bacterial communities (Figure 4). Over time, the total abundance of these four most abundant phyla remained consistent across all five groups, ranging from 93.15 to 99.92%.

At the family level, 32 were common to all 5 groups, and their relative abundance was overall significantly affected by the leafy vegetable (Supplementary Table S13). Of the 20 most abundant families, 11, 3, 8, and 0 families showed significant changes in relative abundance over time for spinach F1 Trumpet, spinach F1 Cello, rocket Buzz, and rocket Esmee, respectively (Supplementary Tables S14–S17).

Spinach F1 Cello and Trumpet shared 17 out of the 20 most abundant families, whereas rocket varieties Esmee and Buzz only shared 14 families, of which the relative abundances of 10 were significantly different ($p < 0.05$). Since only four (days 7 and 9) kale samples were obtained with a sufficient number of reads for analysis, comparisons at the family level for kale were avoided.

Overall, *L. monocytogenes* populations for both spinach and rocket varieties exhibited only one common negative correlation with the *Sphingomonadaceae* and one common positive correlation with the *Pseudomonadaceae* (Table 4 and Supplementary Tables S14–S17). *L. monocytogenes* populations in spinach F1 Trumpet showed a strong positive correlation with *Pseudomonadaceae*, while a strong to very strong negative correlation was identified with six other families (Table 4). *L. monocytogenes* populations of spinach F1 Cello had a strong and very strong positive correlation with *Flavobacteriaceae* and *Pseudomonadaceae*, respectively, whereas a strong negative correlation was identified with four families. *L. monocytogenes* populations of rocket Buzz had a strong positive correlation with five families and a strong to very strong negative correlation with seven families. For rocket Esmee, a strong positive correlation with families *Pseudomonadaceae* and *Xanthomonadaceae* and a strong to very strong negative correlation was observed with eight families (Table 4 and Supplementary Tables S14–S17).

Spinach F1 Cello had an average higher, although not significant, *Pseudomonadaceae* content (19.0%) compared to spinach F1 Trumpet (9.6%). Rocket Esmee had a significantly ($p < 0.05$) higher average *Pseudomonadaceae* content (28.1%) compared to rocket Buzz (10.8%). However, at the genus level, absolute numbers (based on total heterotrophic counts) of *Pseudomonas* sp. are only clearly higher at days 2, 5, and 7 in variety Esmee when compared to Buzz (Supplementary Table S18). For both spinach varieties and rocket Buzz, *Pseudomonadaceae* content appeared to drastically and significantly increase (3.3–5.5-fold) over time. *Pectobacteriaceae* content (genus *Dickeya*) of polytunnel spinach F1 Trumpet produce displayed an increasing trend in relative abundance from days 0–9 (3.8–13.6%) compared to spinach F1 Cello produce, which displayed a decreasing trend (28.5–6.4%) for the same period. Moreover, the *Pectobacteriaceae* content (genus *Dickeya*) of polytunnel rocket Buzz from days 0–9 remained consistent (1.9–2.6%), whereas it increased substantially on rocket Esmee from 1.0 to 6.9%. Spinach F1 Cello had

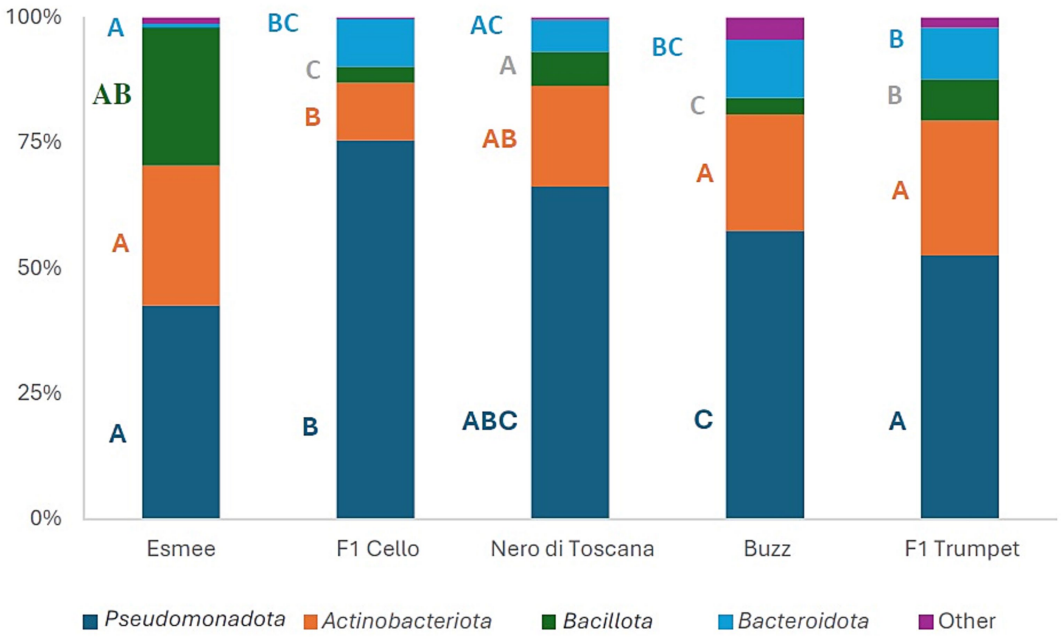

FIGURE 4
Mean relative abundances (%) of the four most abundant phyla of the 16S gene of the polytunnel produce: rocket Esmee, spinach F1 Cello, kale Nero di Toscana, rocket Buzz, and spinach F1 Trumpet, with rarefaction applied. All remaining lower abundant phyla are combined in "Other," Letters A to C indicate significant differences between groups.

a *Lactobacillales* (order level) content of 0.03% compared to 0.35% for spinach F1 Trumpet. The *Lactobacillales* content of rocket Esmee and rocket Buzz was similar to F1 Cello (0.01 and 0.06%). The average *Carnobacteriaceae* content of spinach F1 Cello (*L. monocytogenes* growth potential = 1.84 $\log_{10}$ cfu g^{-1}) was 0.03% and significantly different compared to 0.26% (0.69 and 0.33% at days 7 and 9, respectively) for spinach F1 Trumpet ($p = 0.048$). The *Carnobacteriaceae* content of the remaining groups ranged from 0.00 to 0.01%. *Listeria* (genus) content was only 0.01% for rocket Esmee and spinach F1 Cello, and not detected in rocket Buzz or spinach F1 Trumpet. When samples with a low number of reads were included in the analysis, *Listeria* was identified in 14 out of all 20 kale Nero di Toscana samples. In stark contrast, *Listeria* was detected in two of 20 samples in rocket Esmee (0.05 and 0.01%), spinach F1 Cello (0.04 and 0.07%), and rocket Buzz (both 0.01%), and in only one of 20 samples belonging to spinach F1 Trumpet (0.02%). Bacillaceae showed a strong negative correlation with *L. monocytogenes* growth in rocket Esmee, but not in Buzz or both spinach varieties. However, differences between Esmee and Buzz were also present at the genus level of Bacillaceae, with *Bacillus* sp. about four-fold higher in Esmee on days 2, 5, and 7 than in Buzz (Supplementary Table S18).

Similarly to findings at 3.1.3, the majority of bacterial phyla and families were detected on spinach, rocket, and kale. Similarly, specific taxa and their change in abundance over time appear to be correlated with *L. monocytogenes* growth.

3.3 Comparison 3 (seasonality)

This section describes how alpha and beta diversities were shaped by seasonality (winter and summer) in spinach Trumpet and how diversities evolved during storage. The primary assumption was that winter vs. summer production affects alpha and beta diversities. In turn, differently developing phyllosphere communities were expected to affect *L. monocytogenes* growth over time, as well as the succession of the phyllosphere community over time.

3.3.1 Influence of time of harvest on alpha diversity of a *L. monocytogenes* inoculated spinach phyllosphere

All alpha diversity metrics did not significantly change over time ($p > 0.05$). The number of observed features (ASVs) from summer produce (295–351) and winter produce (308–336) was statistically similar. The same findings were observed for Faith's Phylogenetic Diversity (18.1–24.9). In contrast, the Shannon index was on average significantly greater for winter produce (6.2–6.8, *L. monocytogenes* growth potential = 1.65 $\log_{10}$ cfu g^{-1}), compared to summer produce (5.8–6.4, *L. monocytogenes* growth potential = 2.59 $\log_{10}$ cfu g^{-1}). However, these values did not change significantly over time (days 0–9) for either group ($p > 0.05$). Evenness was also considerably higher for winter produce (0.74–0.81) compared to summer produce (0.72–0.77). Compared to comparisons 1 and 3, here the changes of winter to summer produce had a less pronounced effect on alpha diversity.

3.3.2 Influence of growing season on beta diversity of a *L. monocytogenes* inoculated spinach phyllosphere

Based on the PCoA plot (Figure 5), separations were visually identified between winter and summer groups over time, evident between all data points, as confirmed by PERMAMOVA ($p = 0.001$). Adjusting the *p*-value significance threshold with

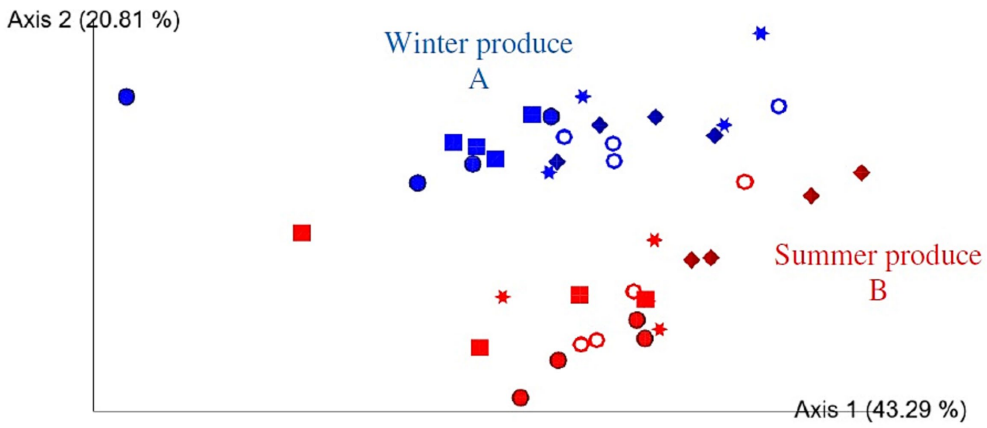

FIGURE 5
Two-dimensional Emperor (PCoA) plots showing beta diversity distances, that is, weighted UniFrac, among the different samples across open field spinach: winter (blue) and summer (red) produce, with rarefaction applied. Shapes revealed separations over time where day 0 = circle, day 2 = square, day 5 = star, day 7 = ring, and day 9 = diamond. A and B indicate significant differences.

Benjamini–Hochberg correction did not alter any significances. Separations for each day 0, 2, 5, 7, and 9 (summer vs. winter) were significant ($p = 0.026–0.048$). A visual separation according to time point within winter and summer produce was also clearly visible (Figure 5). For summer produce, statistically significant separations were observed over time for days 0–9, 2–9, and 5–9 ($p = 0.022–0.032$). For winter produce separations days (0–9, 2–9, 0–7, and 2–7) were significant ($p = 0.026–0.034$). However, after applying Benjamini–Hochberg correction, none of these results remained significantly different. Overall, when compared to alpha diversity, the beta diversity was affected by seasonality.

3.3.3 Influence of time of harvest on phyla and family relative abundance of a *L. monocytogenes* inoculated spinach phyllosphere

For Winter and Summer produce, the most abundant four phyla were *Pseudomonadota*, *Actinobacteriota*, *Bacteroidota*, and *Bacillota*, which comprised 98.94 and 98.86% of the phyllosphere bacterial communities (Supplementary Figure S1). The only significant difference between summer and winter produce at the phylum level was that the *Bacillota* were significantly more abundant in the summer produce ($p < 0.05$). Over time (days 0–9), the total abundance of these four most abundant phyla remained consistent for both groups, ranging from 98.50 to 99.62%.

Winter and summer produce shared 31 families (Supplementary Table S19). However, 20 of those families had significantly different relative abundances between groups ($p < 0.05$). A total of 17 of the most abundant 20 families were shared between both groups, of which eight had significantly different relative abundances, that is, order *Enterobacterales* (family unknown), *Sphingomonadaceae*, *Oxalobacteraceae*, *Rhizobiaceae*, *Caulobacteraceae*, *Nocardioidaceae*, *Rhodanobacteraceae*, and *Nocardiaceae*.

ANCOM revealed 11 differentially abundant families, that is, *Paenibacillaceae*, order *Saccharimonadales* family Unknown, *Myxococcaceae*, *Phormidiaceae*, *Deinococcaceae*, *Rhodobacteraceae*, *Spirosomaceae*, *Moraxellaceae*, *Rhodanobacteraceae*, *Hymenobacteraceae*, and *Nocardioidaceae*, between winter and summer. *Pseudomonadaceae* content was not significantly different between the summer and winter produce ($p = 0.905$) or across all time points from days 0–9 ($p = 0.075$, 0.149, 0.255, 0.051, and 0.527). Similarly, *Lactobacillales* (order level) content was not significantly different between the summer and winter produce ($p = 0.322$) or across days 0–9 ($p = 0.387$, 0.638, 0.773, 0.767, and 0.314). Although *Lactobacillales* relative abundance was less than 1% for all produce, *Lactobacillales* content was on average higher for winter produce (0.34%), compared to summer produce (0.20%). The relative abundance of *Lactobacillales*, that is, *Lactococcus* genus, remained consistent throughout for winter produce, but for summer produce dropped from 0.52 to 0.22 to 0.03% from days 5–9, coinciding with increases in *L. monocytogenes* growth, such levels of *L. monocytogenes* growth which were not observed on winter produce. Moreover, in contrast to polytunnel spinach produce (Comparisons 1 and 2), *Carnobacteriaceae* was not present on open field spinach produce from summer or winter produce (Supplementary Table S19).

L. monocytogenes populations for the summer and winter groups showed five common negative correlations with the families *Sphingomonadaceae*, *Microbacteriaceae*, *Beijerinckiaceae*, *Nocardiaceae*, and *Nocardioidaceae*. Seven common positive correlations were identified with families *Pseudomonadaceae*, *Sphingobacteriaceae*, *Weeksellaceae*, an unknown family (*Enterobacterales* order), *Rhizobiaceae*, *Oxalobacteraceae*, and *Xanthomonadaceae* (Supplementary Tables S20, S21). *L. monocytogenes* populations of winter produce had a strong to very strong positive correlation with six families and a strong to very strong negative correlation with five families. *L. monocytogenes* populations in summer produce showed a strong to very strong positive correlation with six families and a strong to very strong negative correlation with four families (Table 5 and Supplementary Tables S20, S21). Similar to findings at 3.1.3 and 3.2.3, the majority of bacterial phyla and families were detected on spinach summer and winter produce. Again, specific taxa and their change in abundance over time appear to be correlated with *L. monocytogenes* growth. Although detected and enumerated on *Listeria* selective agar, the *Listeria* genus, belonging to the *Lactobacillales* order, was not detected using NGS on either winter or summer open field spinach produce (F1 Trumpet variety).

TABLE 5 Average relative abundance (% ± standard errors) of families (16S rDNA) of the summer open field spinach variety F1 Trumpet produce across days 0, 2, 5, 7, and 9 rarefied with strong to very strong Pearson's correlation coefficient (i.e., the strength and direction of the relationship between that specific family's relative abundances and the corresponding *Listeria monocytogenes* populations over time). Letters a–d indicate significant differences.

	Day 0	Day 2	Day 5	Day 7	Day 9	Pearson's correlation
Winter produce						
Sphingomonadaceae	13.33 ± 3.57^{a}	12.64 ± 0.30^{a}	10.87 ± 1.84^{a}	11.29 ± 1.73^{a}	10.98 ± 1.32^{b}	−0.93, very strong
Pseudomonadaceae	7.35 ± 2.10^{a}	9.86 ± 1.20^{ab}	12.03 ± 2.61^{ab}	19.28 ± 1.00^{b}	19.01 ± 2.05^{b}	+0.89, strong
Microbacteriaceae	7.77 ± 1.13^{a}	8.38 ± 0.62^{a}	6.09 ± 1.42^{a}	5.47 ± 0.67^{a}	5.80 ± 0.56^{a}	−0.82, strong
Beijerinckiaceae	20.05 ± 5.25^{a}	13.57 ± 1.30^{a}	6.45 ± 1.09^{ab}	6.82 ± 1.51^{ab}	7.04 ± 1.13^{ab}	−0.93, very strong
Sphingobacteriaceae	4.08 ± 1.54^{a}	4.90 ± 0.43^{a}	9.18 ± 1.48^{b}	9.37 ± 0.79^{b}	9.10 ± 0.89^{b}	+0.92, very strong
Nocardiaceae	12.51 ± 1.74^{a}	9.58 ± 1.19^{ab}	6.73 ± 1.15^{b}	6.64 ± 1.23^{b}	8.20 ± 0.67^{ab}	−0.84, strong
Unknown family (*Enterobacterales* order)	1.56 ± 0.71^{a}	4.07 ± 1.06^{a}	5.31 ± 1.54^{a}	9.03 ± 3.53^{a}	6.61 ± 0.47^{a}	+0.83, strong
Rhizobiaceae	3.14 ± 0.11^{a}	3.71 ± 0.79^{a}	4.49 ± 1.22^{a}	3.57 ± 0.32^{a}	4.70 ± 0.45^{a}	+0.82, strong
Oxalobacteraceae	1.06 ± 0.35^{a}	1.40 ± 0.16^{a}	1.91 ± 0.36^{a}	1.93 ± 0.33^{a}	1.82 ± 0.37^{a}	+0.91, very strong
Nocardioidaceae	6.24 ± 3.17^{a}	2.93 ± 0.23^{ad}	1.71 ± 0.12^{acd}	1.60 ± 0.32^{bcd}	1.28 ± 0.24^{bc}	−0.93, very strong
Rhodanobacteraceae	1.57 ± 0.37^{a}	2.60 ± 0.39^{a}	2.92 ± 0.64^{a}	2.77 ± 0.88^{a}	2.97 ± 1.07^{a}	+0.91, very strong
Summer produce						
Sphingomonadaceae	19.71 ± 1.10^{a}	17.15 ± 2.12^{a}	16.99 ± 2.12^{a}	16.45 ± 2.59^{a}	12.83 ± 2.03^{a}	−0.95, very strong
Microbacteriaceae	10.27 ± 1.42^{a}	8.01 ± 0.98^{a}	7.37 ± 0.41^{a}	7.31 ± 1.12^{a}	7.25 ± 1.19^{a}	−0.71, strong
Beijerinckiaceae	7.80 ± 1.02^{a}	11.93 ± 3.36^{a}	8.93 ± 2.42^{a}	6.52 ± 1.30^{a}	3.16 ± 0.51^{a}	−0.82, strong
Sphingobacteriaceae	3.09 ± 1.23^{a}	3.62 ± 1.55^{a}	5.80 ± 1.75^{a}	7.06 ± 1.64^{ab}	11.70 ± 1.35^{b}	+0.99, very strong
Weeksellaceae	2.66 ± 1.00^{a}	3.95 ± 1.65^{a}	3.95 ± 0.96^{a}	3.75 ± 0.76^{a}	6.06 ± 0.84^{a}	+0.89, strong
Unknown family (*Enterobacterales* order)	1.13 ± 0.38^{a}	1.69 ± 0.57^{a}	3.00 ± 0.56^{a}	5.56 ± 2.70^{a}	5.56 ± 1.82^{a}	+0.93, very strong
Oxalobacteraceae	2.59 ± 0.46^{a}	2.15 ± 0.61^{a}	2.72 ± 0.55^{a}	2.70 ± 0.78^{a}	4.19 ± 0.71^{a}	+0.88, strong
Xanthomonadaceae	2.09 ± 0.58^{a}	2.64 ± 1.01^{a}	2.17 ± 0.58^{a}	2.91 ± 0.43^{a}	3.21 ± 0.64^{a}	+0.84, strong
Flavobacteriaceae	0.07 ± 0.01^{a}	0.19 ± 0.06^{ab}	1.32 ± 0.31^{ab}	1.71 ± 1.49^{ab}	4.50 ± 2.12^{b}	+0.98, very strong
Hymenobacteraceae	10.01 ± 1.88^{a}	5.61 ± 0.42^{a}	4.31 ± 0.64^{a}	3.19 ± 0.67^{a}	2.09 ± 0.84^{a}	−0.86, strong

4 Discussion

The purpose of this study was to describe the influence of leafy vegetable cultivation conditions (cultivation method, plant species, cultivar, and season of harvest) on the development of the phyllosphere bacteriome and the effect on epiphytic *L. monocytogenes* growth.

4.1 Effects of cultivation conditions (open field vs. polytunnel), plant species (spinach and rocket), and cultivars (varieties)

Previous research assessing the effect of nitrogen fertilizer and leaf mineral content revealed that plant species alone, like spinach and rocket, influence the development of the phyllosphere (Darlison et al., 2019). However, the current study further revealed that the vegetable cultivation method had the strongest influence on the bacterial phyllosphere community structure. At the same time, plant species had a more pronounced effect on the overall abundance of phyllosphere bacteria. Here, polytunnel and open field cultivation of rocket and spinach displayed more similar phyllosphere bacterial communities compared to plant species alone. Additionally, the phyllosphere bacterial communities of various rocket and spinach cultivars were found to be significantly different in the present study. Previous research has identified the presence of microbe-plant variety interactions in field-grown lettuce. Dominated by *Pseudomonadaceae* and *Enterobacteriaceae* families, a clone library of three lettuce cultivars revealed significant differences between the relative abundances of genera belonging to the *Enterobacteriaceae* family, including *Erwinia* and *Enterobacter* (Hunter et al., 2010). While another study of the microbial diversity and structure of the phyllosphere of Alfalfa (*Medicago sativa L.*) identified significant effects of the season and the site where the plants were cultivated in open fields, no significant differences were detected between the two tested varieties (Zhang et al., 2022). While lettuce and alfalfa are bred for cultivation, and both plants are cultivated through a broad range of varieties, only lettuce is bred with the aim of human consumption of the leaves, as is the case for spinach and rocket. One may speculate that the breeding focus of lettuce, spinach, and rocket is primarily on the consumer experience of eating the leaves; thus, different varieties may differ more substantially in their leaf structure than this is the case for other plant varieties that are bred for livestock feeding.

4.2 Correlations between *in situ* phyllosphere taxa and inoculated *L. monocytogenes* growth

A novel aspect of the present study was the identification of the presence or absence of bacteria, and their shifts in relative abundance, which may be of potential importance to the *L. monocytogenes* growth. For example, *Pseudomonadaceae*, which are of high abundance and are associated with the hydrolysis of proteins into amino acids, can induce the stimulation of *L. monocytogenes* growth (Marshall et al., 1992; Zilelidou and Skandamis, 2018). Contrariwise, *Lactobacillales* that were present in low abundance are commonly associated with decreased *L. monocytogenes* survival due to their competitive growth abilities (Østergaard et al., 2014). Indeed, the *L. monocytogenes* growth-enhancing *Pseudomonas* species has previously been associated with spinach leaves of neutral pH (Babic et al., 1996). Additionally, as *Pseudomonas* species are pectolytic, their presence is positively correlated with the degradation and spoilage of such leafy vegetables, which increases during storage, as observed in the present study. Exposure to solar active radiation influenced the relative abundance of the *Betaproteobacteria* and *Gammaproteobacteria*, which is the class level of the *Pseudomonadales* order (Truchado et al., 2017). Relative abundances of *Gammaproteobacteria* were not significantly different with reductions in cumulative photosynthetically active radiation (PAR) from 4,889 to 3,602 μmol m^{-2} s^{-1}, but were substantially higher when cumulative PAR was 3,115 μmol m^{-2} s^{-1}. In the present study, the protection of spinach and rocket produce from PAR by cultivating in a polytunnel setting, compared to an open field, did not lead to significantly higher *Pseudomonadaceae* content.

In the present study, *L. monocytogenes* populations of all groups were positively correlated with *Pseudomonadaceae* content. In particular, *Pseudomonadaceae* content appeared to be most important for *L. monocytogenes* growth on spinach F1 Trumpet produce, especially from day 7 to 9. Relative increases from days 7–9 for open field spinach produce were associated with *L. monocytogenes*' most significant increase during the same period. Conversely, when *Pseudomonadaceae* content decreased from days 7–9 for polytunnel spinach, the *L. monocytogenes* populations remained stationary. Indeed, amino acids hydrolyzed from proteins by *Pseudomonadaceae* are localized within the cellular tissue of leafy vegetables (Koseki and Isobe, 2005; Vacher et al., 2016). Open field spinach produce is likely exposed to more liquids on leaf surfaces due to wetter outdoor climatic conditions, potentially causing higher leaching of those nutrients for *L. monocytogenes* utilization compared to polytunnel produce (Tukey, 1970; Comte et al., 2012; Vacher et al., 2016; Kyere et al., 2019; Zhu et al., 2022). Overall, spinach contained higher total abundances of *Pseudomonadaceae* than rocket. The leaf physiology of rocket, that is, less surface area and fewer stomata (Maylani et al., 2020) might have prevented the release of some nutrients, that is, amino acids (hydrolyzed protein) for *L. monocytogenes* utilization (Culliney and Schmalenberger, 2022), thus limiting the growth of bacteria more than this is the case for spinach. However, at the genus level, higher numbers of *Pseudomonas* sp. on Esmee than on Buzz do not seem to influence the growth potentials of *L. monocytogenes*.

Moreover, a higher *Lactobacillales* content was associated with the lower *L. monocytogenes* growth potential compared to both rocket Buzz and spinach F1 Trumpet produce. A recent study of a mixed spinach salad containing chicken meat identified low levels of *Lactobacillales* content, consisting of only *Carnobacteriaceae* and *Enterococcaceae*, which increased from 0 to 1% at day 7 of storage at 15°C (Söderqvist et al., 2017a). There, the authors did not detect any *Lactobacillales* on plain baby spinach. In another study, storage of romaine lettuce over 14 days revealed a significant increase in *Carnobacteriaceae*'s relative abundance from 1.93 to 52.26% and a non-significant increase in *Pseudomonadaceae* content from 13.38 to 21.20% (Dharmarha et al., 2019). Both bacteriocin-producing, for example, Divercin AS7 and non-bacteriocin-producing species of Carnobacteria, *C. divergens* and *C. maltaromaticum*, have been demonstrated to be effective *in vitro* at minimizing epithelial cell invasion caused by *L. monocytogenes* Scott A (Pilchová et al., 2016) and *Listeria* spp. (Marković et al., 2022). *Carnobacteria piscicola LK5* and *2762* strains suppressed the maximum population density reached by *L. monocytogenes* in brain heart infusion broth (Buchanan and Bagi, 1997). However, little of the *L. monocytogenes* maximum population density suppression was due to the strain's bacteriocin production. Those authors suggested that the suppression potential of the strain *C. piscicola 2762* was not caused by peroxide, pH depression, or oxygen depletion, but was caused by induced nutrient depletion. In the present study, *Carnobacteriaceae* were absent from open field spinach produce but present in significantly higher quantities on polytunnel spinach produce, particularly at days 7 and 9. This may have also inhibited the growth *L. monocytogenes*, leading to its lower growth potential. Moreover, spinach F1 Cello variety had no *Carnobacteriaceae* present, but significantly higher *Pseudomonadaceae* content (+9.43%) compared to spinach F1 Trumpet from the polytunnel setting. Thus, potentially explaining the higher growth potential of the spinach F1 Cello variety. However, albeit a higher *Pseudomonadaceae* content (+5.58%), polytunnel spinach F1 Cello may have caused less leaching of nutrients (hydrolyzed amino acids) due to being less exposed to rain and liquid on surface of the leaf, thus resulting in lower *L. monocytogenes* growth potential for spinach F1 Cello (1.84 $\log_{10}$ cfu g^{-1}) compared to open field spinach F1 Trumpet (2.59 $\log_{10}$ cfu g^{-1}).

4.3 Effect of cultivation conditions (open field, polytunnel, species, and variety) and bacterial taxa abundance on *L. monocytogenes* growth potential

In addition to *Carnobacteriaceae*, polytunnel spinach F1 Trumpet, which displayed a lower growth potential of *L. monocytogenes*, showed an increasing trend in *Pectobacteriaceae* content (genus *Dickeya*). In contrast, open field spinach, which was associated with a larger *L. monocytogenes* growth potential, exhibited a decreasing trend in *Pectobacteriaceae* content (genus *Dickeya*). Additionally, polytunnel spinach F1 Cello, which had a decreasing trend of *Pectobacteriaceae* content (genus *Dickeya*), was associated with a higher *L. monocytogenes* growth potential than polytunnel spinach F1 Trumpet. Similarly, rocket Esmee had an increasing trend in *Pectobacteriaceae* content (genus *Dickeya*), whereas rocket Buzz, with a consistently lower *Pectobacteriaceae* content (genus *Dickeya*), was associated with higher *L. monocytogenes* colonization. *Pectobacteriaceae* spp., in particular the genus *Dickeya*, is a necrotroph that is known to cause soft rot, where deterioration of vegetables occurs from the secretion of plant cell wall-degrading enzymes (Bellieny-Rabelo et al., 2019; Wasendorf et al., 2022). Additionally, *Pectobacterium* spp. are associated with a

type VI secretion system, which also targets plant pathogens lacking cognate immunity proteins by secreting bactericidal effectors and further releasing low molecular weight bacteriocins, that is, carocin, pectocin, and carotovoricin (Shyntum et al., 2019). Moreover, *Pectobacterium*, *Dickeya*, and *Serratia* spp. produce the β-lactam antibiotic carbapenem (1-carbapen-2-em-3-carboxylic acid). However, leafy vegetable isolates of *Pseudomonas* sp., which putatively influenced *L. monocytogenes* growth on spinach in this study, have been found to possess antibiotic resistance genes toward β-lactam antibiotics such as meropenem and colistin (Yin et al., 2022).

4.4 Factors that affect the *L. monocytogenes in situ* growth

In 2016, *Pectobacteriaceae* was added to the *Enterobacterales* order. Prior to this, only a single *Enterobacteraceae* family existed for that order (Adeolu et al., 2016). In the present study, an unknown family from the *Enterobacterales* order was identified, with a relative abundance ranging from 0.00 to 33.46%. While the current study has no particular information on this new taxonomic bacterial group, the *Enterobacteraceae* of the same order possess the ability to produce colicins and microcins (Rebuffat, 2011). Microcins have proven ineffective against *L. monocytogenes*, but colicins produced with the help of the ColE1 gene are highly effective as an anti-listerial agent (Marković et al., 2022). *Enterobacter* spp., particularly *Enterobacter cloacae*, isolated from shredded iceberg lettuce, significantly reduced *L. innocua* colonization due to its nutritional competitiveness (Francis and O'Beirne, 2002).

Darlison et al. (2019) suggested that the influence of phyllosphere diversity on the proliferation of foodborne pathogens such as *L. monocytogenes* should be determined. Indeed, the significantly higher alpha diversity (Shannon index) of produce essentially appears to be correlated with lower *L. monocytogenes* growth potentials in the current study. However, the more diverse polytunnel rocket Buzz variety had more *L. monocytogenes* growth than the rocket Esmee variety. Indeed, higher and increasing *Pectobacteriaceae* content of Esmee, compared to the consistently low *Pectobacteriaceae* content, may be responsible for the 0.22 $\log_{10}$ cfu g^{-1} difference between those two growth potentials.

In the current study, seasonality was a significant driver of phyllosphere development in spinach. Bacterial diversity of the phyllosphere of *Typha latifolia* plants was not meaningfully influenced by short-term perturbations in weather conditions, such as rain events, but somewhat affected by seasonal climatic conditions and leaf-associated changes (Stone and Jackson, 2020). Darlison et al. (2019) suggested that annual variations resulting from varying weather conditions influenced phyllosphere communities of rocket and spinach. Although they could not rule out the effect of site-specific factors, as the produce was sampled in different parts of the same field over the 2 years. The present study accounted for site-specific factors by cultivating from the same location within both field and polytunnel settings, and also observed that weather parameters significantly influenced the spinach phyllosphere. Recently, the spinach phyllosphere has also been shown to be substantially influenced by seasonality (PERMANOVA, $p < 0.003$) (Ibekwe et al., 2021). An additional study revealed that the bacterial colonization of lettuce and rocket phyllosphere is also driven, at least in part, by seasonality (Dees et al., 2015).

4.5 Effects of total abundances of phyllosphere bacteria across plant species

To date, no previous studies have described the kale phyllosphere. Nevertheless, the kale endosphere has been recently studied (McNees et al., 2020). Across three different brands of store-purchased kale, Illumina sequencing of their endospheres revealed two common dominating Operational Taxonomic Units (OTUs) present were *Pseudomonas* and *Enterobacteriaceae*. In the present study, for kale, these, along with *Micrococcaceae* were also dominating families. Kale Nero di Toscana had the most similar content of *Pseudomonadaceae* as spinach F1 Cello. Although it demonstrated higher *L. monocytogenes* growth, due to the lower TBCs of kale (i.e., 2.80–4.74 $\log_{10}$ cfu g^{-1}) and lower diversity, compared to rocket and spinach, less inhibition of the *L. monocytogenes* growth potentially occurs due to less competition for resources required for growth. Utilization of chloroplast-excluding protocols at the polymerase chain reaction (PCR) stage COMPETE (RInvT primer) (McManamon et al., 2019) or BLOCK (pPNA clamp) (Fitzpatrick et al., 2018; Culliney and Schmalenberger, 2024) as employed for open field spinach produce in a recent study, and would have been appropriate for rocket Esmee and kale Nero di Toscana. Their chloroplast-to-total DNA content was high, ranging from 58.00 to 97.30% (rocket Esmee) and 92.27 to 99.75% (kale Nero di Toscana), and thus could have prevented the exclusion of the 7 and 16 samples, respectively. Using the NGS approach, *Listeria* content was regularly detected on kale, but rarely occurred for rocket and spinach produce. The high TBCs of spinach and rocket may have contributed to this observation. Moreover, cultivation methods may not have detected cells that were at their viable but not-culturable (VBNC) stage (Müller and Ruppel, 2014). Thus, TBCs for all produce, including kale, may have been underestimated and, therefore, their total DNA content may have been associated with higher actual abundances. For example, a previous study used quantitative PCR (qPCR) and culturable techniques (TSA) to analyze lettuce samples from the same field and revealed that only 0.1–8.4% of TBCs were culturable bacteria (Rastogi et al., 2010). qPCR methods may be used in the future to enumerate TBC for this reason. However, qPCR-based quantifications may potentially overestimate bacterial population densities due to chloroplast co-amplification (Culliney and Schmalenberger, 2022) and multiple 16S ribosomal RNA (rRNA) gene copies per bacterial cell (Schmalenberger et al., 2001); hence, cultivation-dependent and independent approaches have biases. Furthermore, primer selection for 16S rRNA gene-based amplicon sequencing may also be responsible for an additional due to primer mismatch, which appears to be the case for *L. monocytogenes* 16S with the popular V3–V4 primers.

4.6 *Pseudomonadaceae* and *Lactobacillales* with putative contradicting effects on *L. monocytogenes* growth

Ibekwe et al. (2021) revealed that the four common dominating phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, which comprised 66.35% of their phyllosphere, were significantly different from the overall abundance, ranging from 88.21 to 99.92% of those four main phyla for spinach in the present study. Additionally, their Pseudomonadaceae content (0.49–11.5%) was,

on average, lower than the *Pseudomonadaceae* content observed on spinach, rocket, and kale produce in this study. With an overall relative abundance of 35–53%, Pseudomonas has been referred to as the most commonly occurring genus in the spinach and rocket phyllospheres, even after being harvested in different seasons (spring and autumn) (Rosberg et al., 2021). Upon closer inspection of the *Pseudomonadaceae* family's relative abundances, potential seasonal effects exist, especially for spinach. However, in the current study, the relative abundances of winter and summer open field spinach did not show significant differences. However, there was still a large difference of 0.94 $\log_{10}$ cfu g^{-1} between their *L. monocytogenes* growth potentials. LAB are more commonly detected on leafy produce cultivated in spring and summer compared to autumn and winter (Caponigro et al., 2010); however, the opposite was true in the current study. With a relative abundance of less than 1%, *Lactobacillales* may have been responsible for the significant growth potential difference. The *Lactobacillales* decreased from 0.52 to 0.22 to 0.03% from days 5–9 for summer produce, which was correlated with large increments in *L. monocytogenes* growth, which did not occur when *Lactobacillales* remained constant and on average in higher relative abundance for winter produce. More specifically, winter produce with lower *L. monocytogenes* growth had a significantly higher content of the *Lactococcus* genus (*Streptococcaceae* family; *Lactobacillales* order). Indeed, *L. lactis* subsp. *lactis* has been previously isolated from rocket leaves and is known as a bacteriocinogenic strain due to its ability to produce lantibiotic, which is an antimicrobial nisin variant that is highly effective as an anti-listerial agent on food products, including iceberg lettuce (Franz et al., 1997; Kruger et al., 2013; Ho et al., 2018; McManamon et al., 2019; Ho et al., 2021).

The remainder of phyllosphere-associated bacteria, which showed positive and negative correlations with *L. monocytogenes* populations identified in this study, did not appear to be potentially responsible for the conflicting epiphytic *L. monocytogenes* growth on spinach or rocket leaves. Correlations were determined using Pearson's correlation, which is primarily used for linear relationships between two continuous variables, due to the normal distribution and increasing *L. monocytogenes* populations over time. However, a recent study revealed that Pearson's can also be more efficient in testing a monotonic nonlinear relation compared to Spearman's (van den Heuvel and Zhan, 2022). Future studies may use Spearman's correlation as it evaluates the monotonic relationship between two continuous variables (Schober et al., 2018). Indeed, this approach is most often used for bacterial growth curves, which reach the stationary phase. In either case, such correlations must be interpreted with caution. For example, Zhao et al. (2021) revealed that association means that one variable provides information about another, whereas correlation means that two variables show an increasing or decreasing trend. Therefore, correlation implies an association, but not causation. Additionally, due to the absence of absolute numbers upon sequencing (Gloor et al., 2017), comparing relative abundances could lead to inaccurate conclusions when comparing phyllosphere microbiome over time or when comparing different phyllosphere communities, for example, kale or spinach, which have considerably different absolute cfu data, as relative data reflect a different amount of absolute numbers. Future studies should conduct correlations between absolute cfu data, that is, total bacterial populations and relative abundances from NGS datasets that are converted into absolute values via an additional qPCR step.

5 Conclusion

This study identified a link between leafy vegetable species, variety, and environmental growth conditions and the bacterial communities present on the leaf surface, that is, *Pseudomonadaceae*, *Pectobacteriaceae*, and *Lactobacillales*, such as *Streptococcaceae* and *Carnobacteriaceae*. Together, these factors are important in determining the growth potential of *L. monocytogenes*. However, the *Pseudomonadaceae* content appeared to be less critical for plant species with specific leaf surface characteristics, such as a narrow leaf surface area and a smaller number of stomata (e.g., rocket). Therefore, future studies should include leaf surface analyses in growth studies of *L. monocytogenes* on leafy vegetables. Due to the limitations of second-generation sequencing technologies in determining species-level identification of bacteria, a sequencing approach using third-generation amplicon sequencing techniques, as well as true metagenomics approaches, may reveal further insights into the functions of certain bacterial taxa in the phyllosphere and their abilities to aid or retard the *L. monocytogenes* growth.

Advancing aspects of microbial food safety for leafy vegetables may include future selection of varieties that are not only preferred due to their taste and sensory input during consumption but also due to their beneficial natural microbiome. Similarly, one could imagine a future where leafy vegetables are treated with probiotic foliar applications, where beneficial microbes are designed not only to be helpful for digestion but also helpful in suppressing foodborne pathogens.

EURL's guidance document requires three batches for assessment of the growth potential of RTE products. These three batches are recommended to be from different production days. Although based on results from this study, this should be further updated to reflect produce with different seasonality. Moreover, as identified in the present study for spinach and rocket, the presence of certain phyllosphere or microbiome members could provide more in-depth information regarding *L. monocytogenes* growth potentials on RTE food products than TBC. Thus, the inclusion of NGS techniques could be considered an essential tool for assessing future challenges.

Microbiologists looking to describe the phyllosphere of kale or rocket (Esmee variety) should consider the use of chloroplast amplification blocking methods. This will reduce the number of samples discarded due to low bacterial reads, as occurred in the present study, thereby providing more detailed descriptions of phyllosphere-associated bacteria.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA1177234.

Author contributions

PC: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. AS: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1516740/full#supplementary-material

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