International Journal of Food Microbiology 360 (2021) 109438

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Genomic characteristics of *Listeria monocytogenes* isolated during mushroom (*Agaricus bisporus*) production and processing

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ARTICLE INFO

Keywords:
Frozen sliced mushroom processing
MLST
cgMLST
Antibiotic susceptibility
Benzalkonium chloride susceptibility

ABSTRACT

Listeria monocytogenes is a foodborne pathogen ubiquitously found in nature and which has been isolated from food and food processing environments. This study aimed to characterize *L. monocytogenes* strains isolated from the production and processing environments of frozen sliced mushrooms (*Agaricus bisporus*). An analysis was executed along the mushroom processing chain including one mushroom grower and two mushroom processing factories. A total of 153 *L. monocytogenes* strains were isolated, which could be grouped in three PCR serogroups, namely, serogroup 1/2a-3a (39.2%), serogroup 1/2b-3b-7 (34.0%) and serogroup 4b-4d-4e (26.8%). A selection of 44 *L. monocytogenes* strains isolated from the processing environment after cleaning and disinfection (C&D) and from frozen sliced mushrooms was genotyped by whole genome sequencing (WGS), because these strains pose a potential risk for product contamination after C&D and for human consumption. Multilocus sequence typing (MLST) revealed 11 clonal complexes (CCs), with strains belonging to CC1, CC4, CC37 and CC87 being detected in both processing factories. Comparative WGS analysis of the 44 strains showed the presence of *Listeria* pathogenicity island 1 (LIPI-1) with a disrupted version of *actA* in all CC1, CC4, CC5, CC59 strains, and all but one CC224 strains. Notably, both *inlA* and *inlB* were detected as full-length loci in every strain, except for *inlA* in a CC6 strain that harbored a three amino acid deletion. LIPI-3 was detected in all CC1, CC4, CC6 and CC224 strains, while LIPI-4 was detected in all CC4 and CC87 strains. In addition, antibiotic susceptibility tests showed susceptibility towards fourteen antibiotics tested. The *bcrABC* operon was found in one CC5 strain, that showed a higher tolerance towards benzalkonium chloride than any other strain tested with confluent growth till 12.5 μg/ml for the CC5 strain compared to 2.5 μg/ml for the other strains. This study highlights that the ecology of *L. monocytogenes* in the frozen sliced mushroom production chain is highly diverse, and shows the importance of hygienic measures to control *L. monocytogenes* along the frozen sliced mushroom production chain.

1. Introduction

Listeria monocytogenes is a major foodborne pathogen that can cause listeriosis in humans. This disease is especially relevant for sensitive population groups (the elderly, immunocompromised persons, pregnant women and infants). Infection with *L. monocytogenes* could lead to spontaneous abortion in pregnant women, septicemia or meningitis and infections have led to a high case fatality rate of 17.6% in the EU in 2019 (EFSA and ECDC, 2021).

L. monocytogenes has been isolated in food products such as ready-to-eat (RTE) vegetables and frozen vegetables (Moravkova et al., 2017). Presence of *L. monocytogenes* on food products may be attributed to its ubiquity in nature (Sauders et al., 2006; Sauders et al., 2012) and its robustness to cope with different stresses as *L. monocytogenes* is able to grow at low pH, high salt concentrations and at refrigeration temperatures (Van der Veen et al., 2008; Walker et al., 1990). These characteristics could be of serious concern for the RTE foods industry, as RTE foods are intended by the producer for direct consumption without the

Abbreviations: RTE, ready-to-eat; ST, sequence type; CC, clonal complex; BC, benzalkonium chloride; LIPI, *Listeria* pathogenicity island; inl, internalin; SSI, stress survival islet; WGS, Whole Genome Sequencing.

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https://doi.org/10.1016/j.ijfoodmicro.2021.109438
Received 7 June 2021; Received in revised form 5 October 2021; Accepted 8 October 2021
Available online 14 October 2021

need for cooking or other type of processing (European Comission (EC), 2014). This concerns the mushroom industry, as the white button mushroom (*Agaricus bisporus*) can be classified as a RTE food. This includes both hand-picked mushrooms that are sold fresh and machine-harvested mushrooms that are sliced and sold frozen, although the frozen mushrooms are usually not classified and sold as RTE food by the producer.

L. monocytogenes has been detected in the *A. bisporus* mushroom production environment (Pennone et al., 2018), on a variety of edible mushrooms (*A. bisporus* not included) (Chen et al., 2018) and on frozen mushrooms (Willis et al., 2020). *L. monocytogenes* strains isolated from *A. bisporus* mushroom production environments were demonstrated to form biofilms on materials used in the mushroom processing industry (Dygico et al., 2020). Detection of *L. monocytogenes* has led to a limited number of mushroom recalls, including recalls of fresh enoki mushrooms in Europe (Pennone et al., 2018) and fresh sliced white mushrooms in Canada (Anonymous, 2012). To date, in spite of no reports of listeriosis cases associated with the consumption of *A. bisporus* mushrooms, a listeriosis case associated with the consumption of fresh enoki mushrooms has been reported in a multistate outbreak in the USA that led to 36 diseased individuals of which 31 were hospitalized and four died (Anonymous, 2020).

Various typing methods can be applied to characterize and group different *L. monocytogenes* strains. The multiplex polymerase chain reaction (PCR) serogrouping technique discriminates between groups of serotypes, namely, serotypes 1/2a and 3a, serotypes 1/2b, 3b and 7, serotypes 1/2c and 3c, and serotypes 4b, 4d and 4e (Doumith et al., 2004). Most *L. monocytogenes* strains have been described to belong to lineages I and II, which contain serotypes 1/2b, 3b, 4b, 4d, 4e and serotypes 1/2a, 1/2c, 3a, 3c, respectively. Most listeriosis cases to date have been attributed to serotypes 1/2a, 1/2b and 4b (Orsi et al., 2011). Further strain discrimination could be applied with PFGE analysis, but whole genome sequencing (WGS) has demonstrated higher discriminatory power (Pietzka et al., 2019) enabling the detection of a common cause of a listeriosis outbreak (Pettengill et al., 2020). WGS data can be applied for multilocus sequence typing (MLST) that has been used to cluster *L. monocytogenes* strains into clonal complexes (Ragon et al., 2008). WGS data could also be used for the core genome MLST (cgMLST) technique, which is a method with a high discriminatory power that uses the sequence variation of 1748 core genome genes of *L. monocytogenes* (Moura et al., 2016). Analyzing large amounts of *L. monocytogenes* genomic data has led to the identification of hypervirulent and hypovirulent clonal complex types, that were overrepresented among clinical and food isolates, respectively (Maury et al., 2016), as well as grouping of CCs into hypovirulent, medium virulent and hypervirulent clonal complex groups based on clinical frequency (Fritsch et al., 2018).

A wide variety of *L. monocytogenes* virulence genes have been described so far, including the *Listeria* pathogenicity island 1 (LIPI-1). LIPI-1 genes encode for virulence factors that are involved in important processes in the intracellular life cycle of *L. monocytogenes*. This includes *actA*, which was shown to be involved in actin recruitment for intracellular movement and intercellular spreading (Vázquez-Boland et al., 2001). LIPI-3 encodes a cytotoxic and a hemolytic factor that has been shown to contribute to the virulence of *L. monocytogenes* (Cotter et al., 2008) and LIPI-4 is associated to infection of the central nervous systems and to maternal neonatal infections (Maury et al., 2016). In addition, internalins have been identified in *L. monocytogenes* with roles in pathogenicity, except for the InlI protein (Bierne et al., 2007). An important internalin is the surface protein InlA of *L. monocytogenes* that plays a key role in the epithelial cell entry (Nikitas et al., 2011). Mutations in *inlA* could lead to a pre-mature stop codon (PMSC) and it has been shown that PMSCs in *inlA* are associated with attenuated virulence (Nightingale et al., 2005).

Various *L. monocytogenes* stress defense and survival strategies have been described including resistance to environmental stresses, disinfectants and antibiotics. Genes encoded on stress survival islet 1 (SSI-1) have been shown to enhance growth at low pH and high salt concentrations (Ryan et al., 2010), while SSI-2 encoded functions were shown to enhance survival of *L. monocytogenes* in alkaline and oxidative stress conditions (Harter et al., 2017). Presence of the *bcrABC* resistance gene cassette in *L. monocytogenes* has been attributed for growth at elevated levels of benzalkonium chloride (Elhanafi et al., 2010). In addition, resistance to antibiotics that are commonly used for treatment of *L. monocytogenes* infections, such as ampicillin and penicillin, has been reported and this has raised concerns since antibiotic treatments could become less effective in case multiple antibiotic resistant strains arise (Olaimat et al., 2018). Additionally, an ongoing discussion concerns the possible persistence of pathogens to cleaning and disinfectants used in food industry. Particularly because the resistance mechanisms involved in survival to industrial disinfectants may provide cross resistance to antibiotics used in clinical settings (Donaghy et al., 2019).

To date, no chain-wide analysis has been performed that characterized *L. monocytogenes* strains present in mushrooms from the growing farms up to the frozen sliced mushrooms in mushroom processing factories. Therefore, this study aims at characterizing *L. monocytogenes* strains isolated from industrial equipment surfaces after cleaning and disinfection, as well as isolates from frozen sliced mushrooms. The complementary genetic and phenotypic typing approaches gave new insights into the presence, diversity and virulence repertoire of the *L. monocytogenes* strains from frozen sliced mushroom production facilities.

2. Materials and methods

2.1. Listeria monocytogenes isolation

Isolates of *L. monocytogenes* were collected during an analysis in the spring of 2018 in the Netherlands. Samples were taken in a chronological order following one particular batch of mushrooms, from the filling of the growing room at the mushroom grower's facility to the frozen sliced mushrooms at the mushroom processing factory (Supplementary Table S1). Casing soil was sampled at the grower's facility. This was followed by sampling mushrooms and underlying casing soil during the first and second harvest, and by swabbing harvest equipment during the second harvest and after cleaning and disinfection (C&D). Mushrooms were transported to the factory and were sampled before processing and after processing as frozen sliced mushrooms. Moreover, processing equipment in the factory was swabbed during mushroom processing and after C&D treatment. In addition, frozen sliced mushrooms were sampled before and after the aforementioned batch of grower 1 and included mushrooms from multiple growers. Also, in spring of 2018, samples were taken from the frozen sliced mushrooms of processing factory 2, which uses mushrooms from multiple growers. Lastly, samples were taken of fresh mushrooms at factory 1 during previous years (2016 and 2017) including mushrooms from multiple growers. Surface sampling was executed using cotton swabs (CLASSIQSwabs, 165KS01, Copan) moisturized in 10 ml half Fraser broth containing 0.1% Tween80. They were used to swab a surface area of 100 cm^2 after which the swab was resuspended in the half Fraser broth suspension. All casing soil samples and mushroom samples were diluted 1:10 in half Fraser broth by adding 25 g of sample in 225 ml half Fraser broth.

Samples of casing soil, mushrooms and surface swabs were analyzed for presence of *L. monocytogenes* following the ISO protocol NEN-EN-ISO 11290-1:2017 (International Organization for Standardization, 2017). After incubation, a loopful of the half Fraser broth and the full Fraser broth were streaked on Agar Listeria according to Ottaviani-Agosti (ALOA) plates (Biomérieux) and plates were incubated for 24–48 h at 37 °C. Presumptive *L. monocytogenes* colonies, blue-green colonies with an opaque halo (one colony per positive sample), were purified by restreaking on ALOA and incubated for 24 h at 37 °C. Subsequently, a single colony was restreaked on Brain Heart Infusion agar (BHI) (Becton

Dickinson and Company, Difco) supplemented with 1.5% agar (Oxoid) and incubated for 24 h at 30 °C followed by another streak on BHI agar plates incubated for 24 h at 30 °C to ensure isolate purity. Pure isolates were cultured in BHI broth incubated statically for 17 h at 30 °C for preparing −80 °C stock cultures with a final concentration of 25% glycerol (Sigma-Aldrich).

2.2. *L. monocytogenes* confirmation

Each isolate was streaked on BHI agar followed by incubation for 24 h at 30 °C. Presumptive *L. monocytogenes* isolates were confirmed by heamolysis tests using blood (defibrinated sheep blood, Biotrading) agar. For that purpose, colony material from BHI agar plates was streaked on 6% (v/v%) blood agar plates and incubated for 24 h at 37 °C. Carbohydrate utilization was tested by taking colony material from BHI agar plates and inoculating three tubes, each containing carbohydrate utilization medium with 0.5% of carbohydrate (mannitol, xylose or rhamnose). Tubes were incubated for 24–48 h at 37 °C and *L. monocytogenes* was confirmed when a color change had occurred for rhamnose only (NEN-EN-ISO 11290-1:2017) (International Organization for Standardization, 2017).

Further confirmation of the *L. monocytogenes* isolates was executed using multiplex PCR analysis with *Listeria* spp. specific primers (*prs* primer set targeting *prs* gene) (Doumith et al., 2004) and *L. monocytogenes* specific primers (*isp* primer set targeting *isp* gene) (Rawool et al., 2016). Several colonies per isolate were transferred from the BHI agar plate and were resuspended in 100 µl InstaGene Matrix (Bio-Rad) and the manufacturer's protocol was followed for DNA extraction. The PCR reaction mixture contained 0.5 µl genomic DNA, 0.2 µM of *prs* primer set for *Listeria* spp. determination (Doumith et al., 2004), 0.2 µM of *isp* primer set for *L. monocytogenes* determination (Rawool et al., 2016), 2.5 µl of 10× Taq buffer (including 20 mM $MgCl_2$, Thermo Scientific), 0.2 mM dNTP mix (Thermo Scientific), 0.6 U Dreamtaq DNA polymerase (Thermo Scientific), in a total volume of 25 µl. The PCR cycle was performed in a Veriti 96-well Thermal Cycler (Applied Biosystems) and included an initial denaturation step at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 0.40 min, annealing at 56 °C for 1.15 min and extension at 72 °C for 2 min followed by final extension step at 72 °C for 10 min. Five microliter of PCR product was mixed with 1 µl 6× DNA loading dye (TriTrack, Thermo Scientific) and samples were examined in a 1% agarose (SeaKem LE agarose, Lonza) gel containing 1× TAE buffer (Bio-Rad) and DNA safe stain (SYBR Safe DNA Gel Stain, Invitrogen). Gels were run in 1× TAE buffer and DNA bands were visualized with ultraviolet light (Uvitec, Cambridge). Isolates confirmed to be *L. monocytogenes* were used in further analyses and an overview is listed in Supplementary Table S1.

2.3. *L. monocytogenes* serogroup determination

Confirmed *L. monocytogenes* isolates were PCR serogrouped using the multiplex PCR protocol (Doumith et al., 2004) with some modifications. The components of the reaction included 0.5 µl genomic DNA, 0.4 µM of three primer sets (*lmo0737*, *orf2819*, *orf2110*) and 0.6 µM of one primer set (*lmo1118*), 2.5 µl 10× Taq buffer, 0.2 mM dNTPs, 0.6 U Dreamtaq DNA polymerase, in a total volume of 25 µl. The PCR cycle was adapted from Doumith et al. (2004) and PCR products were visualized as described in Section 2.2.

2.4. Genomic DNA isolation, library preparation, sequencing and genome annotation

A selection of 44 strains isolated from frozen sliced mushrooms and from swab samples taken after C&D was used for whole genome sequencing. These locations were selected because strains on frozen sliced mushrooms pose a higher risk due to probability of consumption and strains surviving on processing equipment after C&D practicing pose a potential risk for product contamination during processing. Selection of the strains was based on the serogroup abundance in a location and the number of selected strains per serogroup reflected the relative abundance of that serogroup. If a particular serogroup was more abundant, a higher percentage of strains of this serogroup were selected for WGS. If a particular strain was less abundant, a lower percentage of this serogroup was selected for WGS. This strain selection included 31 strains from factory 1, namely 11 strains from frozen sliced mushrooms that were supplied by multiple growers, 12 strains from frozen sliced mushrooms when mushrooms of grower 1 were processed, and eight strains from processing equipment after C&D. Moreover, 13 strains were selected from the frozen sliced mushrooms processed at factory 2.

Extraction of genomic DNA was performed using the DNeasy Blood & Tissue kit (Qiagen), according to the protocol provided by the manufacturer with some modifications. Strains were grown from frozen stock cultures by streaking on BHI agar and plates were incubated at 30 °C for 24 h. Colonies were collected and resuspended in 10 ml BHI broth and grown statically for 17 h at 30 °C. Two milliliter overnight cultures were centrifuged for 2 min at 16,000 ×*g* and pellets were washed in one ml peptone physiological salt (PPS, Tritium Microbiologie) and resuspended in one ml enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% (w/v) Triton-X-100, 5 mg/ml lysozyme, pH 8.0). Then, solutions were incubated at 37 °C for one hour after which 10 µl RNAse was added followed by incubation at room temperature for 30 min. Subsequently, 62.5 µl proteinase K and 500 µl AL buffer were added and the mixtures were incubated at 56 °C for one hour. Five hundred microliter ethanol (96% ethanol, Merck) was added to the mixtures and the solutions were transferred to the spin columns provided by the kit for DNA isolation. The mixtures were left on the columns for 10 min and centrifuged for 1 min at 6000 ×*g*. Five hundred microliter ethanol (96%) was added to the columns, left for 10 min and centrifuged for 1 min at 6000 ×*g* and this step was repeated one time. Additionally, the columns were washed two times with 500 µl AW1 and two times with 500 µl AW2 by centrifuging 1 min at 6000 ×*g* and unloaded columns were centrifuged for 3 min at 16,000 ×*g* to dry the membranes. DNA was eluted by adding two times 50 µl MilliQ followed by incubation at room temperature for 10 min and centrifugation for 1 min at 6000 ×*g*. DNA concentrations and quality were determined using a nanodrop (ND-1000 Spectrophotometer).

Library preparation and paired-end 2 × 150 bp short-reads were generated using the INVIEW resequencing of bacteria service from Eurofins GmbH (Constance, Germany) using Illumina Miseq chemistry. Read quality control was performed using FastQC 0.11.5, after which reads were de novo assembled via SPAdes 3.13.1 (Bankevich et al., 2012) with the careful option and k-mer values of 21, 33, 55, 77. Assemblies were checked using QUAST (Mikheenko et al., 2018). Annotation of genomes was done using Prokka (Seemann, 2014). The raw sequences of all strains were deposited in the Sequence Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/sra under BioProject PRJNA726944.

2.5. MLST and cgMLST determination and tree construction

MLST was performed using the sequences of seven housekeeping genes to assign strains to sequence types (STs) and clonal complexes (CCs) (Ragon et al., 2008). This was determined using the analysis platform of Institut Pasteur (bigsdb-*Lm*; https://bigsdb.pasteur.fr/listeria/listeria.html, last accessed: 22 January 2021) (Moura et al., 2016). In addition, a phylogenetic tree was constructed using the 44 strains based on the concatenated DNA sequences of the seven MLST genes. The tree was constructed using Clustal W - Simple Phylogeny (Madeira et al., 2019) with default setting and using UPGMA clustering method. The tree was visualized using the Interactive Tree Of Life (iTOL) version 5 (https://itol.embl.de, last accessed: 22 January 2021) (Letunic and Bork, 2019).

In addition, the core genome MLST (cgMLST) of all 44 strains was performed using 1748 conserved loci and cgMLST profiles were

determined using the analysis platform of Institut Pasteur (bigsdb-*Lm*; https://bigsdb.pasteur.fr/listeria/listeria.html, last accessed: 22 January 2021) (Moura et al., 2016). The genome sequencing approach was not able to cover the whole genome and to assign numbers to all 1748 loci of every strain. Unidentified alleles were marked as NA and excluded from every strain. This yielded a total of 1463 cgMLST alleles, out of 1748 cgMLST alleles, for which allele numbers were assigned that were used to construct the minimum spanning tree. A minimum spanning tree of the 44 strains was created using Phlyloviz online (http://online2.phyloviz.net/index, last accessed: 22 January 2021) (Nascimento et al., 2017; Ribeiro-Gonçalves et al., 2016). Supplementary Table S2 provides a detailed list of the characteristics of the 44 strains.

2.6. Genetic characterization of L. monocytogenes strains

Assembled genomes were screened for the presence of virulence factors, stress survival islets, cleaning and disinfectant resistance markers, biofilm genes and antibiotic resistance genes. Genes of interest were extracted and mapped to every strain for gene visualization, after which presence, absence and gene modifications were assessed. Alignments were performed against the *L. monocytogenes* EGD-e genome (RefSeq = NC_003210.1, NCBI), except for alignment of LIPI-3, LIPI-4, and *bcrABC* for which the genomes of *L. monocytogenes* PNUSAL000019 (RefSeq = NZ_CP054040.1), *L. monocytogenes* Clip80459 (RefSeq = (NC_012488.1) and *L. monocytogenes* N1-011A (RefSeq = NC_022045.1, derived from the plasmid) were used, respectively.

2.7. Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using a range of antibiotics that have been reported in treatment of listeriosis patients (Temple and Nahata, 2000). Antimicrobial susceptibility testing of the selected *L. monocytogenes* strains using the disc diffusion method was performed by streaking the stock cultures on BHI agar followed by incubation for 24 h at 30 °C. After incubation, several colonies were picked and resuspended in a tube containing nine ml PPS. The homogenized suspension was streaked with a sterile cotton swab (CLASSIQSwabs, 165KS01, Copan) on Mueller-Hinton agar (MH) (Oxoid). The antibiotic susceptibility discs (Oxoid) were placed on top of the agar surface and plates were incubated at 37 °C for 24 h. Different antibiotics (disc content) were tested, namely, ampicillin (10 μg), gentamycin (10 μg), sulfamethoxazole-trimethoprim (23.75 μg + 1.25 μg), erythromycin (15 μg), penicillin G (10 units), streptomycin (10 μg), tetracycline (30 μg), vancomycin (30 μg), rifampin (5 μg), imipenem (10 μg), ciprofloxacin (5 μg), amoxicillin-clavulanic acid (20 μg + 10 μg), linezolid (30 μg) and chloramphenicol (30 μg). Nalidixic acid (30 μg) was included as a positive control. Inhibition zones of the disk diffusion method were measured after incubation and compared with the inhibition zones of *L. monocytogenes* (EUCAST, 2020) and of *Staphylococci* spp., recommended by the Clinical and Laboratory Standards Institute (CLSI) (*CLSI M100-S22*, 2012), since not all tested antibiotics had breakpoints available for *L. monocytogenes*. The inhibition zones for two antibiotics, streptomycin and vancomycin, have not been reported for *Staphylococci*, but were taken from *Enterobacteriaceae* and *Enterococcus* spp., respectively. Experiments were performed with two biological replicates.

2.8. Resistance determination according to benzalkonium chloride

Benzalkonium chloride resistance was determined according to a previously published procedure (Elhanafi et al., 2010) with slight modifications. Briefly, strains were grown on BHI plates as described before, after which colonies were resuspended in 200 μl MH broth. Five microliter of suspension was spotted in quintuplicate onto MH agar plates supplemented with benzalkonium chloride (Acros Organics) in different concentrations, namely, 0, 2.5, 5, 7.5, 10, 12.5 and 15 μg/ml. Plates were incubated for four days at 25 °C and bacterial growth was scored as 'no growth', 'single colony growth' and 'confluent growth'. Experiments were performed with two biological replicates.

3. Results

3.1. Prevalence and serogrouping of Listeria monocytogenes isolates

Samples were taken in a chronological order following one particular batch of mushrooms from the grower's facility up to the processing factory, resulting in the collection of 133 *L. monocytogenes* isolates. Sampling started with raw materials at the mushroom grower's facility (grower 1) up to the final frozen mushrooms, i.e. frozen sliced mushrooms at mushroom processing factory 1. In addition, 13 isolates were collected from frozen sliced mushrooms at mushroom processing factory 2, resulting in a total of 146 *L. monocytogenes* isolates. Sampling at grower 1 resulted in 49 *L. monocytogenes* isolates. None of the 100 casing soil samples was found to be positive for *L. monocytogenes* during filling of the growing room. Although, six out of 60 casing soil samples were found positive at the time of harvest, no *L. monocytogenes* was detected in the mushroom samples taken above the sampled casing soil. After mechanical harvesting and transporting the mushrooms over the distribution line at grower 1, *L. monocytogenes* was detected on the processing equipment during processing (11 out of 44 samples) on the processing equipment after C&D (seven out of 31 samples) and on mushrooms transported to factory 1 (25 out of 50 samples). Sampling in the processing factory resulted in 84 isolates. *L. monocytogenes* was detected on processing equipment during processing mushrooms of grower 1 (five positive samples) and on the frozen sliced mushrooms originating from grower 1 (29 out of 99 samples). Furthermore, *L. monocytogenes* was detected on the frozen sliced mushrooms that were sampled before and after processing the mushrooms of grower 1 (34 positive samples). *L. monocytogenes* was also detected on processing equipment of factory 1 after C&D (16 out of 74 samples). Seven additional isolates were included, which were isolated in 2016 and 2017 from fresh mushrooms after mechanically harvesting, resulting in 153 isolates in total.

Serogroup typing of the 153 isolates revealed that 39.2% belonged to group 1/2a-3a, 34.0% belonged to group 1/2b-3b-7 and 26.8% belonged to group 4b-4d-4e. Serogroup 1/2a-3a and serogroup 4b-4d-4e were detected at the grower's facility and also in factory 1 in fresh mushrooms, frozen sliced mushrooms and in environmental samples. Serogroup 1/2b-3b-7 was first detected in fresh mushrooms from grower 1 at factory 1 with a prevalence of 4%. The prevalence of this serogroup increased through the production chain, reaching a maximum of 72% on frozen sliced mushrooms. The same three serogroups were also detected in the frozen sliced mushrooms of factory 2. Additionally, the isolates from fresh mushrooms of previous years belonged to serogroup 1/2a-3a and 4b-4d-4e (Supplementary Table S1).

3.2. Characterization of L. monocytogenes strains by MLST analysis

The 44 selected *L. monocytogenes* strains, isolated from frozen sliced mushrooms and from processing equipment after C&D, were included in MLST analysis to further characterize the strains that survived C&D regimes, and strains that were present in the frozen sliced mushrooms that could reach the consumer phase. The MLST analysis revealed 11 sequence types (STs) and 11 clonal complexes (CCs) (Fig. 1). Six CCs (CC5, CC6, CC7, CC29, CC59, CC451) were isolated from one of the four places of isolation, once or twice. The other five CCs were isolated from multiple places and included CC1, CC4, CC37, CC87 and CC224. CC224 was the most frequently found CC among the samples with a total of 15 out of 44 samples. CC87 appeared to be the only CC observed in all four places (Fig. 1). The strains were grouped in lineage I and II, with a higher number of strains belonging to lineage I (84%) than to lineage II (16%) (Fig. 1).

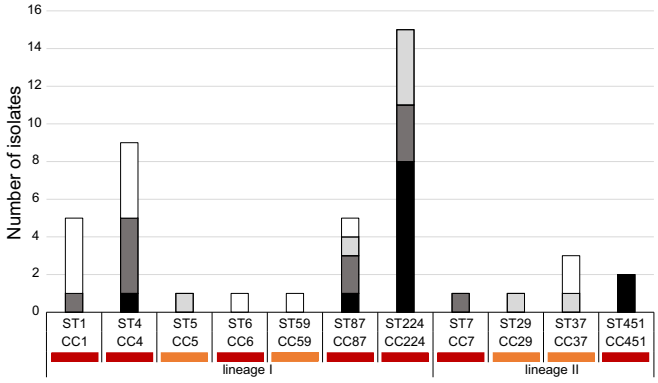

Fig. 1. Distribution of clonal complexes and sequence types among the 44 *L. monocytogenes* strains. Strains were isolated from four locations in the mushroom production and processing chain (frozen sliced mushrooms of factory 1 from mushrooms of grower 1 (black bars, 12 samples), frozen sliced mushrooms of factory 1 from mushrooms of multiple growers (dark grey bars, 11 samples), surface samples after C&D at factory 1 (light grey bars, eight samples) and frozen sliced mushrooms of factory 2 from mushrooms of multiple growers (white bars, 13 samples)). The colored bars in the figure indicate the identification of hypervirulent clones (red) and medium virulent clones (orange), according to the classification used by (Fritsch et al., 2018; Maury et al., 2016; Maury et al., 2019). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Following the identification of hypervirulent, medium virulent and hypovirulent clones (Fritsch et al., 2018; Maury et al., 2016; Maury et al., 2019), 38 out of 44 strains (86%) and six out of 44 (14%) were hypervirulent and medium virulent, respectively. The CCs of the groups CC1, CC4, CC6, CC7, CC87, CC224, CC451 are considered to be hypervirulent and CC5, CC29, CC37, CC59 are considered to be medium virulent (highlighted in red and orange respectively, Fig. 1). Notably, CC1, CC4 and CC87 types were observed in the frozen sliced mushrooms at both factories, while CC224 type was found in a high abundance in frozen sliced mushrooms of factory 1, while it was not observed in frozen sliced mushrooms of the factory 2.

3.3. Distribution of the L. monocytogenes strains

The minimum spanning tree constructed using all 44 *L. monocytogenes* strains (Fig. 2) revealed that strains were distributed in three serogroups (Fig. 2a) and eleven clonal complexes (Fig. 2b). Strains belonging to the same serogroup clustered together based on their cgMLST profile in which the lineages are clearly separated from each other (Fig. 2a). In addition, strains comprising the same clonal complex based on MLST are closely linked to each other (Fig. 2b).

The cgMLST profiles of the selected strains were obtained by including all available loci per strain, leading to a total of 18 cgMLST profiles. The 31 strains of factory 1 were assigned to ten cgMLST profiles and the 13 strains of factory 2 were assigned to ten cgMLST profiles as well. The two factories had two overlapping cgMLST profiles and these included strains belonging to CC87 and CC4. CCs comprising of multiple strains displayed either one cgMLST profile (CC451 and CC87), or two to four cgMLST profiles (CC1, CC4, CC37 and CC224). High heterogeneity was observed in cgMLST profiles for CC1 and CC4 of factory 2, yielding both three cgMLST profiles for four *L. monocytogenes* strains. Not all similar named cgMLST profiles had a unique cgMLST loci numbering and were therefore displayed by multiple circles instead of one circle (18 cgMLST profiles and 21 unique loci numbering profiles) (Fig. 2). This is due to one locus mismatch (two cgMLST profiles of CC4) or two loci mismatches (one cgMLST profile of CC37) between similar named cgMLST profiles, leading to 21 unique loci numbering profiles in total. The highest representative cgMLST profile belonged to the CC224 strains, in which 14 out of 15 CC224 strains belonged to the same cgMLST profile.

3.4. Presence of virulence genes and biofilm genetic markers in L. monocytogenes strains

The WGS results showed the presence of *Listeria* pathogenicity island 1 (LIPI-1), LIPI-3, and LIPI-4 in 100%, 68% and 32% of the 44 strains, respectively (Fig. 3). The virulence genes of LIPI-1, consisting of *prfA*,

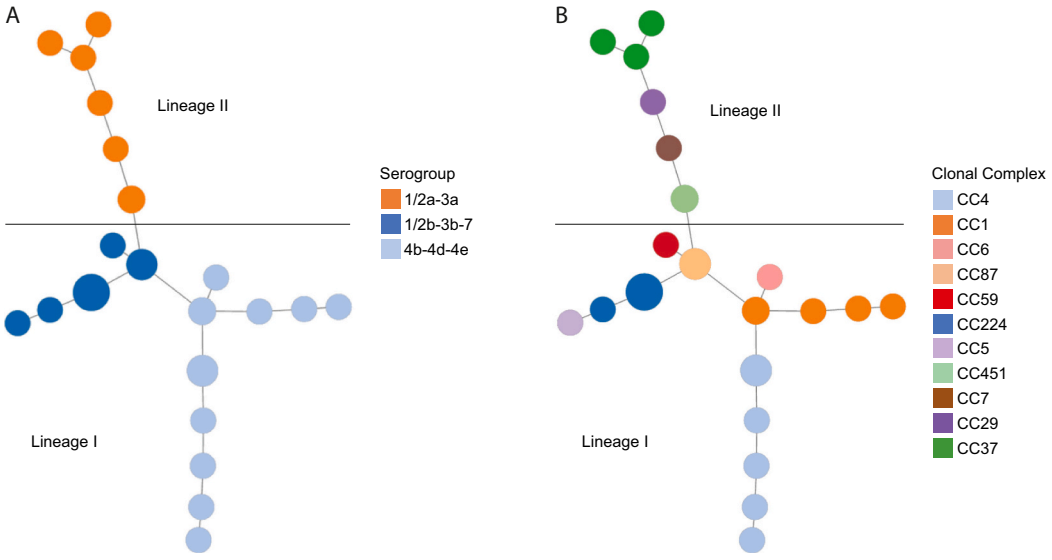

Fig. 2. Minimum spanning tree based on the allelic profiles of the cgMLST of the 44 *L. monocytogenes* strains. Strains were isolated from four locations in the mushroom production and processing chain (frozen sliced mushrooms of factory 1 from mushrooms of grower 1 (12 samples), frozen sliced mushrooms of factory 1 from mushrooms of multiple growers (11 samples), surface samples after C&D at factory 1 (eight samples) and frozen sliced mushrooms of factory 2 from mushrooms of multiple growers (13 samples)).
Allelic profiles are based on a total of 1463 (out of 1748) cgMLST genes. The size of the circles is proportional to the number of strains and the distances between circles indicate the allelic relationship. The colors of the circles represent (a) the three serogroups and (b) the eleven clonal complexes. The line separates the lineage I from the lineage II strains.

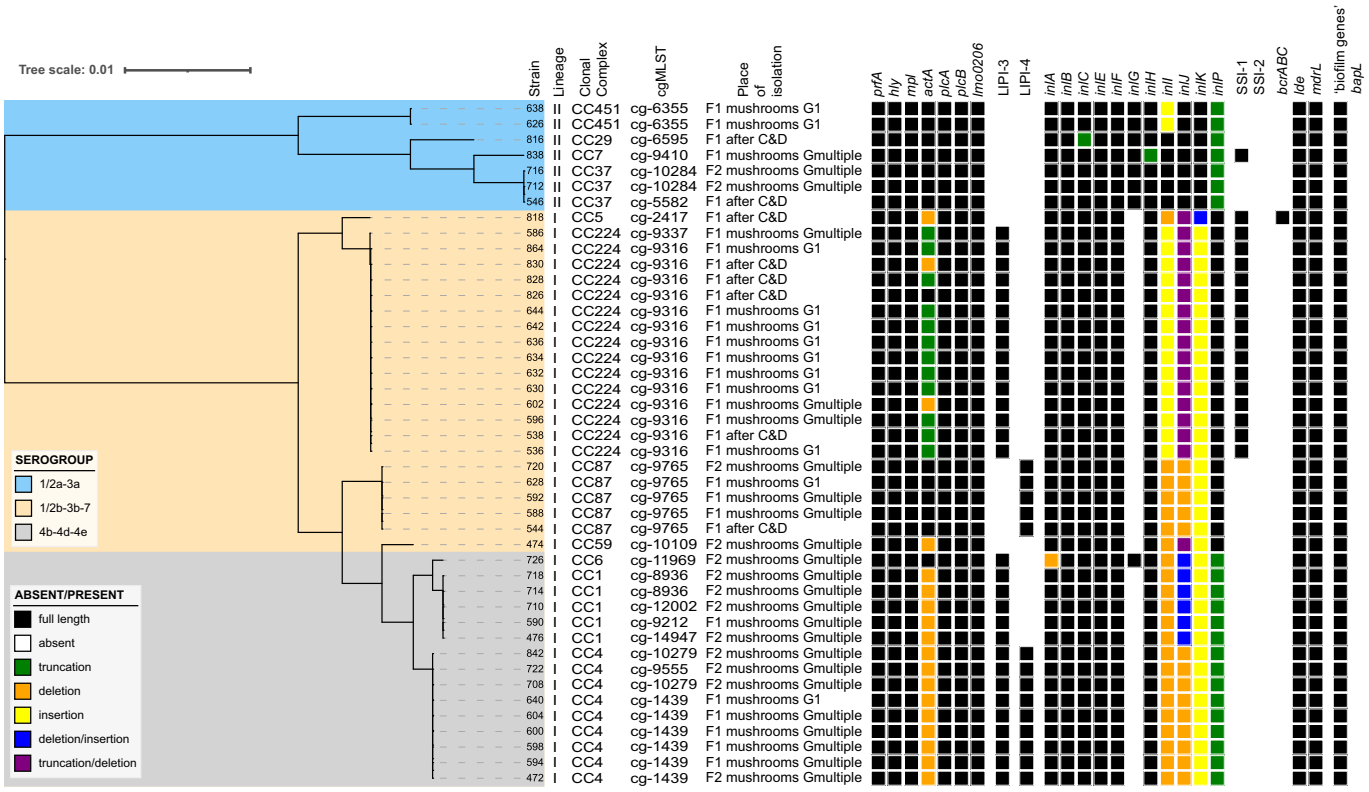

Fig. 3. Phylogenetic tree of the 44 *L. monocytogenes* strains determined with concatenated sequences of seven housekeeping genes derived from MLST. The serogroups are highlighted by color; serogroup 1/2a-3a is highlighted in blue, serogroup 1/2b-3b-7 is highlighted in orange and serogroup 4b-4d-4e is highlighted in grey. The strains were isolated from two mushroom processing companies, indicated by factory 1 (F1) and factory 2 (F2). Strains were isolated from four locations in the mushroom production and processing chain 'F1 mushrooms G1' are frozen sliced mushrooms of factory 1 from mushrooms of grower 1 (12 samples), 'F1 mushrooms Gmultiple' are frozen sliced mushrooms of factory 1 from mushrooms of multiple growers (11 samples), 'F1 after C&D' are surface samples after C&D at factory 1 (eight samples) and 'F2 mushrooms Gmultiple' are frozen sliced mushrooms of factory 2 from mushrooms of multiple growers (13 samples). Gene products including virulence factors, disinfectants resistance mechanisms and biofilm genes were screened for their presence. In case of presence, the matrix shows the full length (black box), truncations (green box), deletions (orange box), insertions (yellow box), deletion/insertion (blue box) and truncation/deletion (purple box). The box of the 'biofilm genes' are presumed biofilm genes and include *agrABCD*, *luxS*, *relA*, *yneA*, *hpt*, *prfA*, *secA2*, *degU*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hly, *mpl*, *actA*, *plcA*, *plcB* and *lmo0206* were present at full length in all 44 strains, except for *actA*. Although this gene was present in every strain, 14 out of 44 strains harbored a full-length gene, 18 out of 44 harbored a 35 amino acid (AA) internal deletion and 12 out of 44 had a large truncation. Genes of the LIPI-3 cluster were found in all 4b-4d-4e strains containing CC1, CC4 and CC6, and in all CC224 strains belonging to serogroup 1/2b-3b-7. The LIPI-4 cluster was observed in all CC4 (serogroup 4b-4d-4e) and CC87 (serogroup 1/2b-3b-7) strains.

A total of 11 internalin genes were observed among the strains. Internalin A (*inlA*) and B (*inlB*) were present in full length among all the tested strains, with the exception of *inlA* in the CC6 strain that harbored a three AA deletion within the sequence, but which did not result in an *inlA* PMSC. Also other members of the internalin gene family were present in the strains, with *inlC*, *inlE*, *inlF*, *inlH*, *inlI*, *inlJ*, *inlK*, *inlP* being present in all tested strains, while *inlG* was present in the lineage II strains and in the CC6 strain of lineage I (Fig. 3).

In addition, WGS analysis revealed the presence of several biofilm associated genes. This included *prfA* (virulence factor and regulator of flagella biosynthesis), *secA2* (involved in cell aggregation, biofilm formation and biofilm structure), *luxS* (involved in the inhibition of biofilm formation with the involvement of autoinducer 2), *agrABCD* (involved in adherence and regulation of early stages of biofilm formation), *relA* and *hpt* (regulators of the starvation response), *yneA* (involved in SOS response) and *degU* (response regulator for the activation of biofilm formation) (Abee et al., 2011; Kocot and Olszewska, 2017). All these genes were present in full length in all tested strains. On the other hand, *bapL*, encoding biofilm associated protein required for cell attachment (Abee et al., 2011; Kocot and Olszewska, 2017) was not found in any of the tested strains (Fig. 3).

3.5. Presence of resistance markers in L. monocytogenes strains

The stress survival islet 1 (SSI-1) has been associated with higher tolerance to low pH and high salt concentrations (Ryan et al., 2010) and was observed in 17 out of 44 strains (39%). The islet was observed in the CC7 strain (serogroup 1/2a-3a), and all CC5 and CC224 strains (serogroup 1/2b-3b-7). The SSI-2 was not observed in any of the strains.

3.6. Antimicrobial susceptibility testing

Antimicrobial susceptibility tests showed that all 14 antibiotics were effective against the 44 *L. monocytogenes* strains tested. All the strains showed clear inhibition zones for every antibiotic tested, and the inhibition zones were above the sensitivity threshold except for one antibiotic. For penicillin G, the inhibition zones of 15 strains were one mm below the breakpoint for sensitivity classification for only one of the two replicates. For two strains, the inhibition zone was one mm below the breakpoint for both of the replicates. Due to the clear inhibition zones, small differences and lack of exact *L. monocytogenes* breakpoints, the strains were also classified being sensitive to penicillin G. On the other hand, WGS analysis showed presence of the antimicrobial resistance genes *fosX*, *sul*, *norB* and *lin* in all sequenced strains, possibly conveying

resistance towards fosfomycin (inhibition of peptidoglycan biosynthesis), sulfonamides (inhibition of folic acid synthesis), quinolones (inhibition of DNA synthesis) and lincosamides (inhibition of protein synthesis) (Peach et al., 2013).

3.7. Benzalkonium chloride determination

The *bcrABC* resistance cassette, in which all three genes are essential to provide resistance to benzalkonium chloride (Elhanafi et al., 2010), was found in one strain belonging to CC5 (strain 818, serogroup 1/2b-3b-7). Indeed, this strain was found to be more resistant to benzalkonium chloride compared to all other strains that lacked this cassette. Confluent growth was observed up to 12.5 μg/ml benzalkonium chloride for strain 818. The strains lacking this *bcrABC* cassette showed confluent growth at 2.5 μg/ml, and single colony growth at 5.0 μg/ml. Depending on the strain, single colony growth or no growth was observed with 7.5 μg/ml benzalkonium chloride, and no growth was observed at values of 10 μg/ml benzalkonium chloride and higher (Table 1). Other efflux pump systems, namely *lde* and *mdrL*, were present in all 44 sequenced strains.

4. Discussion

This study evidenced the high diversity of *L. monocytogenes* within the mushroom production chain, from the grower to frozen sliced mushrooms. Remarkably, two of the three serogroups (1/2a-3a and 4b-4d-4e) were already detected at the grower's facility, indicating that the high diversity originates already at the first stage of the production chain. Three serogroups (1/2a-3a and 1/2b-3b-7 and 4b-4d-4e) were detected in the frozen sliced mushrooms at both factories, indicating that the high diversity is conserved at the end of the chain. The relative prevalence of each serogroup of *L. monocytogenes* is comparable to a previous study also focused on *L. monocytogenes* in *A. bisporus* mushrooms in Ireland (Pennone et al., 2018). These authors grouped 30.1% of the isolates to serogroup 1/2b-3b-7, 40.8% to serogroup 1/2a-3a and 29.1% to serogroup 4b-4d-4e. In the current study, 34.0% of isolates were grouped to serogroup 1/2b-3b-7, 39.2% were grouped to serogroup 1/2a-3a and 26.8% to serogroup 4b-4d-4e. On the other hand, a study that focused on a fresh mushroom processing environment in the United States grouped 3.5% to serogroup 1/2b-3b, 2.9% to serogroup 1/2a-3a and 93.6% to serogroup 1/2c-3c (Murugesan et al., 2015), while the last serogroup was not detected in the current study or the study in Ireland. Analysis of the frozen sliced mushrooms showed up to 43% (13 positive out of 30 samples, detection in 25 g) of the samples to be positive for *L. monocytogenes*. This value was comparable with a previous study done in England where it was found that 50% (five positive out of 10 samples, detection in 25 g) of the frozen sliced mushrooms were contaminated with *L. monocytogenes* (Willis et al., 2020).

WGS analysis showed the high genetic diversity among the 44 selected strains, isolated either from surface samples after C&D or from the frozen sliced mushrooms, with 11 different CCs/STs identified. The identified CCs of this study have been reported in other studies that focused on other types of processed foods. Studies showed presence of CC1, CC4, CC5, CC6, CC7, CC29, CC37, CC451 on frozen vegetables and fruits (Willis et al., 2020), CC1, CC4, CC5, CC6, CC7, CC29, CC37, CC59, CC87, CC224, CC451 in milk and milking equipment (Kim et al., 2018), CC1, CC4, CC5, CC6, CC7, CC29, CC37, CC59, CC451 from various food products (Ebner et al., 2015), indicating a wide spread of the CCs in food production environments. High genetic diversity was also observed when the cgMLST profiles of the 44 strains were determined, which led to a total of 18 cgMLST profiles. For CC4 and CC87, the same cgMLST profiles in the frozen sliced mushrooms were observed in both factories, which may be the result of introduction events by raw materials from a common source. On the other hand, 14 of the 15 CC224 strains isolated at factory 1 displayed a single cgMLST profile. These CC224 strains were isolated from different batches of the frozen sliced mushrooms and of surface samples after C&D. Also CC87 was present in multiple sampling locations with the same cgMLST profile across these locations. This could suggest a better biofilm forming capability of these possibly persistent CC87 and CC224 strains. However, the biofilm formation capacity depends not only on genetic biofilm markers, but also on the type of surface (Magalhães et al., 2017). Therefore the different types of surfaces in the mushroom industry will also influence the biofilm forming capacity of *L. monocytogenes*. Various biofilm genes were identified in the isolated strains, factors that may contribute to the

Table 1
Growth of the 44 *L. monocytogenes* strains exposed to various concentrations of benzalkonium chloride using the agar spot plating method. Experiment was performed in duplicate for 44 strains and each experiment was performed with quintuplicate spots on the agar. The *L. monocytogenes* strains are indicated with their number (No.), their clonal complex and the presence/absence (+/−) of the *bcrABC* cassette. Growth on the agar plate is indicated as confluent growth (dark grey), single colony growth (light grey) and no growth (white boxes). Confluent growth was defined as continuous growth of *L. monocytogenes* within the originally spotted droplet. Single colony growth was defined when at least one colony of *L. monocytogenes* was observed in one of the spots in one replicate.

No.	CC type	*bcrABC* cassette	Benzalkonium chloride (μg/ml) 0.0	2.5	5.0	7.5	10.0	12.5	15.0
626	CC451	–							
638	CC451	–							
816	CC29	–							
838	CC7	–							
716	CC37	–							
546	CC37	–							
712	CC37	–							
818	CC5	+							
586	CC224	–							
864	CC224	–							
830	CC224	–							
828	CC224	–							
826	CC224	–							
644	CC224	–							
642	CC224	–							
636	CC224	–							
634	CC224	–							
632	CC224	–							
630	CC224	–							
602	CC224	–							
596	CC224	–							
536	CC224	–							
538	CC224	–							
720	CC87	–							
628	CC87	–							
592	CC87	–							
544	CC87	–							
588	CC87	–							
474	CC59	–							
726	CC6	–							
718	CC1	–							
714	CC1	–							
710	CC1	–							
476	CC1	–							
590	CC1	–							
842	CC4	–							
722	CC4	–							
708	CC4	–							
640	CC4	–							
604	CC4	–							
600	CC4	–							
598	CC4	–							
472	CC4	–							
594	CC4	–							

presence and prevalence of *L. monocytogenes* in mushroom production environments. Whether this high genetic diversity among the isolated strains affects biofilm forming capacity on different surface materials remains to be elucidated.

A high percentage (86%) of the strains could be classified as hypervirulent strains using the CC virulence association (Fritsch et al., 2018; Maury et al., 2016; Maury et al., 2019). These hypervirulent CC types have previously been found in other food types, such as milk and milking equipment (Kim et al., 2018) and RTE food samples (Chen et al., 2020). Every strain showed full length genes of the LIPI-1 cluster, except for *actA* in some strains, which is involved in actin recruitment and intercellular spreading (Vázquez-Boland et al., 2001). The *actA* sequence showed an internal deletion of 35 AA (AA position 265-299) in all CC1, CC4, CC5, CC59 and two out of 15 CC224 strains. In addition, one out of 15 CC224 strain had a truncation of 282 AA and 11 out of 15 CC224 strains had a truncation of 317 AA. Disrupted versions of *actA* were previously described in the aforementioned CCs (Maury et al., 2016) and disrupted versions of this gene could have an effect on the intracellular motility. The amino-terminal region (AA position 128-151) has been found to be essential for *actA* in actin filament recruitment, while the proline rich repeats (AA position 265-390) are involved in higher efficiency for the recruitment of filamentous actin (Pistor et al., 1995). Effects of mutations in *actA* on *L. monocytogenes* virulence and pathogenicity in humans are not fully understood, because disrupted versions of *actA* are also present in hypervirulent strains. LIPI-3 was absent in all lineage II strains, but was present in a subset of the lineage I strains involving CC1, CC4, CC6 and CC224 and presence of LIPI-3 in these CCs had already been reported previously (Kim et al., 2018). These results are in accordance with a previous study mentioning the presence of LIPI-3 in a subset of the lineage I strains, but absence in lineage II and lineage III *L. monocytogenes* strains (Cotter et al., 2008). LIPI-4 was observed in all CC4 and CC87 strains of this study, which is in accordance with other studies, which reported the presence of the LIPI-4 cluster for CC4 strains (Maury et al., 2016) and for both the clonal complexes (Kim et al., 2018).

All 44 *L. monocytogenes* strains sequenced in this study had a full length *inlA* and *inlB* coding sequence, except for the CC6 strain that had a nine nucleotide deletion in the *inlA* sequence at position 2212 to 2220. This resulted in a three AA loss in the protein sequence. The same deletion is previously mentioned as characteristic for CC6 strains with the deletion located at the pre anchor region of *inlA* and it is not expected that this deletion would affect the *inlA* mediated action (Cantinelli et al., 2013). On the contrary, other studies described *inlA* PMSCs in 10.8% to 20.8% of the *L. monocytogenes* strains that were isolated from a variety of food products and from meat and meat processing environments (Chen et al., 2020; Kurpas et al., 2020). Clonal complexes containing an *inlA* PMSC included CC5 among others, although not all CC5 strains harbored a PMSC in *inlA* (Chen et al., 2020; Kurpas et al., 2020). Other internalins detected in every strain analyzed in this study included *inlC*, *inlE*, *inlF*, *inlH*, *inlJ*, *inlK*, *inlP*, which had already been detected previously in strains from lineage I and lineage II of refrigerated RTE food samples, except *inlF* in one CC121 strain (Chen et al., 2020). Gene *inlF* was identified in all lineage I and II strains in this study, in accordance with previous study reporting its presence in the majority of strains belonging to lineage I and lineage II (Chen et al., 2020; Kurpas et al., 2020). Gene *inlG* was identified in all lineage II strains in this study and in the CC6 strain belonging to lineage I, which is in accordance with previous research in which *inlG* was observed in the majority of the lineage II strains and not in lineage I, except for CC6 strains that were isolated from RTE meat and meat processing environments (Kurpas et al., 2020) and refrigerated RTE food (Chen et al., 2020). The high abundance of virulence factors in the *L. monocytogenes* strains isolated from the mushroom production and processing chain indicate the virulence potential of these strains.

The SSI-1 cluster was present in 39% of the 44 *L. monocytogenes* strains, while SSI-2 cluster was absent in every strain. The SSI-2 cluster was not observed in this study, possibly due to the absence of CC121 strains isolated, as this CC was previously identified as the main group containing this islet (Harter et al., 2017). On the other hand, our results showed the presence of the SSI-1 cluster in the CC7 strain of lineage II and in CC5 and all CC224 strains of lineage I, in line with previous data on *L. monocytogenes* strains obtained from food and food processing environments and from sporadic human listeriosis (Hingston et al., 2017). Functions encoded by this islet have previously been shown to enhance growth at suboptimal conditions/environmental stresses, as low pH and high salt concentrations (Ryan et al., 2010), but contradictory results have been reported on stress tolerance related to SSI-1 (Hingston et al., 2017). However, the presence of SSI-1 in 39% of our strain selection may point to a selective advantage for growth and prevalence in conditions encountered in the production of frozen sliced mushrooms.

All selected strains were classified as sensitive against the antibiotics tested, including those commonly used in clinical settings for listeriosis patients. Additional analysis showed no inhibition zones upon exposure to nalidixic acid, in line with the previously reported results of natural resistance of *L. monocytogenes* to this compound (Olaimat et al., 2018). Hence, although *L. monocytogenes* could be found in the mushroom production and processing chain, no multidrug resistance types were found in our study.

Both the presence and high genomic diversity of the *L. monocytogenes* strains isolated in this study underlines the importance of hygienic measures to control *L. monocytogenes* along the whole mushroom production and processing chain. Resistance towards the disinfectant benzalkonium chloride (BC) is linked to the *bcrABC* resistance cassette (Elhanafi et al., 2010; Minarovičová et al., 2018). Our results support this hypothesis, as the CC5 strain harboring this cassette had a higher tolerance to BC than the rest. Indeed, the presence of the *bcrABC* cluster in *L. monocytogenes* strains has been described and associated with CC5 strains (Meier et al., 2017). On the other hand, strains that did not harbor the *bcrABC* genes showed single colony growth at 5 μg/ml of BC and a subset of strains also at 7.5 μg/ml BC, which might indicate natural resistance to low concentrations of BC. This could be due to the presence of additional efflux pumps. Efflux pumps *mdrL* and *lde* were present in all tested strains in which *mdrL* is partly responsible for BC resistance, but *lde* is not (Romanova et al., 2006). Other studies show higher percentages of BC resistance in *L. monocytogenes* strains isolated from food production, which could be derived either from *bcrABC* or *qacH*. BC resistance was reported for 18% of the strains isolated from food matrices in Switzerland (Ebner et al., 2015) and 22% of the strains from a meat processing facility in Slovakia (Minarovičová et al., 2018). The fact that only one out of 44 *L. monocytogenes* strains contained the *bcrABC* resistance cassette could be due to the lack of selection pressure for BC resistance since quaternary ammonium compounds are not applied in cleaning and disinfection regimes at the selected mushroom production and processing companies.

5. Conclusion

Our analysis demonstrated that *L. monocytogenes* is present in the mushroom production and processing chain, with low prevalence at the grower's facility and higher prevalence in the frozen sliced mushrooms. Genotypic analysis showed high diversity in serogroups, CCs and cgMLST types among the strains. Two of the three serogroups (1/2a-3a and 4b-4d-4e) were already detected at the grower's facility, indicating a rather high diversity already in the first stage of the mushroom production chain. The diversity increased further in the end product i.e. the frozen sliced mushroom products. The majority of strains (86%) isolated from the processing environment and the frozen sliced mushrooms were assigned hypervirulent CCs and a wide diversity of virulence genes was present among the strains, yet all were sensitive towards a wide range of antibiotics.

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.ijfoodmicro.2021.109438.

CRediT authorship contribution statement

Frank B. Lake: Conceptualization, Methodology, Writing – original draft, Writing – review and editing, Visualization, Data Curation, Validation, Investigation, Formal analysis

Leo S. van Overbeek: Conceptualization, Methodology, Writing – review and editing, Supervision, Funding acquisition, Project administration, Validation

Johan J.P. Baars: Conceptualization, Methodology, Writing – review and editing, Supervision, Resources

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Heidy M.W. den Besten: Conceptualization, Methodology, Writing – review and editing, Supervision, Project administration, Validation, Formal analysis, Resources

Tjakko Abee: Conceptualization, Methodology, Writing – review and editing, Supervision, Validation, Formal analysis

Declaration of competing interest

None.

Acknowledgement

The authors thank Patricia van der Zouwen for her excellent contribution to the sampling analyses.

In addition, the authors thank all project partners for their contributions and feedback to the project.

This work was funded in cooperation with private and public partners and included the topsector TKI (under number TU 16031) (Horticulture and starting materials) and companies from the mushroom sector.

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